Estudi transcripcional i funcional de les ribonucleotidil reductases de *Pseudomonas aeruginosa*

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Memòria presentada per Anna Crespo Puig per optar al títol de doctor per la Universitat de Barcelona

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Abreviatures

(d)NDP	(Desoxi)ribonucleòtid difosfat
(d)NTP	(Desoxi)ribonucleòtid trifosfat
Δ	Delta - deleció
ADNc	Àcid desoxiribonucleic complementari
AdoCbl	5’ Adenosil cobalamina (vitamina B_{12})
AdoMet	5’ Adenosil metionina
Amp	Ampicil·lina
Arg	Arginina
ARNm	Àcid ribonucleic missatger
Asn	Asparagina
ATP	Adenosil trifosfat
B_{12}	Vitamina B_{12}
bd	Bi-destil·lat
C	Citosina/Carboni
c-di-GMP	Diguanilat cíclic
C-terminal	Part carboxi-terminal d’una proteïna
CFTR	*Cystic Fibrosis Transmembrane regulator*
CLSM	*Confocal laser scanning microscopy*
Cm	Cloramfenicol
Co	Cobalt
CT	*Cycle threshold* – cicle umbral
Cys	Cisteïna
DMSO	Dimetilsulfòxid
DO	Densitat òptica
DPA	Assaig de *diphenylamine*
DTT	Ditiotreitol
EMSA	*Electrophoretic Mobility Shift Assay*
Fe	Ferro
FQ	Fibrosi quística
GFP	*Green fluorescence protein* – proteïna verda fluorescent
Gm	Gentamicina
GR	Glutatió reductasa
Grx	Glutaredoxina
GSH	Glutatió
GSNO	*S-nitrosoglutathione*
H_2O_2	Aigua oxigenada o peròxid d’hidrogen
His	Histidina
hpf	Hores post-fecundació
hpi	Hores de post-infecció
HU	Hidroxiurea
Ile	Isoleucina
IPTG	Isopropil-β-D-1-tiogalactopiranòsid
Km	Kanamicina
LB	Medi Luria Betrani
LBN	Medi Luria Betrani amb KNO_3
Lys	Lisina
MCS	*Multipe cloning site* - Lloc de clonació múltiple
MIC	Mínima concentració inhibitòria
MM	Medi mínim
MMN	Medi mínim amb KNO_3
Mn	Manganés
MPOC	Malaltia Pulmonar Obstructiva Crònica
N-terminal	Part amino-terminal d’una proteïna
N_2	Nitrogen
NADPH	Nicotinamida adenina dinucleòtid fosfat
NO_2	Nitrit
NO_3	Nitrat
nrd	*Nucleotide reduction* - gen que codifica per a una RNR

ON	Òxid nítric
O/N	*Over/night*–tota la nit
ORF	*Open reading frame*
P*algD*	Promotor del gen *algD*
P*algR*	Promotor del gen *algR*
PFL	Piruvat format liasa
Phe	Fenilalanina
P*nrdA*	Promotor del gen *nrdA*
P*nrdD*	Promotor del gen *nrdD*
P*nrdJ*	Promotor del gen *nrdJ*
P*nrdR*	Promotor del gen *nrdR*
P*orpF*	Promotor del gen *oprF*
P*topA*	Promotor del gen *topA*
qRT-PCR	PCR quantitativa a temps real
QS	*Quorum sensing*
RFU	*Relative fluorescence units* – unitats relatives de fluorescència
RNR	Ribonucleotidil reductasa
ROS	*Reactive oxygen species* –especies reactives d'oxigen
RT-PCR	Reacció en cadena de la polimerasa amb transcriptasa reversa
SD	Desviació estàndard
Tc	Tetraciclina
Tm	Temperatura de fusió o *melting*
TrxR	Tioredoxina reductasa
Tyr	Tirosina
ufc	Unitats formadores de colònies
UTR	*Untranslated region*- regió no traduïda
XDR	Soques amb resistència a antibiòtics extensa - *Extensively Drug Resistant*
Zn	Zinc

Introducció

1.1. L'enzim Ribonucleotidil Reductasa

L'any 1960, un estudi liderat pel Professor Peter Reichard del *Karolinska Institute* (Suècia) va descobrir una reacció essencial per sintetitzar els precursors de l'ADN (dNTP). Aquest estudi va consistir en la identificació de l'enzim RiboNucleotidil Reductasa (RNR) [1].

Les ribonucleotidil reductases són metal·loenzims essencials per la vida que catalitzen la reducció dels ribonucleòtids (NDP/NTP) als seus corresponents desoxiribonucleòtids (dNDP/dNTP) necessaris per a la síntesi i reparació de l'ADN, i per tant, per la divisió cel·lular [2]. Aquesta reacció enzimàtica consisteix a reduir el grup 2'-OH de la ribosa a un hidrogen (H) per tal de generar els desoxiribonucleòtids fosfats (dATP, dTTP, dCTP i dGTP) [2] (Figura 1).

Els dNTPs es poden sintetitzar a través de dues vies: via *de novo* (depenent de RNR) o via *salvatge* (independent de RNR). En la via *salvatge*, els dNTP s'obtenen a partir de la reutilització dels desoxiribonucleòtids monofosfats i la posterior fosforilació d'aquests. Aquesta via independent de les RNRs s'utilitza en alguns compartiments cel·lulars, com ara en els mitocondris de les cèl·lules eucariotes [3]. Tot i així, el mecanisme principal d'obtenció de dNTP per a la replicació i la reparació de l'ADN dels organismes és mitjançant la catàlisi dels ribonucleòtids a través de l'enzim RNR (síntesi de *novo*).

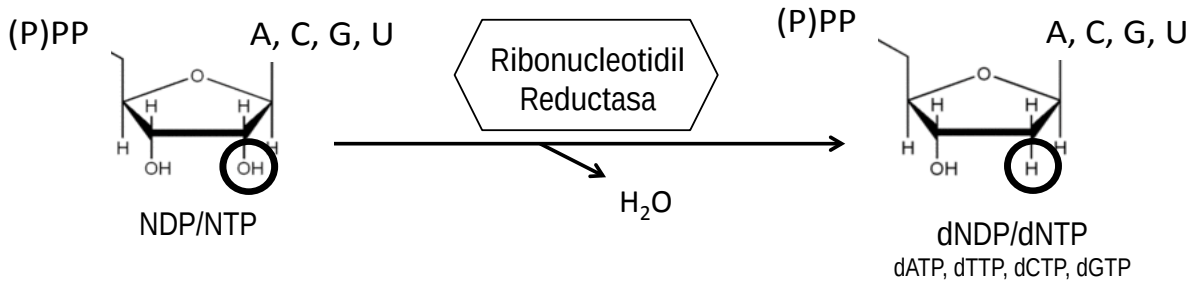

Figura 1: Reacció enzimàtica simplificada de l'enzim RNR.

L'evolució ha permès l'aparició de diferents classes de l'enzim RNR. Totes les classes de RNR catalitzen la mateixa reacció tot i presentar diferent activitat segons les condicions ambientals. A continuació es descriuen breument les diferents classes de RNR fins ara estudiades.

1.1.1. Les classes de l'enzim RNR

L'enzim RNR es divideix en tres classes diferents: la classe I, la classe II i la classe III. La classificació de les RNRs depèn de la generació del radical proteic, l'estructura tridimensional, el cofactor requerit, el centre metàl·lic i la dependència a l'oxigen per poder dur la reacció (Taula 1). Les diferents classes de RNR es troben codificades per unitats transcripcionals diferents i comparteixen molt poca similitud en l'estructura primària (10-20%) [4], tot i així, comparteixen el mateix mecanisme de reacció.

Els primers estudis de les classes de l'enzim RNR es van realitzar principalment en el bacteri *Escherichia coli.* Es va determinar la RNR d'*E. coli* com a prototip de la RNR de classe I [1]. Posteriorment, l'any 1964 es va descobrir i caracteritzar un segon enzim a *Lactobacillus leichmannii* amb un mecanisme catalític diferent. Aquesta nova classe de RNR requereix la vitamina B_{12} per tenir activitat enzimàtica, i va ser el prototip de la RNR de classe II [5]. Uns anys més tard, l'any 1989, a *E. coli* es va detectar activitat RNR en condicions d'anaerobiosis estricta per part d'una RNR que requeria de S-adenosilmetionina per la seva reacció, sent el prototip de la RNR de classe III [6, 7]. Aquestes tres classes (la classe I, la classe II i la classe III) es troben distribuïdes de manera combinada a diferents organismes (http://rnrdb.pfitmap.org/count_matrix) (Apartat 1.1.7.) [8-10].

Actualment, la RNR classe I es subdivideix en les subclasses Ia, Ib i Ic. La RNR de classe I descoberta a *E. coli* correspon a la RNR de classe Ia. Als anys 90 es va descobrir, a *Salmonella thyphimurium*, una nova classe de RNR diferent a la classe Ia d'*E. coli*. Aquesta nova classe correspon a la subclasse Ib [11, 12]. Per últim, recentment, a *Chlamydia trachomatis*, s'ha identificat una nova subclasse anomenada Ic [13]. Per tant, les classes de RNR fins a l'actualitat descobertes són; la classe I (Ia, Ib i Ic), la classe II i la classe III. Les característiques generals per a cada classe de RNR es troben detallades en la Taula I.

Totes tres classes de RNR comparteixen l'estructura tridimensional de la subunitat catalítica, en el centre actiu, altament conservada per a realitzar la mateixa reacció. A més, cada classe de RNR redueix els quatre nucleòtids (A, U, C i G) en un mateix centre actiu. Per tant, ha d'existir una regulació molt acurada de cada classe de RNR, tant a nivell transcripcional, com a nivell proteic (regulació al·lostèrica), imprescindible pel manteniment equilibrat dels dNTP a la cèl·lula. Una desregulació dels nivells de dNTP indueix a un augment de la taxa de mutació durant la síntesi de l'ADN afectant a la divisió cel·lular [14]. A nivell de proteïna, la regulació al·lostèrica de l'activitat de l'enzim es dóna en dos centres al·lostèrics conservats, el centre de l'especificitat i el de l'activitat [15, 16]. La regulació al·lostèrica de l'especificitat permet la reducció específica del ribonucleòtid unit (substrat) per la unió d'un determinat desoxiribonucleòtid (efector), mentre que la regulació

al·lostèrica de l'activitat regula la unió del substrat al centre actiu o no, per la unió d'ATP (enzim actiu) o dATP (enzim inactiu), en una zona anomenada *ATP-cone* (Apartat 1.1.5.).

Taula 1: Característiques de les diferents classes de RNR.

	Classe I			Classe II	Classe III
	Classe Ia	**Classe Ib**	**Classe Ic**		
Requeriments d'oxigen	Aeròbica	Aeròbica	Aeròbica	Independent	Anaeròbica estricta
Estructura	$\alpha_2\beta_2$	$\alpha_2\beta_2$	$\alpha_2\beta_2$	α o α_2	$\alpha_2 + \beta_2$
Gens	*nrdAB*	*nrdHIEF*	*nrdAB*	*nrdJab*	*nrdDG*
Proteïna	NrdAB	NrdEF	NrdAB	NrdJ	NrdDG
Radical	Tyr…Cys	Tyr…Cys	Phe…Cys	AdoCbl…Cys	AdoMet…Gly/Cys
(Metall)Cofactor	Fe^{III}-O-Fe^{III}	Mn^{III}-O-Mn^{III} Fe^{III}-O-Fe^{III}	Mn^{IV}-O-Fe^{III}	Co (AdoCbl)	4Fe-4S (+AdoMet)
Substrat	NDP	NDP	NDP	NDP/NTP	NTP
Reductant	Tioredoxina Glutaredoxina	NrdH Glutaredoxina	Tioredoxina	Tioredoxina Glutaredoxina	Format Tioredoxina
Regulació al·lostèrica					
- Especificitat	SI	SI	SI	SI	SI
- Activitat	SI	NO	SI	NO	SI

La RNR de classe I, fins ara la més estudiada, està present en tots els organismes eucariotes, bacteris, virus i bacteriòfags. L'estructura quaternària de l'enzim està formada per dues subunitats homodimèriques anomenades R1 o α (de major pes molecular), i R2 o β (de menor pes molecular). Aquestes dues subunitats donen lloc a l'estructura $\alpha_2\beta_2$ com a estructura predominant. En la subunitat R1 es localitzen el centre actiu i els dos centres de regulació al·lostèrica (el de l'especificitat i el de l'activitat). En la subunitat R2 s'hi troba el cofactor metàl·lic essencial per iniciar la reacció enzimàtica. Aquest cofactor metàl·lic necessita l'oxigen per oxidar-se i generar un radical tirosil en una tirosina de la subunitat R2 (Tyr 122 a *E. coli)* que a través d'una cadena de transport d'electrons es transfereix al centre actiu. Per últim, es genera un radical transitori tiil en les cisteïnes de la subunitat R1 per iniciar el radical proteic necessari per dur a terme el procés de reducció. En requerir l'oxigen, aquesta classe de RNR només tindrà activitat en condicions aeròbies.

Els gens implicats en codificar les tres subclasses de RNR de classe I (Ia, Ib i Ic) són *nrdA* (subunitat R1) i *nrdB* (subunitat R2) per les RNRs de classe Ia i Ic, i els gens *nrdE* (subunitat RIE) i *nrdF* (subunitat R2F) per la RNR de classe Ib. Els gens *nrdE* i *nrdF* es troben formant un operó amb els gens *nrdI* i *nrdF*. El gen *nrdI* codifica per una flavoredoxina i el gen *nrdH* per una glutaredoxina-like específica del sistema [12, 17]. La diferència principal entre les tres subclasses de classe I es troba en el centre metàl·lic localitzat en la subunitat R2 (Taula 1). La RNR de classe Ia conté un centre metàl·lic del tipus Fe^{III}-O-Fe^{III}, la RNR de classe Ib conté un centre metàl·lic del

tipus Mn^{III}-O-Mn^{III} (*in vivo*) [18, 19], tot i que, també es pot trobar el centre Fe^{III}-O-Fe^{III} [20], i per últim, la RNR de classe Ic conté un centre metàl·lic de Mn^{IV}-O-Fe^{III} [21].

La RNR de classe II es troba en bacteris, arquees i bacteriòfags. L'estructura quaternària de la proteïna consta d'una única subunitat homodimèrica (α_2) o monomèrica (α), equivalent a la subunitat R1 de la RNR de classe I. Aquesta subunitat de la RNR de classe II necessita l'adenosilcobalamina (AdoCbl) o vitamina B_{12} per a generar el radical tiil que duu a terme la reacció. Per tant, l'activitat d'aquesta classe és completament independent d'oxigen, ja que no en requereix per generar el radical, però en canvi, necessita la vitamina B_{12} [22, 23]. El gen que codifica aquesta subunitat és el gen *nrdJ* (excepcionalment en alguns organismes, com *Pseudomonas aeruginosa*, la subunitat es troba codificada en dos *open reading frames* (ORF), *nrdJa* i *nrdJb* [24]). A més, la RNR de classe II, en la majoria de microorganismes, com a *P. aeruginosa*, tan sols conté el centre de regulació al·lostèric d'especificitat, i no el d'activitat. Tot i així, excepcionalment en altres microorganismes, la RNR de classe II sí que pot ser regulada pels nivells d'ATP/dATP [15, 25].

La RNR de classe III està composta per dues proteïnes homodimèriques codificades per *nrdD* i *nrdG* formant una estructura $\alpha_{2+}\beta_2$. En la proteïna NrdD es localitza el centre catalític de l'enzim i els dos centres de regulació al·lostèrica (el d'especificitat i el d'activitat). En canvi, NrdG actua com una *activasa*, ja que és capaç d'activar NrdD sense tenir una unió permanent a aquesta, i una vegada ha generat el radical en NrdD, pot generar diferents cicles de reducció de substrat [26]. Tot i així, es necessita la unió de la S-adenosilmetionia (SAM) al centre metàl·lic de NrdG, format per 4Fe i 4S, per la generació del radical glicil. Aquest radical és extremadament sensible a l'oxigen, així doncs, es considera la RNR més ancestral evolutivament [10], ja que, la RNR de classe III només té activitat en condicions anaeròbiques i tan sols es troba codificada en organismes que creixen en absència d'oxigen.

Per últim, les tres classes de RNR també es diferencien pel sistema donador d'electrons, necessari per a reduir un nou cicle de reducció d'un NTP, ja que, en la RNR de classe III es dóna pel format o per tioredoxines, a diferència de les classes I i II que tan sols utilitzen ditiols provinents de tioredoxines i glutaredoxines [4, 27, 28] (Apartat 1.1.4.).

1.1.2. Estructura tridimensional de l'enzim

Les tres classes de RNR revelen una estructura tridimensional de la subunitat catalítica que segueix una topologia predominant en hèlix α i fulles β, sobretot en la RNR de classe I i II on la identitat a nivell d'estructura primària és >25 % [29, 30]. A més, existeix una alta conservació dels

residus del centre actiu que determina un mateix sistema catalític. En canvi, la subunitat catalítica de la RNR de classe III (NrdD) és la que més difereix, ja que no té una homologia significativa amb el centre α/β de les RNRs de classes I i II encara que l'estructura tridimensional del centre actiu és molt semblant [31].

1.1.2.1. La RNR de classe I

L'estructura quaternària de la RNR de classe Ia d'*E. coli* va ser la primera estructura tridimensional dels enzims RNR que es va determinar i, en aquest treball, s'ha pres com a referència. Presenta dues subunitats homodimèriques diferents, NrdA (α) i NrdB (β), ($\alpha_2\beta_2$)..

La subunitat catalítica R1A o NrdA d' *E. coli*, conté 761 aminoàcids (85.7 kDa) els quals es diferencien en tres dominis diferents (Figura 2) [4, 32, 33]:

1) un domini hèlix α format per 220 residus aminoacídics, en l'extrem N-terminal.
2) un domini format per 480 residus que donen lloc a 10 làmines α/β en forma de barril, en el centre actiu de l'enzim.
3) un domini αβααβ de 70 residus en la part C-terminal.

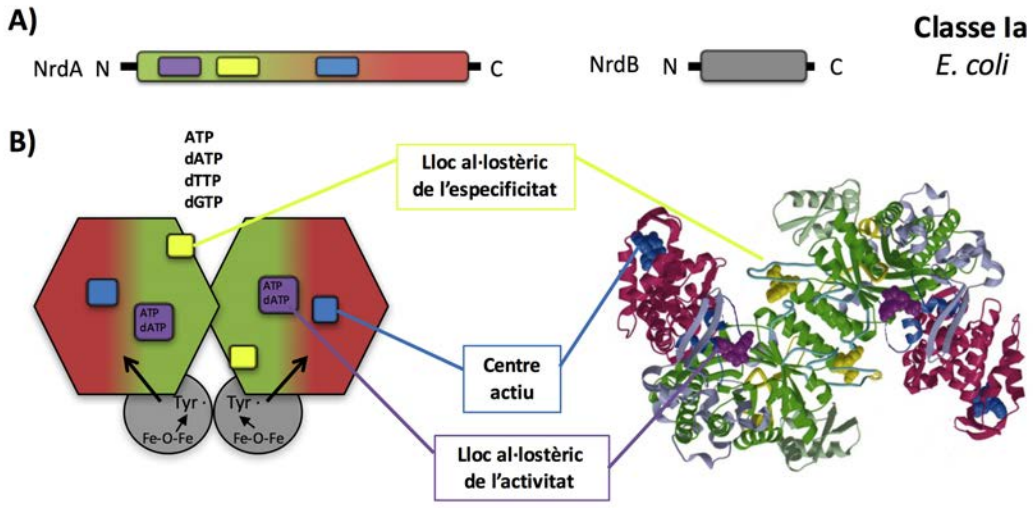

Figura 2: Esquema i dibuix dels dominis de la RNR de classe Ia d'*E. coli*. La subunitat R1 (NrdA, α) i la subunitat R2 (NrdB, β) es troben representades esquemàticament, tant de forma lineal (A) com tridimensional **(B).** El domini N-terminal es troba representat en color verd, mentre que el C-terminal en vermell. Els centres de regulació al·lostèrics s'indiquen en lila (lloc al·lostèric d'activitat) i en groc (lloc al·lostèric d'especificitat) i el centre actiu en blau. Per últim, la subunitat β (NrdB) es troba en gris, i s'indica el radical i el centre metàl·lic (Figura adaptada de [32]).

En el centre actiu de la RNR de classe Ia (NrdA), es troba un residu cisteïna (Cys 439) on es transfereixen els electrons generats en NrdB per formar el radical tiil i dos residus de cisteïnes (Cys

462 i Cys 225) que permetran la reducció del nucleòtid. Els nucleòtids difosfats s'uneixen al centre catalític o actiu, entre les dues cisteïnes (Cys 225, posició proximal, i Cys 462, posició distal) i el radical tiil (Tyr 122, en NrdB), per tal de ser reduïts. Aquestes tres cisteïnes són necessàries pel procés de reducció dels nucleòtids (Apartat 1.1.3.) i, a través del sistema donador d'electrons (tioredoxines o glutaredoxines (Apartat 1.1.4.), s'oxiden i permeten realitzar més cicles de reducció de NDP. A més, existeixen altres residus importants que interaccionen amb els grups OH de la ribosa posicionant el substrat: Glu 441 i Asn 437.

El lloc d'unió dels efectors (dNTP) per a la regulació al·lostèrica de l'especificitat es troba en la zona de dimerització de les subunitats NrdA (o *loop* 2), a prop del centre actiu (Figura 2). La conformació del *loop* 2 varia segons si s'uneix un efector o un substrat, per tal de regular l'activitat de l'enzim. La unió de l'efector al lloc de regulació de l'especificitat estabilitza els *loops* (altament flexibles) per permetre la interacció de la RNR amb el substrat.

En la regió N-terminal hi ha situada una regió hèlix α que pertany al domini de la regulació al·lostèrica de l'activitat, anomenat també *ATP-cone*, per tal de regular l'activitat general de l'enzim (Figura 2) [4]. En canvi, en la part C-terminal, la subunitat R1 es troben localitzades dos residus tirosines (Tyr 730 i Tyr 731), que permetran la transferència dels dos electrons provinents dels donadors externs d'electrons (les tioredoxines o glutaredoxines, Apartat 1.1.4.), cap a la zona activa de l'enzim per reduir les Cys 225 i Cys 462, necessaris per duu a terme un altre cicle de reducció de nucleòtids [34].

La subunitat petita o R2 (NrdB) de la RNR de classe Ia d'*E. coli* (375 aa i 43.4 kDa) està formada per 12 hèlixs α i 2 fulles β i conté un centre metàl·lic de ferro. En aquesta subunitat es forma el radical tirosil estable en la tirosina Tyr122. Aquest radical es troba molt a prop del centre metàl·lic difèrric situat en un ambient molt hidrofòbic [35].

L'estructura quaternària de l'enzim de la RNR de classe Ia d'*E. coli* predominant és la forma homodimèrica ($\alpha_2\beta_2$). La dimerització es dóna en una zona no conservada hidrofòbica d'hèlix α de les dues subunitats de NrdA quan s'uneix l'ATP al centre de regulació de l'activitat de l'enzim [35]. En canvi, la unió de dATP produeix la inhibició general de l'enzim, per l'oligomerització de les subunitats en una estructura $\alpha_4\beta_4$ provocant la pèrdua de l'estructura activa ($\alpha_2\beta_2$) [36-38].

L'estructura quaternària de la RNR de classe Ib és també homodimèrica ($\alpha_2\beta_2$) i va ser estudiada a *S. typhimurium* [39, 40]. La subunitat catalítica de l'enzim és NrdE o R1E (720 aa, 80.5 kDa), i la subunitat encarregada de generar el radical és NrdF o R2F (319 aa, 36 kDa). El centre metàl·lic de la subunitat R2F està format per Mn^{III}-O-Mn^{III}, tot i que, també es pot trobar un centre metàl·lic de Fe^{III}-O-Fe^{III} [20]. L'estructura del centre actiu de la RNR de classe Ib està situat en la subunitat R1E i és molt semblant a l'estructura tridimensional del centre actiu de la subunitat R1A

de la RNR de classe Ia d'*E. coli*. En aquesta classe de RNR, el centre de regulació al·lostèrica de l'especificitat es troba conservat però, en canvi, no conté el centre de regulació al·lostèrica de l'activitat.

Per últim, en la RNR de classe Ic, descoberta recentment a *C. trachomatis*, la subunitat catalítica (NrdA) de 119 kDa (1047 aa), conté 33 kDa (286 aa) més que la subunitat catalítica de classe Ia d' *E. coli* [13, 41]. Aquests aminoàcids addicionals corresponen a la seqüència Val-X-Lys-Arg-Asp-Gly, que pertany a una duplicació del lloc al·lostèric de l'activitat (*ATP-cone*). La subunitat NrdB d'aquesta classe de RNR es caracteritza per tenir un centre metàl·lic de Mn^{IV}-O-Fe^{III} i una substitució de la tirosina Tyr122 de la RNR de classe Ia per una fenilalanina [42, 43].

1.1.2.2. La RNR de classe II

L'estructura tridimensional de la RNR de classe II o NrdJ (738 aa i 81.9 kDa) va ser estudiada per primera vegada en *L. leichmanni* [29]. En aquest microorganisme, de manera atípica, l'enzim RNR de classe II és actiu en forma monomèrica (α). En canvi, en altres microorganismes com ara *Thermotoga maritima* o en *Thermoplasma acidophila*, la forma activa és dimèrica (α_2) (Figura 3) [15, 29, 30]. La dimerització dels dos monòmers es dóna de manera similar a la dimerització que trobem en les RNRs de classe I.

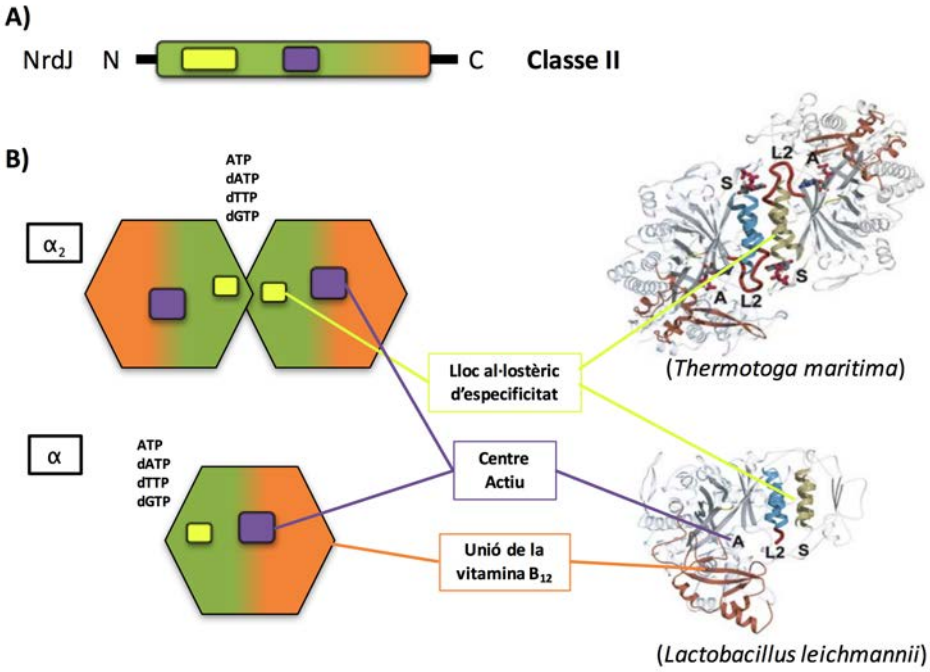

Figura 3: Esquema i dibuix dels dominis de la RNR de classe II de *T. maritima* (dimèrica) i *L. leichmannii* (monomèrica). A) Estructura lineal i B) Estructura tridimensional. En verd es troba representada la subunitat catalítica, en la qual es localitza: el centre actiu, en lila, i el lloc de regulació al·lostèrica de l'especificitat (*loop 2*), en groc. La zona d'unió de la vitamina B_{12} es troba en taronja. (Figura adaptada de [15]).

L'estructura tridimensional del domini central de la RNR de classe II està composta per 10 fulles α/β en forma de barril, semblant al que trobem en la RNR de classe Ia i en la RNR de classe III. En el centre actiu de la RNR de classe II de *L. Leichmannii* també trobem tres cisteïnes involucrades en la catàlisi; Cys 119, Cys 408 i Cys 419, corresponents a les cisteïnes Cys 225, Cys 439 i Cys 462 de la subunitat R1 de la classe Ia d' *E. coli.* Aquests residus de cisteïna del centre actiu de la classe II, interaccionen amb l'AdoCbl o Vitamina B_{12} generant un radical transitori (radical desoxiadenosil) per captar l'hidrogen i produir un seguit de canvis conformacionals que permetran la reacció catalítica de l'enzim [29, 44]. El domini d'unió a la vitamina B_{12}, situat en C-terminal, segueix un motiu estructural Asp-X-His-XX-Gly de fulles β i d'hèlix α, diferent al domini d'altres proteïnes dependents de la vitamina B_{12} [45, 46]. La RNR de classe II utilitza les cisteïnes Cys 731 i Cys 736 per interaccionar amb els donadors externs d'electrons o redoxines.

En la part N-terminal de la proteïna, es troba situat el centre de regulació al·lostèrica de l'especificitat de la RNR de classe II. La unió del substrat o de l'efector en aquesta regió produeix un canvi conformacional de la proteïna de manera específica per reduir un ribonucleòtid [30]. A diferència de les altres classes de RNR, majoritàriament, la RNR de classe II no conté el domini de regulació de l'activitat en la part N-terminal. En canvi, excepcionalment, *Thermoplasma acidophilum* conté dos llocs al·lostèrics, un d'especificitat i un altre d'activitat, tot i així, altes concentracions de dNTP no inhibeixen l'enzim [15, 25].

1.1.2.3. La RNR de classe III

L'estructura quaternària de la RNR de classe III és $\alpha_2 + \beta_2$, sent la subunitat α (NrdD) l'encarregada de la catàlisi i la subunitat β (NrdG) una *activasa*, que activa la subunitat NrdD (Figura 4). La primera estructura identificada i estudiada de la RNR de classe III va ser a *E. coli*. Tot i així, una altra RNR de classe III molt estudiada, i detallada a continuació, és la RNR del bacteriòfag T4, que comparteix una similitud de 70 % amb la RNR de classe III d'*E. coli* [31, 47].

La proteïna NrdD de la classe III del bacteriòfag T4 (T4NrdD, 608 aa i 618 kDa) té la mateixa estructura tridimensional del centre actiu que la NrdA de la classe I i la NrdJ de la classe II, tot i així, comparteixen tan sols un 10 % de l'estructura primària [10, 31]. A més, la zona de dimerització també és diferent a la que trobem a la resta de classes en la RNR, com també ho és el lloc de regulació d'especificitat, que es troba més allunyat del centre actiu. Aquestes diferencies vénen donades per una rotació de 90º de les dues subunitats de la RNR de classe III. En general, les RNRs de classe III comparteixen una alta homologia amb altres enzims com; el piruvat format liasa (PFL), el glicerol deshidratasa (GD), el 4-hidroxifenilacetat descarboxilasa (4-HPAD) i el

benzilsuccinat sintasa. Tots aquests enzims comparteixen amb la RNR de classe III un radical glicil altament sensible a l'oxigen i essencial per l'activitat de l'enzim [48-50]. El radical glicil es troba localitzat en la part C-terminal de la subunitat NrdD (en l'aminoàcid Gly 580 del bacteriòfag T4 i en Gly 681 d'*E. coli*) [51, 52]. En l'estructura del centre actiu la RNR de classe III del bacteriòfag T4 conté dues cisteïnes conservades, la Cys 290 i la Cys 90, corresponents a la Cys 439 i Cys 225 de la subunitat R1A de la RNR de classe Ia d' *E. coli.* A diferència de la Cys 462 d'*E. coli* de la subunitat R1A, en el bacteriòfag T4, trobem una asparagina, Asn 311, que interaccionarà amb el format, com a donador d'electrons [47].

La subunitat petita o NrdG, anomenada també *activasa* (156 aa, 34 kDa), conté un clúster metàl·lic de 4Fe-4S que junt amb la S-adenosilmeteonina (SAM) i NADPH, genera el radical glicil en NrdD [53, 54]. Aquest clúster de 4Fe-4S es destrueix en presència d'oxigen, ja que s'oxida provocant el trencament de la cadena polipeptídica. Per tant, en condicions aeròbies no es podrà formar el radical glicil i la subunitat NrdD perdrà l'activitat [55-57]. A més, existeixen 4 cisteïnes (Cys543, Cys546, Cys562 i Cys564) formant un clúster Cx2C-Cx2C en la subunitat NrdD del bacteriòfag T4 que són essencials per mantenir l'estructura i el clúster de 4Fe-4S de la subunitat NrdG [47].

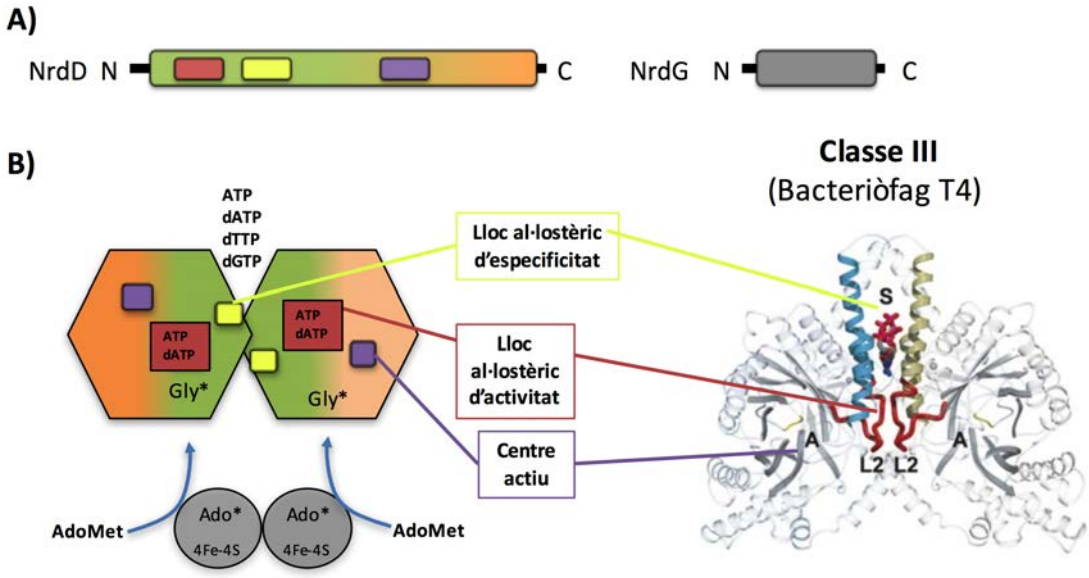

Figura 4: Esquema i dibuix dels dominis de la RNR de classe III del bacteriòfag T4. La subunitat α (NrdD) i la subunitat β (NrdG) es troben representades esquemàticament, tant de forma lineal (A) com tridimensional (B). En la subunitat catalítica NrdD, la part N-terminal es troba representada en verd, i en taronja, la C-terminal. La subunitat *activasa* o NrdG es troba en gris. El centre actiu es troba en lila, el lloc de regulació al·lostèrica d'especificitat (*loop 2*) es troba en groc, i el lloc regulació al·lostèrica es troba en vermell (Figura adaptada de [15]).

Recentment, una segona estructura de la RNR de classe III va ser determinada en *Thermotoga maritima* (TmNrdD). Aquesta estructura, sorprenentment, no conté la cisteïna conservada (Cys 290)

sinó una isoleucina (Ile359) [58]. A més, la seqüència on la cisteïna del bacteriòfàg T4 inicia la reacció es troba lluny del centre actiu a *T. maritima*. Tot i així, TmNrdD s'uneix al substrat i als efectors amb una conformació semblant a la que es troba en les altres RNR de classe III. Els autors han proposat una nova subunitat anomenada NrdDIIIh o bé, l'existència d'un nou mecanisme d'activació. Paral·lelament, l'estructura identificada mitjançant cristal·lografia de la subunitat NrdD a *Neisseria bacilliformis* (NbNrdD) mostra una identitat del 30% amb la subunitat de TmNrdD. A diferència de TmNrdD i T4NrdD, NbNrdD conserva les tres cisteïnes en el centre actiu i utilitza tioredoxines en lloc de format com a donadors d'electrons (Apartat 1.1.4.), com a la RNR de classe Ia d'*E. coli* [27].

1.1.3. Activitat catalítica de l'enzim

El mecanisme catalític de les RNRs es pot dividir en dos processos:

1) El procés de generació del radical (depèn del radical tirosil de la RNR de classe I, del cofactor vitamina B_{12} de la RNR de classe II o del radical glicil de la RNR de classe III en absència d'oxigen).
2) El procés de reducció del ribonucleòtid (en el centre actiu de la subunitat catalítica de les tres classes de RNR).

La reacció de reducció dels ribonucleòtids és semblant per a totes les classes de RNR, ja que conserven la cisteïna encarregada d'acceptar el radical (Cys439 en la RNR de classe Ia *d'E. coli*, Cys408 en la RNR classe II de *L. leichmannii* i Cys290 en la RNR de classe III del bacteriòfag T4). Per tant, les diferències del mecanisme catalític entre les diferents classes de RNR, es troben a l'inici de la catàlisi o en la generació del radical.

A continuació es detallen els dos processos de la reacció de les classes de RNR.

Procés de generació del radical

La generació del radical de la RNR de classe Ia es produeix en la subunitat NrdB. Aquesta subunitat conté un residu tirosina (radical tirosil), que en presència d'oxigen en el medi, genera el radical tiil, en el residu Tyr122 [59]. L'oxigen permet reduir el centre metàl·lic di-fèrric Fe^{III}-O-Fe^{III} i generar una cadena d'electrons fins interaccionar el radical tiil amb la cisteïna Cys439 de la subunitat R1 i doncs, iniciar la reacció de reducció dels nucleòtids.

El procés de generació del radical en la RNR de classe Ib és el mateix que l'utilitzat en la RNR de classe Ia d'*E. coli*, a diferència del centre metàl·lic de la subunitat NrdF, que pot ser Mn^{III}-O-Mn^{III} o Fe^{III}-O-Fe^{III} en presència d'oxigen [39]. Específicament, en el mecanisme catalític de la RNR de classe Ib, hi participen dues proteïnes més, NrdI i NrdH, que reduiran la RNR [4]. NrdI és

una flavodoxina que interacciona amb NrdF i permet l'oxidació del centre metàl·lic [60, 61], transferint dos electrons, si el centre metàl·lic és de Mn^{III}, o un electró, si és de Fe^{III}.

La classe Ic es diferència de la resta perquè el radical que es genera en la subunitat NrdB no és un tirosil sinó un radical en el residu de fenilalanina (Phe127 en *C. trachomatis*), una vegada oxidat el centre metàl·lic de Mn^{IV}-Fe^{III} [43].

La RNR de classe II inicia la reacció de catàlisi utilitzant el cofactor 5' deoxiadenosilcobalamina (AdoCbl)/vitamina B_{12} per generar el radical tiil en el centre actiu. Aquesta classe de RNR no codifica cap subunitat R2 com la RNR de classe I, sinó que la AdoCbl s'uneix al centre actiu de la RNR de classe II, en un motiu molt conservat, per generar el radical. La ruptura homolítica de l'enllaç entre el cobalt i el carboni de la deoxiadenosilcobalamina produeix un radical 5'-desoxiadenosil que donarà lloc al radical tiil en el centre actiu [23, 29, 30, 44]. El radical tiil interaccionarà amb la cisteïna conservada de la subunitat NrdJ i amb les cisteïnes que interaccionen amb el sistema de tioredoxines i glutaredoxines, per tal de reduir el ribonucleòtid.

El procés de generació del radical proteic de la RNR de classe III, situat en la subunitat NrdD, requereix d'un sistema de reducció compost per: la subunitat NrdG o *activasa*, el sistema flavodoxina (NADPH, flavodoxina i flavodoxina reductasa), i la S-adenosilmetionina [53]. Inicialment, en condicions anaeròbies estrictes, el clúster de 4Fe-4S de la subunitat NrdG és reduït pel sistema flavodoxina. Posteriorment, l'electró provinent de la flavoredoxina s'uneix a la S-adenosilmetionina i la trenca formant una metionina i un radical 5'deoxiadenosil. Finalment, el radical 5'desoxiadenosil format, generarà el radical glicil en la NrdD (Gly580 en bacteriòfag T4 o Gly681 a *E. coli*) [54]. La subunitat NrdG tan sols transfereix l'electró de la flavoredoxina a la S-adenosilmeteonina, per aquest motiu se l'anomena *activasa*. Una vegada format el radical glicil en la RNR de la classe III, el mecanisme catalític en NrdD del bacteriòfag T4 és bastant similar al que es realitza en la RNR de classe I i II.

Procés de reducció del ribonucleòtid

L'enzim RNR de classe I redueix els ribonucleòtids difosfats (NDP) a desoxiribonucleòtids difosfats (dNDP). La classe III només redueix ribonucleòtids trifosfats (NTP) a desoxiribonucleòtids trifosfats (dNTP). En canvi, la classe II pot reduir ribonucleòtids difosfats o trifosfats (NDP o NTP) a desoxiribonucleòtids difosfats o trifosfats (dNDP o dNTP) segons l'organisme.

El mecanisme de la reducció dels nucleòtids s'ha estudiat amb més detall en la RNR de classe Ia d'*E. coli*. En aquesta reacció, tots els quatre ribonucleòtids mantenen la mateixa posició en el centre actiu de NrdA per a ser reduïts. Principalment, els NDPs es redueixen a dNDPs mitjançant la

transferència d'electrons en tres cisteïnes conservades de la subunitat R1 (Cys 439, Cys 225 i Cys 462) per forces Van der Waals [62].

En la RNR de classe Ia, la reducció de ribonucleòtids s'inicia quan el centre metàl·lic di-fèrric (Fe^{III}-O-Fe^{III}) de la subunitat R2, en presència d'oxigen, produeix un radical tiil (–S) transitori (en la Tyr 122) que interacciona amb la cisteïna conservada de la subunitat R1 (Cys 439) (procés de generació del radical) [59, 63]. Aquesta cisteïna reduïda (Cys 439) interacciona amb l'àtom d'hidrogen del carboni 3 (C3) de la ribosa del nucleòtid, i comença el procés de reducció (Figura 5). Seguidament, el carboni 2 (C2) de la ribosa capta un hidrogen de la cisteïna de la posició més propera a la ribosa, la Cys 225. La cisteïna distal, Cys 462, finalment, redueix el carboni 3 (C3) i es genera un pont disulfur S-S entre les dos cisteïnes, Cys 225 i Cys 462. En la reacció s'allibera una molècula d'H_2O [15, 20, 64].

La reducció del nucleòtid en la RNR de classe II és similar a la reducció explicada anteriorment en la classe Ia d'*E. coli*.

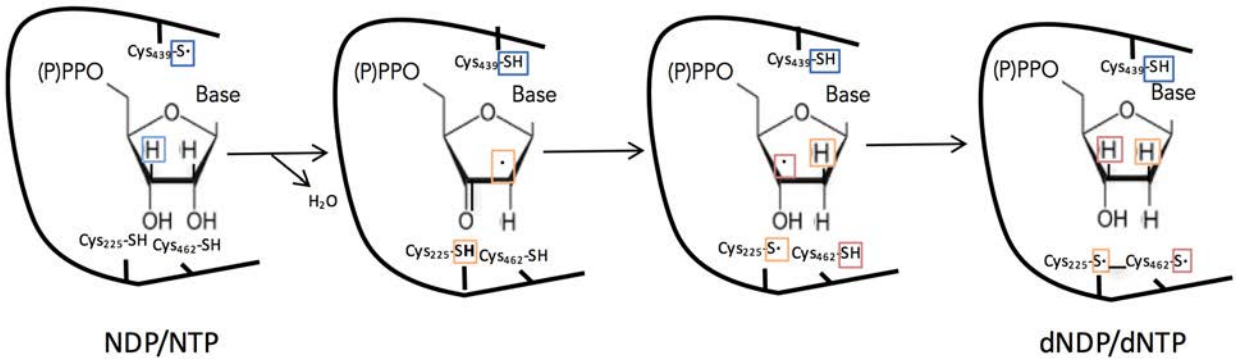

Figura 5: Esquema del mecanisme de reducció del nucleòtid de la RNR de classe I. Es troben assenyalats aquells àtoms que participen en cada pas de la reacció (Figura adaptada [65]).

A diferència de la reducció de nucleòtids de la RNR classe Ia *d'E. coli* i de la RNR de classe II, el centre actiu de NrdD en el bacteriòfag T4 es troba definit per dues cisteïnes conservades, Cys 290 i Cys 79 (Cys 225 d'*E. coli*). Tot i així, principalment la cisteïna Cys 290 és l'encarregada de generar el radical i la Cys 79, més proximal, reacciona amb l'hidrogen situat al C3 de la ribosa alliberant una molècula d'aigua. Existeix una altra RNR de classe III, la RNR de NrdDIIIh, a *T. marítima*, que en el centre actiu conté una isoleucina enlloc de la cisteïna conservada (Cys 290) [58]. La unió del substrat és estructuralment similar a les RNRs de classe I i II.

El procés de reducció de ribonucleòtids es podrà reproduir en el mateix enzim gràcies al sistema donador d'electrons que reduirà les cisteïnes involucrades en la reacció.

1.1.4. Sistemes donadors d'electrons

El sistema de donador d'electrons de les RNRs permeten reduir les cisteïnes conservades del centre actiu per tal de catalitzar un altre cicle de reducció dels ribonucleòtids. Aquest poder reductor, principalment el proporciona un sistema de reductases tiol-depenent constituït per unes proteïnes petites (9-16 kDa), anomenades tioredoxines (Trx) i glutaredoxines (Grx), que reduiran el grup disulfit (S-S) de les cisteïnes del centre actiu de les RNRs [66]. Tant les tioredoxines com les glutaredoxines, contenen un motiu conservat CXXC en el centre actiu [67, 68]. La principal diferència entre les tioredoxines i les glutaredoxines ve donada per la manera de reduir-se: mentre que les tioredoxines ho fan directament mitjançant una tioredoxina reductasa (TrxR) i el NADPH, les glutaredoxines ho fan via la proteïna glutatió reductasa (GSH), glutatió i NADPH [28].

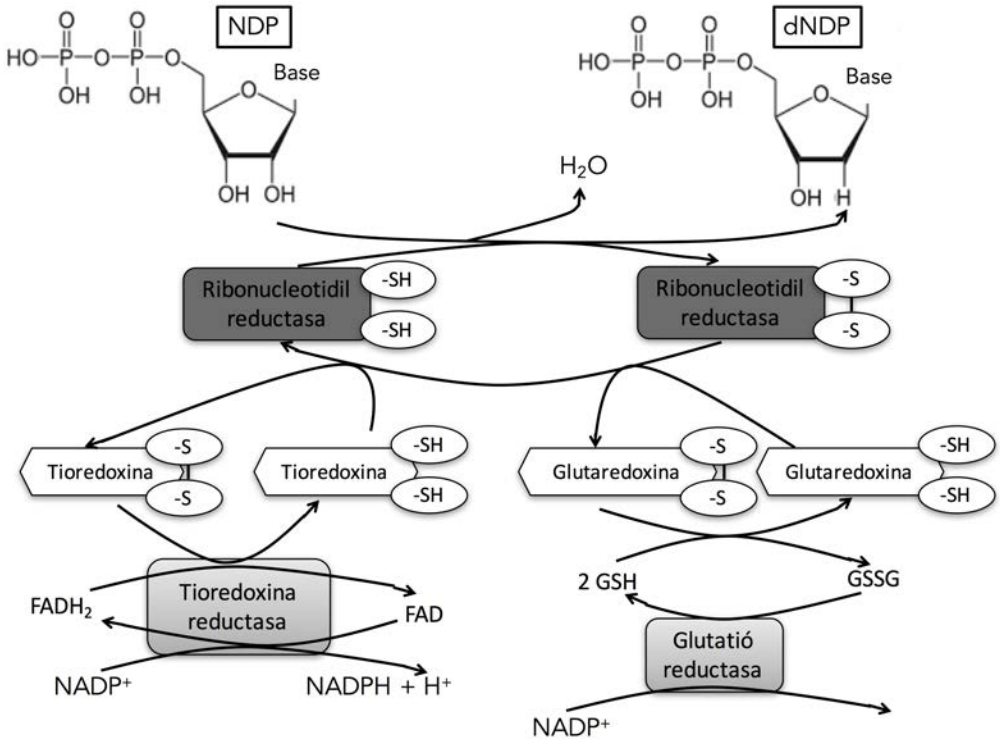

Figura 6: Mecanisme d'acció de les tioredoxines i glutaredoxines en el procés de reducció de les cisteïnes de les RNRs. (Figura adaptada de [69]).

La tioredoxina va ser la primera proteïna identificada com a reductant de les ribonucleotidil reductases a *E. coli* [70, 71]. El sistema de tioredoxines participa en diferents processos del metabolisme cel·lular [72]. Aquest sistema està constituït per tioredoxines (Trx), tioredoxines reductasa (TR) i NADPH. La reacció consisteix en reduir les tioredoxines utilitzant la tioredoxina reductasa a través del NADPH, el qual permetrà la reducció de les cisteïnes de les RNRs (Figura 6).

El sistema de glutaredoxines està constituït per les glutaredoxines (Grx), el glutatió (GSH), la glutatió reductasa (GR) i NADPH. La seva funció principal també és la reducció de les RNRs. En

aquest cas, la glutatió reductasa, a través de NADPH, redueix el glutatió, el qual transfereix el poder reductor a la glutaredoxina i aquesta reduirà les RNRs (Figura 6).

E. coli codifica per dues tioredoxines (Trx1 i Trx2), tres glutaredoxines (Grx1, Grx2 i Grx3) i dues glutaredoxines-like (Grx4 i NrdH), encara que, la Trx1 és la preferent per a la reducció de la RNR de classe Ia d'*E. coli*. La reducció de les tioredoxines és menys eficient, unes 8 vegades inferior que les glutaredoxines [73], tot i així, les Trx també poden actuar com a donadores d'electrons en proteïnes importants contra l'estrès oxidatiu [74]. També, a *E. coli*, l'operó de la RNR de classe Ib codifica a més de NrdE i NrdF, una flavoredoxina (NrdI) i una glutaredoxina (NrdH) essencials per a l'activitat d'aquesta classe. La NrdH és una glutaredoxina pròpia de la RNR de classe Ib que segueix el domini conservat CXXC [19].

La reducció de la RNR de classe II utilitza tant les tioredoxines com les glutaredoxines [75]. En canvi, la RNR de classe III principalment es redueix a través del format, encara que en alguns organismes, com *N. bacilliformis*, utilitzen les tioredoxines com a reductants de la RNR de classe III (ja que no codifiquen per els enzims encarregats de la síntesi del format) [27]. El format, a *E. coli* i *L. lactis,* s'obté a través de la fermentació de sucres utilitzant la piruvat-format liasa (PFL) [76].

A nivell catalític, s'han descrit tres subtipus de RNR de classe III en relació amb el seu metabolisme (NrdD1, NrdD2 i NrdD3) [27]. NrdD1 (*L. lactis, E. coli* i T4) es troba distribuïda en microorganismes fermentatius que utilitzen el format a través de PFL. NrdD2 es troba en microorganismes amb un metabolisme anaeròbic ampli (*N. bacilliformis i T. maritima*) i obté el poder reductor de la tioredoxina que interacciona amb les tres cisteïnes i un àcid glutàmic. Per últim, NrdD3, situat en bacteris metanògens, conté les tres cisteïnes però no l'àcid glutàmic, i no utilitza el sistema de tioredoxines ni format, doncs, el mecanisme reductor de NrdD3 no està encara definit. Alguns bacteris codifiquen la RNR de classe III amb diferents mecanismes de reducció (NrdD1, NrdD2 o NrdD3) tot i així, cada mecanisme s'utilitzarà específicament segons les condicions de creixement del microorganisme.

1.1.5. Regulació al·lostèrica de l'enzim

La regulació al·lostèrica de les RNRs permet que una sola proteïna sigui capaç de reduir els quatre NDP/NTP diferents (A, T, C, G) a dNTP/dNDP en un mateix centre actiu, segons les necessitats de la cèl·lula, i mantenir els nivells de dNTP de manera equilibrada. Aquesta regulació va ser estudiada en la subunitat R1 d'*E. coli* l'any 1969 [77], i ve donada a dos nivells diferents; a nivell de l'especificitat, regulant l'especificitat al substrat, i a nivell de l'activitat, regulant l'activitat

general de l'enzim. La regulació dependrà de la unió d'efectors o substrats, ja que tant la unió dels nucleòtids efectors, com la dels substrats, indueixen canvis conformacionals diferents. La proteïna adaptarà l'estructura per poder reduir un substrat determinat donant lloc a una catàlisi enzimàtica específica en el centre actiu [15, 30].

El lloc al·lostèric de l'especificitat es va estudiar en la subunitat R1A d'*E. coli* i posteriorment, es va trobar una regulació similar en les RNRs de classe II i III. Tots els efectors mostren un patró d'unió similar formant ponts d'hidrogen entre l'hèlix αβ i el *loop* 2 [30]. Aquest *loop* 2 flexible (en els residus 292-301 d'*E. coli*) permet la unió de l'efector al centre de l'especificitat i al centre actiu. Els diferents efectors que s'uneixen són dGTP, dTTP, ATP i dATP, i permetran un canvi conformacional específic per a un substrat concret. Tot i així, el canvi conformacional del lloc al·lostèric de l'especificitat es donarà tan sols quan s'uneixi tant l'efector com el substrat, de manera específica. Així doncs, la unió d'ATP o de dATP permetrà la reducció dels substrats CDP i UDP, la unió de dGTP reduirà ADP, i la unió de dTTP, reduirà el substrat GDP [77]. En la RNR de classe I i II els complexes dTTP-GDP, dGTP-ADP, ATP/dATP-UDP, ATP/dATP-CDP donen lloc a un canvi conformacional semblant. Però, en la RNR de classe III del bacteriòfag T4, el lloc d'unió de l'efector es troba a una distància més llunyana del centre actiu realitzant un canvi conformacional diferent [15, 78].

La unió dels efectors a les RNRs afecta també a l'afinitat entre subunitats R1 (α_2) i R2 (β_2) de la RNR de classe Ia. En absència d'efectors, l'afinitat entre subunitats és baixa (0.4 μM), mentre que la unió de substrat i l'efector específic a les RNRs augmenta l'afinitat entre les subunitats unes cinc vegades [79].

El lloc de regulació de l'activitat o de baixa afinitat, en canvi, només es troba en algunes classes de RNR i permet regular l'activitat general de l'enzim segons la unió d'ATP o dATP, per tal d'obtenir uns nivells dNTP equilibrats en la cèl·lula. Aquesta regió, en l'extrem N-terminal de l'enzim, també se l'anomena domini *ATP-cone* i segueix una seqüència Val-X-Lys-Arg-Asp-Gly. Aquest domini, rarament el trobem en les RNRs de classe II (7%), és més comú en la RNR de classe I (47%) i la majoria es troba en les RNRs de classe III (76%) [79]. Tot i així, la RNR de classe Ib, com la majoria de les RNRs de classe II, no posseeixen aquesta regió *ATP-cone*.

El mecanisme de regulació al·lostèrica de l'activitat de l'enzim consisteix en la unió de dATP a les RNRs induint la inhibició de l'enzim, mentre que l'ATP activa l'enzim. En els organismes eucariotes, el mecanisme de inhibició de l'enzim es dóna mitjançant la formació de dos complexes diferents de la subunitat R1 (α_6) segons si s'uneix ATP o dATP [79, 80]. En ratolí, s'ha demostrat que a altes concentracions de dATP queda afectada la transferència del radical de la subunitat R2 a la subunitat R1 [81]. En organismes procariotes, en canvi, s'utilitza un altre mecanisme de inhibició

general de l'enzim. El mecanisme d'inhibició per dATP a *E. coli*, consisteix en un augment de l'oligomerització de l'enzim (de $\alpha_2\beta_2$ a $\alpha_4\beta_4$) evitant doncs que es produeixi el canvi conformacional de l'enzim per la unió del complex efector-substrat [4, 36, 82] i per tant, no es pot generar la cadena d'electrons correctament entre les subunitats [37]. Tot i així, el dATP té 10 vegades més afinitat de unió al lloc de regulació al·lostèrica de l'especificitat que al de l'activitat, doncs no serà fins arribar a una concentració de dATP molt elevada, quan el dATP actuarà inhibint l'enzim [77, 83].

1.1.6. Regulació transcripcional

Fins ara, s'ha descrit la regulació al·lostèrica de cadascun dels enzims, a nivell enzimàtic, però també existeix una forta regulació a nivell transcripcional, ja que els gens de les diferents classes de RNR s'expressaran de manera diferencial sota diferents condicions de creixement. A més, durant el procés de la replicació de l'ADN és necessari mantenir els nivells de dNTP alts, ja que tots quatre dNTP són els precursors per a la síntesi de la molècula d'ADN. Per tant, durant el procés de replicació i reparació cel·lular dels organismes procariotes i eucariotes es produeix un increment de l'expressió de l'enzim fortament regulat pel cicle cel·lular.

En aquest apartat, s'hi resumeix la regulació transcripcional eucariota i procariota dels gens de les tres classes de RNR i específicament, parlarem de NrdR, com a principal factor transcripcional regulador dels gens *nrd* (Figura 7).

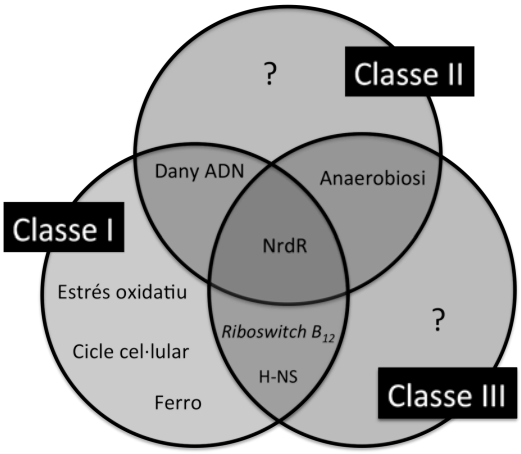

Figura 7: Esquema general dels factors transcripcionals (descrits) que regulen les tres classes de RNR en diferents organismes. (Figura adaptada de [2]).

1.1.6.1. Regulació transcripcional de la RNR de classe I

La regulació transcripcional de la RNR de classe Ia procariota, ha sigut profundament estudiada a *E. coli*. L'operó format pels gens *nrdA* (2286 pb) i *nrdB* (1131 pb) d' *E. coli* es troba sota un promotor actiu en situacions de replicació cel·lular i d'estrès produït pel dany en l'ADN.

Existeix una relació entre l'expressió dels gens *nrdAB* i l'inici de la replicació cel·lular, ja que s'ha vist que la proteïna DnaA, encarregada d'iniciar la replicació cel·lular s'uneix al promotor i regula l'expressió dels gens *nrdAB* [84, 85]. El regulador DnaA-ATP s'uneix a dues caixes consens de 9 pb, situades a -48 i -36 pb des de l'inici de la transcripció de *nrdA* [84, 86, 87]. La proteïna Hda hidrolitza l'ATP unit a DnaA, donant lloc a Dna-ADP, per tal d'iniciar la transcripció de *nrdAB* [88]. El factor de transcripció IciA (*Inhibitor of Cromosome Initiaton*) també s'uneix al promotor *nrdAB* en tres caixes situades a -209, -269 i -334 pb des de l'inici de transcripció, tot i així, la funció principal de IciA és inhibir la replicació de l'ADN [89-92]. FIS és un altre factor de transcripció que produeix l'activació de l'expressió de *nrdAB* de manera depenent del superenrotllament de l'ADN [93]. FIS s'uneix en les regions -156 i -128 bp del promotor de *nrdAB.* En el promotor de *nrdAB,* també s'ha identificat la unió de CRP (*cyclic AMP Receptor Protein*) a la posició -136 pb de l'inici de transcripció, tot i que, actualment no se sap el seu paper regulador [94]. Recentment, en el nostre laboratori, s'ha identificat la unió de H-NS (*Histone-like nucleotid-structurating protein)* en el promotor de *nrdAB* d'*E. coli,* situat en -425 pb, encarregat d'inhibir la transcripció de la RNR de classe Ia [95]. També, s'ha identificat el factor de transcripció NrdR com a repressor sobre la RNR de classe Ia i sobre totes les altres classes de RNR d'*E. coli* (*nrdHIEF* i *nrdDG)* [96] (Apartat 1.1.6.4.).

Una estructura que regula l'expressió de la RNR de classe Ia és el *riboswitch.* S'ha vist a *Streptomyces coelicolor* que la vitamina B_{12} s'uneix a la regió 5' UTR (*untranslated region)* regulant de manera post-transcripcional i modulant la traducció de la RNR de classe Ia via B_{12}-*riboswitch* [97].

Tenint en compte que la RNR de classe Ia d' *E. coli* va ser la primera RNR descrita, pocs estudis transcripcionals s'han realitzat per saber la regulació de la RNR de classe Ib i Ic. Tot i així, existeixen organismes que codifiquen per més d'una classe de RNR en el seu genoma, com *E. coli* o *S. typhimurium,* que codifiquen tant per la RNR de classe Ia (*nrdAB)* com Ib *(nrdHIEF)* i la classe III (*nrdDG),* fet que fa pensar que ha d'existir una regulació transcripcional diferencial. L'any 2000, es va trobar que en la regió promotora de la RNR de classe Ib, hi havia una caixa Fur (*Ferric uptake regulator*) en la posició -61 pb [98]. L'absència d'aquest factor de transcripció indueix un augment de l'expressió de la RNR de classe Ib (actuant com a repressor), a més de regular la captació de ferro, la quimiotaxi i virulència [99, 100]. En condicions d'estrès oxidatiu per la presència de H_2O_2, a *E. coli* s'indueix l'expressió de la RNR de classe Ib i, no tant significativament, la RNR de classe Ia [101]. No se sap el mecanisme directe d'inducció però indirectament, s'ha vist que a través de OxyR s'indueix el gen *mntH*, un transportador de manganès [102, 103], que permetrà augmentar els nivells de manganès de la cèl·lula i, doncs l'activitat de la RNR de classe Ib.

Per una altra banda, la RNR de classe Ia eucariota més estudiada és la RNR de ratolí, descrita com a model de mamífer. El gen *nrdA* (R1) es troba codificat en el cromosoma 7, mentre que el gen *nrdB* (R2) es troba en el cromosoma 12. Principalment, la RNR de ratolí (la RNR de classe Ia) es troba regulada pel cicle cel·lular. Els nivells màxims d'expressió es donen durant la fase S del cicle cel·lular, mentre que els nivells més baixos durant la fase G [15, 104]. L'activitat de l'enzim RNR ve determinada per la regulació de l'expressió de *nrdB* tant a nivell transcripcional com post-transcripcional, per degradació enzimàtica, en canvi, l'expressió de *nrdA* és constant durant el procés de divisió cel·lular. La unió del factor de transcripció E2F4 al promotor de la RNR de classe Ia reprimeix la transcripció de R2 o NrdB, mentre que el factor de transcripció E2F l'activa. La subunitat R2 té una vida mitja curta, ja que la proteïna Cdh1 provoca la seva degradació durant la mitosi [105]. A més, el dany a l'ADN indueix la subunitat R2 a través del factor de transcripció p53, per tal de subministrar els dNTP durant la reparació de l'ADN [106-108].

A més, també s'ha estudiat la RNR de classe Ia de llevats, com *Saccharomyces cerevisiae*. A *S. cerevisiae*, la subunitat R1 es troba codificada en dos gens diferents RNR1 (Y1) i RNR3 (Y3). La subunitat R2, també es troba codificada en dos gens diferents, RNR2 (Y2) i RNR4 (Y4). L'estructura activa de la subunitat petita és un heterodímer Y2Y4 (β_2). RNR1 participa en el cicle cel·lular, i tant RNR1 com RNR3, estan regulades per Sml1 quan hi ha dany de l'ADN. El control de l'activitat es dóna també per la proteïna Sml1, que regula negativament els nivells de dNTP si s'uneix a Y1. Mitjançant la degradació de Sml1, les RNRs s'activen i permeten un augment dels nivells de dNTP durant la reparació de l'ADN [15, 109]. Aquests mecanismes reguladors participen també en la replicació de l'ADN cel·lular i mitocondrial.

1.1.6.2. Regulació transcripcional de la RNR de classe II

Poc se sap sobre la regulació transcripcional de les RNRs de classe II. Tot i així, a *P. aeruginosa* s'ha demostrat que l'expressió de *nrdJ* augmenta en fase estacionària i en anaerobiosi [24, 110]. Alguns estudis transcripcionals globals han vist una desregulació de l'expressió del gen *nrdJ* degut a factors com; l'anaerobiosi, l'absència del factor de transcripció AlgR o per dany a l'ADN [111-114]. Tot i així, no s'ha demostrat una interacció directa de la unió de factors transcripcionals al promotor de la RNR de classe II. S'ha suggerit que aquesta classe de RNR participa en la reparació de l'ADN [24], ja que es produeix la inducció del gen *nrdJ* (fins a 15 vegades) per l'addició d'hidroxiurea (radical *scavenger* (Apartat 1.1.8.)). L'hidroxiurea provoca dany a l'ADN, ja que inhibeix la replicació de l'ADN degut a la inhibició de la RNR de classe I.

A *S. coelicolor*, el gen *nrdJ* es troba en el mateix operó que el gen del factor de transcripció *nrdR*. La deleció de *nrdR* indueix un augment de l'expressió de *nrdJ*, sent doncs, NrdR un repressor

de l'expressió de la RNR de classe II a *S. coelicolor* [115]. A més, a *S. coelicolor*, la vitamina $B_{12,}$ s'uneix a una regió *riboswitch* en la classe Ia, en canvi no s'ha trobat cap inhibició post-transcripcional de la vitamina B_{12} sobre el gen *nrdJ* (possiblement degut a que la vitamina B_{12} és essencial per l'activitat de la RNR de classe II) [24, 116].

1.1.6.3. Regulació transcripcional de la RNR de classe III

La RNR de classe III es troba codificada en l'operó format pels gens *nrdDG*. No existeixen gaires estudis sobre la regulació transcripcional de les RNRs de classe III, tot i així, principalment els estudis han estat relacionats amb la pèrdua de l'activitat de l'enzim en presència d'oxigen. És per aquest motiu possiblement, l'expressió de la RNR de classe III estigui fortament relacionada amb la disponibilitat d'oxigen en l'ambient. Alguns estudis realitzats a *E. coli* han demostrat una inducció de la transcripció del gens *nrdDG* en fase estacionària i en condicions anaeròbies [117, 118]. S'han descrit dues caixes d'unió del factor transcripcional FNR (*fumarate and nitrat reduction*) en el promotor de la RNR de classe III (FNR1 i FNR2). La unió de FNR en l'ADN produeix una inducció de la transcripció dels gens *nrdDG* en condicions anaeròbiques. Les caixes FNR segueixen una seqüència consens $TTGA^{T}/_{C}NNNN^{A}/_{G}TCAA$ en la posició -65 pb (FNR1) i -35 pb (FNR2) *up-stream* del promotor [117, 119, 120].

El factor de transcripció NrdR, també participa en la regulació de la RNR de classe III d'*E coli*. Les caixes d'unió de NrdR al promotor del gen *nrdD* es troben a una posició -10 i +1 de l'inici de transcripció. La deleció de *nrdR* indueix un augment de l'expressió de la RNR de classe III fins a 10 vegades [96]. A *E. coli,* la classe III de RNR també es troba regulada per H-NS, inhibint-la en condicions aeròbies (condició en la qual la RNR de classe III no és funcional), i augmenta l'expressió en condicions anaeròbies [95].

1.1.6.4. El factor transcripcional NrdR

El factor de transcripció NrdR va ser caracteritzat per primera vegada a *S. coelicolor* l'any 2004 com un regulador específic dels gens *nrd* [115]. A *S. coelicolor*, el gen *nrdR* es troba co-transcrit amb el gen *nrdJ* (RNR de classe II). En canvi, en els proteobacteris, com ara *E. coli o P. aeruginosa,* el gen *nrdR* es troba codificat en l'operó dels gens de la síntesi de la riboflavina.

Aquest factor de transcripció és una proteïna d'uns 150-200 aminoàcids, altament conservada i codificada tan sols en organismes procariotes [121]. Conté dos dominis; el domini N-ternimal o *Zinc finger,* que permet la unió a l'ADN en les caixes NrdR del promotor, i un domini C-terminal o *ATP-cone,* on es suggereix que s'uneix ATP o dATP per regular la unió a les caixes NrdR dels

promotors (Figura 8), fet que fa pensar que NrdR respongui als nivells intracel·lulars de NTP i dNTP com un sensor [122, 123].

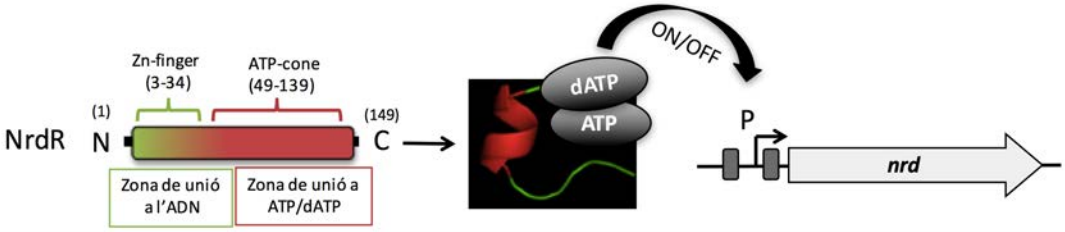

Figura 8: Esquema dels dominis del factor de transcripció NrdR *d'E. coli* i la seva hipotètica regulació al·lostèrica per la transcripció dels gens *nrd* (Figura adaptada de [124]).

L'any 2005, van ser identificades les possibles caixes d'unió de NrdR als promotors dels gens de les tres classes de RNR en diferents organismes [125]. Es van definir dues caixes palindròmiques anomenades *NrdR-box* en els promotors de les tres classes de RNR de diferents organismes, les quals es troben separades per 16 bp i segueixen la seqüència consens: acaCwAtATaTwGtg [125]. Fins al moment, s'ha vist que la regulació dels gens *nrd* per la unió de NrdR provoca una repressió de totes les classes de RNR. Aquesta regulació de NrdR, s'ha demostrat tant a *S. coelicolor* (*nrdAB* i *nrdJ)* [115], com a *E. coli* (*nrdAB, nrdHIEF* i *nrdDG)* [96], *C. trachomatis* (*nrdAB*) [126] o a *S. typhimurium* (*nrdAB, nrdHIEF* i *nrdDG)* [127], però mai en organismes on hi són codificades les tres classes en un mateix organisme (com a *P. aeruginosa*).

Recentment, s'ha vist que aquest factor de transcripció pot regular, de manera indirecta, a altres gens, com ara els gens responsables de la quimiotaxi i motilitat [128, 129]. A més, s'ha vist que la sobreexpressió de NrdR pot afectar a l'adhesió a les cèl·lules epitelials humanes [128].

S'hipotetitza, que NrdR reprimeix l'expressió dels gens *nrd* segons els nivells de NTP i dNTP de la cèl·lula [122]. A *S. coelicolor*, la unió d'ATP o dATP modifica l'estat d'oligomerització de NrdR, i tan sols la proteïna serà activa quan es troba formant un octàmer [122]. L'estructura de la zona d'unió a ATP/dATP de NrdR és molt semblant al sistema de regulació al·lostèric de l'activitat que trobem en les diferents classes de RNR. Tot i així, el sistema *ATP-cone* de NrdR es troba constituït per una llarga estructura hèlix α i posseeix dues tirosines, Tyr 121 i Tyr 128, absents en el domini *ATP-cone* de NrdA, però en canvi, no conté les histidines His 59 i His 88 de la RNR de classe Ia [122].

Un estudi recent ha estudiat la unió de diferents nucleòtids a NrdR com a resposta als nivells de nucleòtids de la cèl·lula, proposant que tan sols els desoxiribonucleòtids monofosfats units a NrdR seran capaços de regular els gens *nrd* mitjançant la unió de NrdR als promotors [130]. En aquest estudi, es demostra que l'activitat de NrdR depèn del nombre de fosfats en els nucleòtids, tenint més afinitat d'unió els monofosfats que els trifosfats. Tot i així, calen més estudis que descriguin el

domini d'*ATP-cone* d'aquest factor de transcripció i si afecta la regulació transcripcional dels gens *nrd*.

1.1.7. Distribució i evolució

La distribució de les diferents classes de RNR en els organismes és variada, ja que podem trobar més d'una classe funcional en un mateix organisme. La classe de RNR codificada en el genoma té relació amb el nivell d'adaptabilitat a diferents condicions ambientals, ja que un organisme que codifica per més d'una classe de RNR, podrà adaptar-se a més d'una condició ambiental. El fet de codificar la RNR de classe III permetrà la divisió cel·lular en condicions anaeròbies, codificar la RNR de classe II facilitarà la divisió cel·lular durant la transició entre condicions d'aerobiosi i d'anaerobiosi, però estarà limitat per la disponibilitat de la vitamina B_{12} (cofactor). En canvi, la RNR de classe I només serà funcional en organismes que creixin en condicions on l'oxigen sigui present.

Taula 2: Distribució de les diferents classes de RNR en diferents organismes.

	Classe I	Classe II	Classe III
Eucariotes			
Arabidopsis thaliana	Ia		
Homo sapiens	Ia		
Mus musculus	Ia		
Saccharomyces cerevisiae	Ia		
Euglena gracis	Ia	II	
Dictyostelium discoideum	Ia	II	
Virus			
Herpes simplex virus	Ia		
Arquees			
Natromonas pharaonis	Ia	II	
Pyrococcus furiosus		II	III
Procariotes			
Aeromonas hydrophila	Ia	II	III
Bacillus subtilis	Ib		
Burkholderia pseudomallei	Ia	II	III
Campylobacter jejuni	Ia		
Chlamydia trachomatis	Ic		
Escherichia coli	Ia Ib		III
Mycobacterium tuberculosis	Ib	II	
Pseudomonas aeruginosa	Ia	II	III
Salmonella typhimurium	Ia Ib		III
Staphylococcus aureus	Ib		III
Streptococcus pyogenes		II	III
Streptomyces coelicolor	Ia	II	

Els organismes eucariotes complexes tan sols codifiquen per una sola classe de RNR, la classe Ia, limitats per les condicions ambientals aeròbiques. Tot i així, hi ha organismes eucariotes que codifiquen per la RNR de classe II, com *Euglena gracilis* i *Dictyostelium discoideum* [131, 132] (Taula 2). En canvi, en el regne bacterià o arquea, existeix qualsevol combinació de RNR [8, 9]; *Bacillus subtilis* codifica la RNR de classe Ib, *Staphylococcus aureus* codifica les RNRs de classes Ib i III o inclús, *P. aeruginosa* codifica per les tres classes de RNR, la classe Ia, II i III [133].

Evolutivament, es creu que l'ARN era la molècula on s'emmagatzemava la informació genètica en els primers éssers vius. En la transició del món d'ARN al món d'ADN va ser necessari la presència tant de ribosomes (traducció) com d'una molècula per sintetitzar dNTP per permetre la síntesi d'ADN [65, 134]. Tot i així, existeixen vàries hipòtesis sobre l'evolució de l'enzim RNR. Les condicions de l'atmosfera de la Terra primitiva fan pensar que la primera RNR que va existir va ser una RNR que no necessités oxigen per funcionar. Probablement, va existir una hipotètica proteïna reductasa anaeròbica (anomenada "ur-RNR") per tal de sintetitzar l'ADN [135]. És per aquest motiu que es creu que la RNR de classe III és la més primitiva, de la qual van divergir les RNRs de classe I i II [10, 31, 65, 136]. La majoria dels autors mantenen aquesta hipòtesi de divergència, ja que existeix molta similitud a nivell de regulació al·lostèrica, mecanisme catalític i estructura tridimensional entre les diferents classes [31]. A més, existeixen una sèrie de raons que estableixen la RNR de classe III com la més ancestral de la qual van divergir la resta [10, 82, 137]: el clúster Fe-S està relacionat amb el metabolisme primari [138], la S-adenosilmetionina i Fe-S són necessaris en molts enzims essencials [139], hi ha més abundància de Fe que de Co, la simplicitat de síntesi S-adenosilmetionina respecte a la síntesi de l'adenosilcobalamina [137], la classe III té una estructura i funció similar amb PFL (piruvat fosfat liasa) [48] i s'utilitza el format (producte de la reacció PFL) per a la reducció dels ribonucleòtids.

Tot i així, encara que hi hagi arguments a favor de la classe III com la RNR més ancestral, també hi ha investigadors que creuen que les tres classes podrien haver divergit de la RNR de classe II, ja que conserven un centre actiu comú [29, 65]. O inclús, podria existir una altra hipòtesi, que defensaria que cada classe podria haver sorgit de manera independent, per convergència. Aquesta hipòtesi sorgeix a partir de la poca similitud en la seqüència primària que comparteixen les tres classes de RNR, només 10-20%.

1.1.8. Inhibició de les RNRs

Les RNRs són enzims essencials per a la divisió de tota cèl·lula, per tant, les RNRs són una diana clau per a la inhibició de la divisió cel·lular. Els compostos dissenyats que bloquegen aquesta

reacció s'han utilitzat per evitar la divisió de les cèl·lules eucariotes (cèl·lules canceroses) i procariotes on existeix un alt nivell de replicació cel·lular.

Els principals inhibidors de les RNRs es troben classificats segons el mètode d'acció [2, 124]. Podem trobar fins a 4 classes diferents d'inhibidors:

- Inhibidors que interaccionen amb la subunitat catalítica R1, com els anàlegs de substrat (gemcitabina, zacitabina o DMDC) o els anàlegs dels efectors (cisplatino o clofarabina).
- Inhibidors que interaccionen amb metal·lofactor de la subunitat R2, com ara quelants de ferro (deferoxamine DFO o triapine) o els anomenats radical *scavengers* (hidroxiurea, didox o N-methilhidroxilamina).
- Inhibidors que eviten la dimerització o la unió entre subunitats, com els peptidomimetics.
- Inhibidors que eviten la transcripció dels gens RNR mitjançant ARN antisentit.

Un dels inhibidors de les RNRs més utilitzat és la hidroxiurea. Aquest compost és un radical *scavenger* de la RNR de classe I [140], utilitzat en cèl·lules eucariotes com a agent anticancerígen [141, 142]. Un estudi recent ha descobert un altre compost *radical scavenger*, la N-Metil-hidroxilamina, que es podria utilitzar com a un agent antibacterià específic contra les RNRs procariotes de *P. aeruginosa, Mycobacterium bovis BCG* i *Bacillus anthracis*, ja que la toxicitat en les cèl·lules eucariotes és molt baixa [143, 144].

1.2. *Pseudomonas aeruginosa*

P. aeruginosa és un bacteri de la família γ–proteobacteri, gram-negatiu i aeròbic, tot i que, també és capaç de créixer en condicions anaeròbies. En humans, es considera un bacteri patogen oportunista que pot causar principalment, infeccions respiratòries, urinàries i també, infeccions en ferides, i en pacients immunodeficients o hospitalitzats [145, 146].

Es caracteritza per tenir un genoma bastant gran (6.3 Mbp) i una alta variabilitat genètica, que li permet créixer i adaptar-se a condicions ambientals diverses. Gràcies a diferents factors de virulència, la capacitat intrínseca de mutar i de secretar material a la matriu extracel·lular, *P. aeruginosa* sol créixer formant biofilms sobre qualsevol superfície. Aquest creixement li confereix protecció vers al sistema immunitari de l'hoste i resistència a la gran majoria d'antibiòtics comuns.

En cultius *in vitro,* s'identifica fàcilment pel color blau-verdós que presenta, degut a la secreció de pigments, com la piocianina o la pioverdina.

1.2.1. Factors de virulència

P. aeruginosa codifica en el seu genoma per diferents factors de virulència que li permeten colonitzar l'hoste, sigui l'home, animals o plantes. La infecció de *P. aeruginosa* es divideix en diferents fases; la colonització, la invasió i la disseminació, on els factors de virulència seran claus en cadascun dels processos. Alguns d'aquests factors de virulència són: l'hemolisina, l'elastasa, la proteasa alcalina, la piocianina, l'exotoxina, els lipolisacàrids, els rammolipids i les vesícules externes de membrana [145].

En els humans, la colonització de *P. aeruginosa* en l'hoste principalment es dóna per via respiratòria o directament en teixits (ferides o operació quirúrgica). El bacteri necessita unes proteïnes anomenades adhesines i dues estructures anomenades fímbries de tipus IV i flagels per tal de colonitzar l'hoste. A més, els lipopolisacàrids permeten la unió de *P. aeruginosa* a les proteïnes de superfície com ara la CFTR (*cystic fibrosis transmembrane conductance regulator)* [147].

Una vegada colonitzat l'hoste, *P. aeruginosa* secreta a l'exterior cel·lular diferents molècules que li conferiran resistència a les condicions ambientals. El sistema de secreció de tipus III (TSS3) li permet a *P. aeruginosa* alliberar toxines que inhibeixen la síntesi del citoesquelet i la síntesi de proteïnes de l'hoste evitant ser fagocitada [148]. A més, *P. aeruginosa* conté nombrosos sistemes reguladors de dos components que li permeten respondre als canvis de les condicions ambientals (a través de la transferència de la senyal ambiental per fosforilació a una proteïna reguladora) i doncs, adaptar-se al medi [149].

Aquest bacteri sintetitza diferents proteases per induir la disseminació de la infecció en l'hoste. Aquestes proteases, com ara la proteasa alcalina, la proteasa IV, LasA i LasB, permeten la destrucció de la matriu extracel·lular de la cèl·lula hoste i l'evasió del sistema immune. A més, també hi participen altres proteases; com l'exotoxina A, que inhibeix la síntesi de proteïnes; les exotoxines, que formen porus a la membrana de la cèl·lula hoste induint la lisi cel·lular, i la piocianina, que inhibeix la funció ciliar del tracte respiratori i indueix ROS (*reactive oxygen species*) que produeix dany cel·lular [150, 151].

La presència de vesícules extracel·lulars en els hostes infectats indica que aquestes estructures tenen rellevància en la patogenicitat dels bacteris [152]. Existeixen estudis que demostren que hi ha una alteració de la formació de vesícules extracel·lulars en resposta a les condicions ambientals, com ara a l'estrès cel·lular o a la desnitrificació, al sistema *quorum sensing* o al sistema SOS [153, 154]. Tot i així, no s'ha determinat de què depèn el contingut de l'interior de les vesícules.

1.2.2. Metabolisme anaeròbic

P. aeruginosa es considera un microorganisme aeròbic, tot i que, també és considerat anaeròbic facultatiu, ja que és capaç de créixer en condicions anaeròbies gràcies al seu metabolisme respiratori anaeròbic mitjançant la fermentació d'arginina i la desnitrificació de nitrat [155-157].

Durant el procés de fermentació d'arginina, *P. aeruginosa* utilitza els enzims d'arginina desaminasa codificats pels gens, *arcA*, *arcB* i *arcC* [158, 159] i un transportador, *arcD*, que transporta l'arginina per a la fermentació de L-arginina a piruvat [160].

En el procés de desnitrificació, *P. aeruginosa* utilitza com a acceptors d'electrons terminals el nitrat (NO_3), el nitrit (NO_2), l'òxid nítric (NO) i l'òxid nitrós (N_2O) [157]. Els gens implicats en aquest procés són *nar, nir, nor* i *nos*, i codifiquen per reductases que catalitzen la reducció de nitrat a nitrogen (N_2) (Figura 9). La reducció de nitrat a nitrit es produeix a la membrana a través del sistema d'assimilació de nitrat (gens *nar, nir* i *nor*), generant una transferència d'electrons per l'ATP sintasa i generant ATP. Aquest procés tan sols es dóna en condicions anaeròbies, i per tant, en absència de Nar no hi haurà el creixement.

En anaerobiosi, *P. aeruginosa* codifica un gen ortòleg a *fnr* d'*E. coli*, anomenat *anr* (*anaerobic regulator of arginine catabolism and nitrate reductase protein)*, altament sensible a l'oxigen [161]. Anr és el principal sensor d'oxigen el qual desencadena una cascada de regulació seqüencial de tots els gens involucrats en l'anaerobiosi com *nir, nor, nos, azu, arc, narXL* i *dnr*. Anr conté un *clúster* de ferro i sofre (4Fe-4S) coordinat amb quatre cisteïnes, que en absència d'oxigen dimeritza i permet unir-se a l'ADN [162] (estructura semblant a NrdG de la RNR de classe III). Dnr també és capaç de regular altres gens encarregats de la desnitrificació com *nirS, norCB* i *norZ*, i a més, detecta la presència d'òxic nítric (ON) (Figura 9) [163-166].

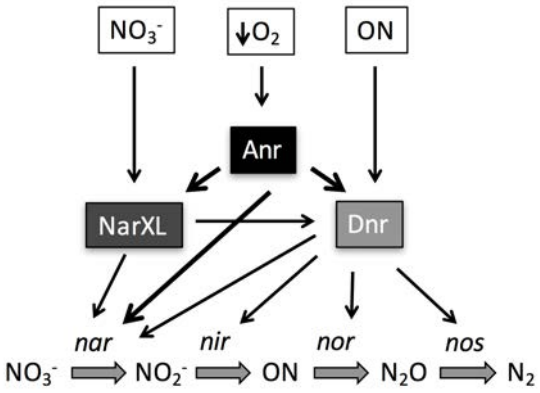

Figura 9: Esquema de la regulació dels gens que participen en el procés de desnitrificació de *P. aeruginosa* (Figura adaptada de [157]).

Tant Anr com Dnr reconeixen una seqüència consens semblant a la que reconeix el factor de transcripció Fnr, localitzada entre 40-50 bp per sobre de l'inici de transcripció [167]. Aquesta seqüència consens ($TTGA^{T}/_{C}NNNN^{A}/_{G}TCAA$) la podem trobar en els promotors de *nir* (*nirSMCF*), *nor* (*norCB*-PA0525), *nos* (*nosZ*), *dnr*, *narXL*, *azu* (azurina), i *arcDABC* (*anaerobic arginine catabolism*) [157, 164].

En condicions de creixement anaeròbies hi ha uns 700 gens expressats de manera diferencial en comparació amb el creixement aeròbic. Principalment, els gens desregulats són els gens de la desnitrificació, del flagel, del biofilm i d'entre ells, també el gen de les RNRs de classe II (*nrdJab*) augmentant aquest, 3 vegades [112, 168-172]. A més, s'ha vist que en soques aïllades de pacients clínics, els gens de la desnitrificació es troben induïts [173]. Específicament, existeix un gen, utilitzat com a marcador de l'anaerobiosi durant el procés de formació de biofilm de *P. aeruginosa*, que està altament expressat en soques aïllades clíniques, l'*oprF* [174, 175]. L'OprF és una proteïna de membrana que sembla estabilitzar l'enzim Nir a la membrana per tal de convertir nitrit (NO_2) a òxid nítric (ON) [176]. Així doncs, el procés de desnitrificació està involucrat en la patogenicitat de *P. aeruginosa* [155, 174].

L'any 2011, un estudi va analitzar el canvi de morfologia de *P. aeruginosa* PAO1 en condicions de creixement anaeròbies [177]. Aquest estudi va associar la filamentació cel·lular de *P. aeruginosa* amb la producció d'òxid nítric (ON) durant el procés de desnitrificació. A més, es va determinar que l'òxid nítric indueix filamentació cel·lular evitant la divisió cel·lular, i la inhibició de l'activitat dels enzims RNR [116, 178, 179]. Alguns estudis han postulat que la filamentació afavoreix la formació de biofilms, ja que la disminució d'òxid nítric al medi indueix la disminució de la formació de biofilm [177, 180]. Tot i així, calen més estudis per demostrar aquesta afirmació.

1.2.3. Biofilm

Un biofilm és un conjunt de bacteris adherits a una superfície caracteritzats per produir una matriu extracel·lular protectora. Aquesta matriu està composta principalment, en el cas de *P. aeruginosa*: d'exopolisacàrids, àcids nucleics i proteïnes. El procés de formació de biofilm s'ha descrit en 4 passos; 1) adhesió a la superfície, 2) formació de microcolònies, 3) formació de biofilm madur i, 4) la dispersió (Figura 10). A continuació es detallen les diferents etapes de la formació del biofilm de *P. aeruginosa.*

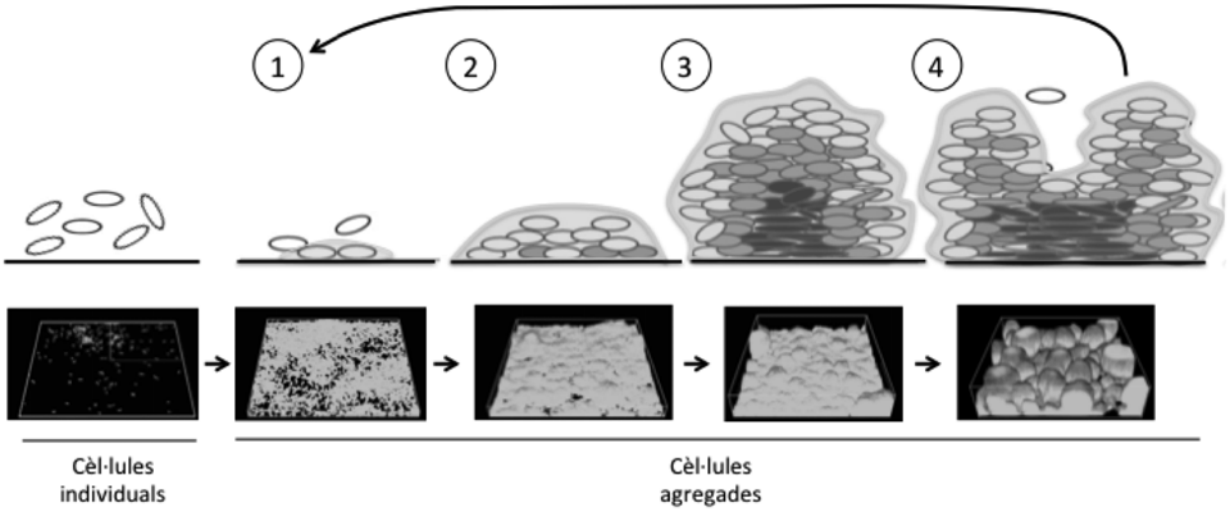

Figura 10: Procés de formació de biofilm de *P. aeruginosa*. Es troben representades les quatre etapes de formació de biofilm: 1) Adhesió a la superfície, 2) Formació microcolònies, 3) Biofilm madur i 4) Dispersió (Imatges del biofilm real extretes de [181]).

1) El procés d'adhesió dels bacteris a la superfície o inici de la formació del biofilm.

P. aeruginosa utilitza diferents molècules per tal d'adherir-se a la superfície, com ara les adhesines, l'alginat, el flagel, les proteïnes de membrana i els pili o fimbries. Es produeixen diferents interaccions cèl·lula-superfície i cèl·lula-cèl·lula, en la fase inicial de formació de biofilm, que permeten a *P. aeruginosa* utilitzar el flagel o el pili tipus IV per tal de moure's per la superfície (moviments tipus *swarming* o *swimming*) fins a agregar-se i adherir-se. Una vegada existeix una mono-capa de cèl·lules, *P. aeruginosa* produeix un altre tipus de moviment anomenat *twitching*, on principalment utilitzarà el pili tipus IV, per tal d'expandir-se [182].

L'adhesió dels bacteris a la superfície produeix canvis en l'estructura de les cèl·lules com ara l'augment de lipopolisacàrids (LPSs) [183] i canvis en l'expressió gènica global. Per exemple, el contacte amb la superfície produeix la pèrdua de motilitat del flagel i augmenta l'expressió de fímbries que permetran la unió a la superfície (pili tipus IV). També augmenten els nivells de c-di-GMP que inhibiran la motilitat i promouran la formació del biofilm [184].

2) La formació de microcolònies.

Una vegada les cèl·lules s'han unit a la superfície es comencen a sintetitzar exopolisacàrids per formar la matriu extracel·lular del biofilm. La densitat cel·lular en el biofilm ve regulada pel mecanisme anomenat *quorum sensing* (QS). El *Quorum Sensing* (QS) és un sistema de senyals extracel·lulars que regula, a més de la densitat cel·lular, la interacció entre cèl·lules i la coordinació

cel·lular durant la formació de biofilm. Existeixen dos tipus de senyals QS principals a *P. aeruginosa*: *N-acyl-L-homoserine lactone* (AHL) i *2-heptyl-3-hydroxy-4-quinolone* (PQS). El sitema QS, a través dels factors de transcripció LasR i RhlR, regula l'expressió dels gens *pel* (*polysaccharide*) i *psl* (*polysaccharide synthesis locus*) [185, 186]. Psl permet atraure altres bacteris mitjançant la inducció dels nivells de c-di-GMP i l'augment dels nivells de Psl de l'altre bacteri. La producció d'exopolisacàrids, també es veu regulada pel gen *mucA* que s'encarrega de la producció d'alginat. La mutació de *mucA* indueix la transició d'una cèl·lula planctònica a una cèl·lula formadora de biofilm, anomenada també mucoide degut a la sobreproducció d'alginat. L'alginat és el polisacàrid principal de la matriu extracel·lular, format per monòmers d'àcid urònic i gluronat [187]. La conversió de *P. aeruginosa* a una soca mucoide (hiperproducció d'alginat) és deguda al sistema sigma/antisigma (*algU/TmucABCD)* capaç de respondre a les diferents condicions ambientals. La producció d'alginat es troba regulada a dos nivells: post-transcripcional (segons estrès ambiental) a causa de la degradació de *mucA* [188, 189] o a nivell genètic, per mutacions cromosòmiques (entre elles, en el gen *mucA)* en soques hipermutables [190]. El resultat de la disminució de *mucA* dóna lloc a una activació de AlgU/T que permetrà la inducció dels gens de la producció d'alginat i de lipoproteïnes [191] i la disminució de l'expressió dels gens del flagel i doncs, també la motilitat cel·lular per afavorir la formació de biofilm [192]. Existeix un altre factor transcripcional, l'AlgR, que també regula els nivells d'alginat, com també altres gens importants per a la virulència de *P. aeruginosa* [113]. El promotor de l'*algR* respon a la unió de RpoS, Vfr [193] i d'AlgU (sigma factor de la família de RpoE), per l'augment d'osmolaritat i de nitrat en el medi [193]. Les soques de *P. aeruginosa* aïllades clíniques de pacients de fibrosi quística formadores de biofilm es consideren soques mucoides, ja que no tenen motilitat, i solen tenir mutacions en el gen *mucA* produint alts nivells d'alginat i alteracions en el sistema de desnitrificació [194]. La majoria d'aquestes soques mucoides són resistents als actuals antibiòtics comuns, ja que l'augment d'alginat també produeix resistència a antibiòtics, com ara la tobramicina [195]. La resistència als antibiòtics en un biofilm també es pot obtenir per un augment d'expressió de canals de membrana, d'enzims i de mutacions en la proteïna diana [196].

3) Formació d'un biofilm madur.

P. aeruginosa indueix l'expressió de síntesi de EPS (*extracellular polimeric substances)*, proteïnes extracel·lulars i ADN extracel·lular, una vegada establert el biofilm en la superfície. Aquestes molècules donaran estabilitat i consistència al biofilm per evadir-se de la resposta immune de l'hoste i produir resistència als antibiòtics [146, 197-199]. A més, es creu que l'ADN

extracel·lular d'un biofilm aporta estabilitat al biofilm, ja que s'ha demostrat que una disminució d'ADN extracel·lular produeix la disminució del biofilm [200].

En un biofilm madur existeix heterogeneïtat estructural, química i biològica a conseqüència d'una difusió dels nutrients al llarg de tot el biofilm [199, 201]. Tot i així, s'ha vist que existeixen canals d'aigua que poden transportar soluts a l'interior o a l'exterior del biofilm. La difusió de nutrients provoca que les cèl·lules dins del biofilm siguin diferents i heterogènies, doncs tenen diferents patrons d'expressió genètica espaial-temporal. El sistema de reparació *mismatch* és essencial per evitar mutacions però, en un biofilm la disminució de la regulació dels gens *mutS*, *mutL* i *uvrD* promou un augment de la diversificació genètica i la resistència als antibiòtics. Aquesta diversificació genètica bacteriana dins d'un biofilm altera els reguladors transcripcionals globals que influeixen a l'expressió de factors de virulència [202, 203] i a l'expressió de gens específics per sobreviure a condicions ambientals on l'oxigen és limitant (condicions aeròbiques o anaeròbiques), com *vfr* i *oprF* afavorint la formació del biofilm [174, 175, 204, 205].

Específicament, el gradient d'oxigen és la principal causa de l'heterogeneïtat bacteriana dins d'un biofilm, ja que, l'oxigen és capaç de penetrar a les capes superiors però, a partir de 10 µm de biofilm, l'oxigen comença a disminuir i es començaran a expressar els gens de l'anaerobiosi [201, 206]. En les capes més profundes del biofilm, l'oxigen és pràcticament inexistent i, *P. aeruginosa* creixerà en condicions anaeròbies. Per tant, l'establiment de gradients de concentració d'oxigen, nutrients, productes de rebuig i substàncies extracel·lulars, indueix a l'heterogeneïtat bacteriana en els biofilms [201].

4) Dispersió cel·lular.

Algunes cèl·lules del biofilm esdevenen mòtils i tornen a créixer de forma planctònica fins que s'uneixen a una altra superfície. L'alginat liasa pot estar implicat en aquest procés per la seva acció en disminuir els nivells d'alginat [207]. Alguns estudis també han vist que la dispersió pot ser induïda per canvis en la composició dels nutrients del medi o a causa de la diversificació cel·lular, així com també, han vist que un augment de c-di-GMP indueix a la dispersió cel·lular de *P. aeruginosa* en un biofilm [184].

1.2.4. Infecció de P. aeruginosa

P. aeruginosa és un bacteri molt versàtil capaç d'infectar qualsevol hoste, sigui humans, animals o plantes. Específicament, *P. aeruginosa* és considerat un patogen molt comú en humans

involucrat en infeccions nosocomials o oportunistes [208]. Durant l'any 2015, el 9% dels pacients ingressats a Espanya van tenir una infecció nosocomial, dels quals el 10.13% eren infeccions de *P. aeruginosa* [209]. Els hospitals són el reservori principal de microorganismes, sent *P. aeruginosa* un dels més importants en les infeccions nosocomials dels pacients immunodeficients com ara pacients tractats amb anticancerígens, inmunosupresors (transplants, VIH...), catèters, pròtesis o amb dificultats respiratòries. *P. aeruginosa* és capaç d'infectar qualsevol part del cos, i les infeccions es classifiquen segons el temps de durada de la infecció (en agudes o cròniques) [210].

En una infecció aguda, *P. aeruginosa* principalment es troba localitzada en un teixit i no sol formar biofilm, com ara en el tracte respiratori, tracte urinari, oïda, còrnia o en qualsevol teixit tou, a causa d'una cremada o d'una post-cirurgia. Generalment, els antibiòtics poden tractar la infecció, ja que solen ser soques no mucoides sensibles als actuals antibiòtics (Apartat 1.2.4.2.). En el cas que no sigui així i s'estableixi la formació d'un biofilm, la infecció persisteix durant anys sent doncs, una infecció crònica [211].

Quan la infecció es torna intermitent, les soques comencen a ser heterogènies (mucoides i no mucoides) fins a arribar a una infecció crònica. En una infecció crònica, *P. aeruginosa* evolucionarà degut a la pressió dels antibiòtics, creant resistències als diferents antibiòtics i formant un biofilm madur en el teixit infectat. *P. aeruginosa* sol formar infeccions cròniques importants en pacients amb dificultat respiratòria com ara malaltia pulmonar obstructiva crònica (MPOC) i fibrosi quística (FQ).

1.2.4.1. Fibrosi quística i malaltia pulmonar obstructiva crònica

En un pacient de fibrosi quística (FQ) es produeix, de manera característica, una colonització/infecció de les vies respiratòries per part de diferents microorganismes com ara *Staphylococcus aureus*, *Burkholderia cepacia* o *P. aeruginosa*. El 80% dels pacients adults de fibrosi quística es troben infectats per *P. aeruginosa* en les vies respiratòries [212]. Inicialment, es produeix una primocolonització o infecció aguda en les vies respiratòries, moment en el qual serà molt importat eradicar *P. aeruginosa* d'aquesta primera infecció. Per tant, s'ha de realitzar un seguiment periòdic per comprovar que no hi hagi una colonització esporàdica o intermitent, que podria donar lloc a una infecció crònica (cultiu recurrent de *P. aeruginosa* durant 6 mesos). Una vegada establerta la infecció crònica, es produeix l'exacerbació o infecció caracteritzada per l'aparició de signes clínics i símptomes d'infecció i una resposta immunitària forta que provoca a la disminució de la funció pulmonar al llarg del temps. La secreció de toxines per part de *P. aeruginosa* durant una infecció crònica també provoca respostes inflamatòries locals i la destrucció del teixit pulmonar. Principalment, les soques de *P. aeruginosa* que infecten els pacients de FQ es

caracteritzen per la capacitat de formar biofilms (soques mucoides) i d'adquirir resistència a antibiòtics, generalment a causa de la facilitat de produir mutacions genètiques en el seu genoma [213, 214].

La malaltia pulmonar obstructiva crònica (MPOC) es caracteritza per l'obstrucció de les vies respiratòries de manera irreversible, disminuint la funció pulmonar del pacient. Aquest fet provoca un augment de les infeccions cròniques en els pulmons dels pacients, principalment de *Haemophilus influenzae* i de *P. aeruginosa.* En aquests pacients, *P. aeruginosa* també és capaç de formar biofilms i destruir el teixit pulmonar (tal com s'ha explicat anteriorment). Per tant, la infecció crònica permanent de *P. aeruginosa* en les vies respiratòres tant dels pacients de FQ com de MPOC provoca la mort de l'individu.

1.2.4.2. Tractament

El tractament de la infecció de *P. aeruginosa* en pacients amb FQ o MPOC es diferència segons si la colonització és inicial, esporàdica, crònica o d'exacerbació [215, 216]. Es considera eradicació de la infecció de *P. aeruginosa* quan durant un any els cultius microbiològics són negatius. Tot i així, quan la infecció ja és crònica, és difícil d'eliminar, i tan sols es controla que hi hagi estabilitat dels pacients, evitant les exacerbacions i la pèrdua de funció pulmonar.

P. aeruginosa és un bacteri resistent als antibiòtics: β-lactàmics, macròlids, tetraciclines i a la majoria de fluoroquinolones. Però, no és intrínsecament resistent a carboxipenicillines (ticarcillina), a ureidopenicillines (piperacillina), a la combinació de β-lactàmics (piperacillina/tazobactam i ticarcillina/clavulànic), a la quarta generació de cefalosporines (cefepima, ceftazidima i cefoperazona), a aminoglicòsids (gentamicina, tobramicina i amikacina), a monobactams (aztreonam), a algunes fluoroquinolones (levofloxacina i ciprofloxacina), a carbapenems (imipenem/cilastatina, meropenem i ertapenem) i a les polimixines (colistina). Tot i així, *P. aeruginosa* és capaç de desenvolupar resistències si hi ha una exposició continua a qualsevol antibiòtic [217, 218]. L'ús simultani de dos antibiòtics pot augmentar l'eficàcia i disminuir l'aparició de resistències [210].

1.2.5. Les RNRs de P. aeruginosa

P. aeruginosa és un dels pocs microorganismes que excepcionalment codifica en el seu genoma per les tres classes de RNR, la classe Ia, la classe II i la classe III [133]. Codificar per a les tres classes de RNR en un mateix genoma, permet a *P. aeruginosa* dividir-se i créixer tant en condicions aeròbies com anaeròbies. Aquest fet excepcional ha donat lloc a estudiar l'essencialitat

de les tres classes de RNR a *P. aeruginosa* per poder entendre perquè un microorganisme, aparentment de manera redundant, codifica per a les tres classes diferents. A continuació es detallen algunes de les característiques específiques de les RNRs de *P. aeruginosa.*

A nivell proteic, l'estructura quaternària de l'enzim RNR de classe Ia de *P. aeruginosa*, en presència d'ATP, forma una estructura enzimàticament activa de $\alpha_2\beta_2$. La RNR de classe Ia d'*E. coli* o de ratolí formen una estructura activa dimèrica ($\alpha_2\beta_2$) i hexamèrica ($\alpha_6\beta_6$), respectivament. Tot i així, en presència de dATP l'estructura canvia i s'inhibeixen enzimàticament formant una estructura $\alpha_4\beta_4$ a *E. coli*, $\alpha_6\beta_2$ o $\alpha_6\beta_6$ a organismes eucariotes o $\alpha_4\beta_2$ a *P. aeruginosa* [79, 219] (Figura 11).

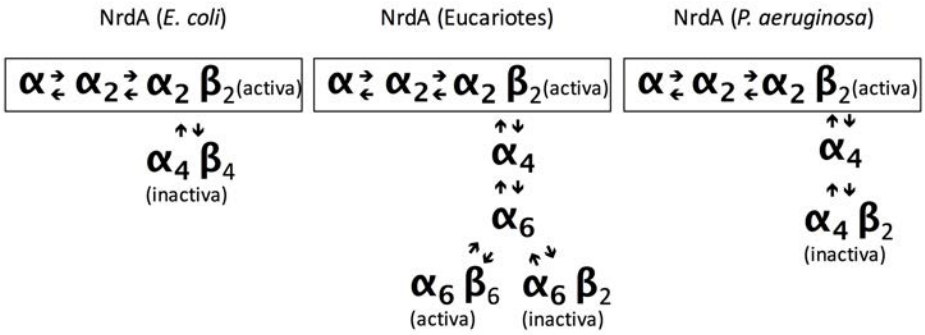

Figura 11: Oligomerització de la classe Ia d'*E. coli*, d'organismes eucariotes i de *P. aeruginosa*. Es mostra la forma activa d'oligomerització i les diferències en la inactivació de l'enzim. (Figura adaptada de [79])

A *P. aeruginosa*, la proteïna NrdA (RNR de classe Ia) conté una seqüència aminoacídica més llarga, d'uns 220-230 aminoàcids extra, situats en la posició N-terminal, que corresponen a una duplicació del domini *ATP-cone* (Apartat 1.1.5). Aquesta duplicació del domini *ATP-cone* no és exclusiu de *P. aeruginosa* sinó que altres β, γ-proteobacteris i la família *Chamydiaceae* que contenen duplicacions o inclús triplicacions del domini. Però, específicament a *P. aeruginosa* s'ha descrit que l'*ATP-cone* situat a N-terminal o *ATP-cone* 1 (ATPc1) està implicat en l'oligomerització i té una funció al·lostèrica, mentre que l'*ATP-cone* intern o *ATP-cone* 2 (ATPc2) no té funció al·lostèrica [219]. Un estudi recent ha revelat l'estructura detallada de la subunitat R1 o NrdA de *P. aeruginosa* [220]. L'estructura de NrdA descrita revela un dímer altament asimètric, trobant-se els dominis *ATP-cone* en orientacions diferents. L'ATPc1, detallat en aquest estudi, correspon al motiu *ATP-cone* descrit anteriorment, altament conservat. Tot i així, s'ha vist que aquest ATPc1 de NrdA és capaç d'unir dos dATP, en lloc d'un sol, com es creia fins ara. En canvi, l'ATPc2 conté pocs residus conservats i tan sols, participa en posicionar el tetràmer [79, 220].

La subunitat NrdB de la RNR de classe Ia de *P. aeruginosa* també és atípica, ja que el radical tirosil que es forma és de curta duració, i tan sols es regenera en presència continua d'oxigen [219].

A *E. coli i L. lactis,* el radical tirosil és més estable i l'oxigen només iniciarà el procés per la formació del radical [12, 221].

Sorprenentment, la seqüència aminoacídica de NrdAB de *P. aeruginosa* mostra una homologia més alta amb la RNR de classe Ia eucariota de ratolí (42% per NrdA i 28% per NrdB d'identitat) que amb la RNR de classe Ia d'*E. coli* (NrdA 27% i NrdB 24% d'identitat). A més, també manté una alta identitat amb la seqüència de NrdAB de *C. trachomatis* (52% NrdA i 57% NrdB).

A nivell transcripcional, el gen *nrdA* conté una regió UTR en 5' d'uns 400 bp que no es tradueix, suggerint una regió reguladora de l'expressió d'aquest gen com ara a través d'una regulació *B_{12}-riboswitch,* com s'ha identificat en altres microorganismes [97, 222]. A més, en la regió flanquejant de l'operó *nrdAB* (PA1156 i PA1155), s'hi troba un gen transposó (PA1153) i un *open reading frame* d'origen fàgic (PA1154). Aquests dos gens poden suggerir una transposició de la RNR de classe Ia al genoma de *P. aeruginosa* via transferència gènica lateral [219].

Per una altra banda, la RNR de classe II de *P. aeruginosa,* està codificada per dos gens o *open reading frames* (2 ORF) anomenats *nrdJa* i *nrdJb* separats per 16 bp. NrdJa conté les regions conservades de l'activitat de l'enzim i del lloc al·lostèric, i NrdJb conté la cisteïna C-terminal que interacciona amb el sistema reductor o la vitamina B_{12}. Tan sols hi ha activitat en la RNR de classe II quan totes dues proteïnes hi són presents [24, 223]. Possiblement, la unió de la vitamina B_{12} es produeixi en la regió que divideix els dos ORF de NrdJa i NrdJb. A més, la RNR de classe II no conté els aminoàcids específics per la unió de dATP a N-terminal (regió ATP-cone), doncs l'enzim no serà inhibit per dATP a *P. aeruginosa* [133]. Així doncs, l'activitat de la RNR de classe II és estrictament dependent de la presència de vitamina B_{12} [24, 110, 133]. Tot i així, a *P. aeruginosa* a diferència d'altres microorganismes, la vitamina B_{12} no sembla regular l'expressió dels gens de les RNRs via *B_{12}-riboswitch* [24].

Per últim, la unió del metall al centre metàl·lic de la RNR de classe III de *P. aeruginosa* en posició C-terminal segueix un domini $CXXHX_9CXXC$, a diferència del domini C-terminal més comú d'altres microorganismes, que segueix una seqüència consens $CXXCX_{14}CXXC$ [2]. Tot i així, la unió del metall de NrdD es dóna en la glicina Gly644 al costat d'un motiu Cys640-His643 conservat, semblant a un domini rubredoxina (proteïnes amb un centre metàl·lic de Fe-S) [2].

El patró d'expressió dels diferents gens de les RNRs és: els gens *nrdAB* (RNR de classe Ia) s'indueixen en fase exponencial i disminueixen unes 6 vegades en fase estacionària inicial, mentre que el gen *nrdJ* (RNR de classe II) augmenta 6-7 vegades en fase estacionària [24]. En canvi, *nrdDG* (RNR de classe III) s'indueix en condicions anaeròbies [110]. Aquesta disminució de l'expressió de la RNR de la classe Ia pot ser deguda a la pèrdua del subministrament d'oxigen constant degut a condicions microaerofíliques.

Finalment a nivell fisiològic, els enzims RNR de *P. aeruginosa* tenen un paper important durant la infecció, ja que és el moment on la divisió cel·lular i per tant els nivells de dNTP equilibrats, són essencials. Un estudi dut a terme en el nostre laboratori l'any 2011, va demostrar que la virulència de *P. aeruginosa* disminueix en absència de RNR de classe II i III durant la infecció en el model animal de *Drosophila melanogaster* [110]. A més, l'expressió de les RNRs de classe II i III augmenta durant la infecció, mentre que la RNR de classe Ia disminueix. Es coneix que la infecció de *P. aeruginosa* en els pulmons de pacients de FQ es produeix en condicions de microaeròfiliques o anaerobiosi [224]. Per tant, els enzims RNR de classe II i III de *P. aeruginosa* són essencials pel procés d'infecció a *D. melanogaster* [110].

Objectius

En aquest treball s'ha aprofundit en l'estudi de l'expressió de les diferents RNRs de *P. aeruginosa* amb la finalitat d'entendre la simultaneïtat de cadascuna de les diferents classes en un mateix microorganisme.

Per tant, els objectius planejats d'aquesta tesi s'exposen a continuació:

1. **Estudiar el factor transcripcional NrdR en la regulació diferencial de l'expressió de les RNRs de *P. aeruginosa*.**

2. **Identificar un nou regulador transcripcional de les RNRs de *P. aeruginosa,* l'AlgR**.

3. **Determinar el paper de les diferents classes de RNR durant la formació de biofilm de *P. aeruginosa* i la seva regulació transcripcional.**

4. **Analitzar l'expressió de les diferents classes de RNR en soques de *P. aeruginosa* aïllades clíniques i la importància de les RNRs en el procés d'infecció de *P. aeruginosa*.**

5. **Determinar les condicions de síntesi de la vitamina B_{12} i establir les condicions d'activitat de la RNR de classe II a *P. aeruginosa*.**

Resultats

Resum

Els resultats obtinguts, corresponents als objectius 1 i 2, es resumeixen a continuació:

Alguns microorganismes codifiquen per més d'una classe de RNR en el seu genoma, com ara *P. aeruginosa* que codifica per les RNRs de classe Ia, II i III [8, 9]. La regulació de cadascun dels gens que codifiquen per les diferents classes de RNR ha de ser molt acurada i precisa per tal d'obtenir una síntesi de dNTP equilibrada durant la replicació i la reparació de l'ADN cel·lular. Un desequilibri en la concentració d'un o un altre dNTP indueix mutacions durant el procés de síntesi de l'ADN. Així doncs, en aquest treball es va proposar l'estudi de la regulació transcripcional dels gens que codifiquen les diferents classes de RNR a *P. aeruginosa* (*nrdAB, nrdJab* i *nrdDG*). A més, es va analitzar la regulació transcripcional diferencial entre cadascun dels gens de les tres classes de RNR per poder entendre la simultaneïtat de les classes de RNR en un mateix genoma.

Inicialment, es va a estudiar el factor transcripcional NrdR. Estudis previs en el nostre laboratori i en altres, demostraven una repressió dels gens *nrd* en diferents microorganismes (*E. coli, C. trachomatis, S. coelicolor, S. typhimurium* [96, 123, 126-128]) mitjançant la unió de NrdR a dues caixes NrdR-box localitzades en les regions promotores dels gens *nrd*.

Específicament en el aquest treball hem analitzat el patró d'expressió del propi factor de transcripció NrdR i la seva regulació transcripcional a *P. aeruginosa*. Es van construir fusions transcripcionals de cadascun dels promotors dels gens que codifiquen per les diferents RNR a la GFP, per tal d'analitzar les diferències d'expressió de cada gen en una soca salvatge (*wild-type*) i una soca mutant isogènica pel gen *nrdR* (Δ*nrdR*). En aquest capítol, s'ha vist que NrdR participa en la regulació dels gens de manera diferencial sota condicions de creixement aeròbies o anaeròbies. A més, també s'ha identificat per primera vegada una caixa d'unió del factor de transcripció NrdR al promotor del gen que codifica per la topoisomerasa I (*topA*), a diferència de les dues caixes NrdR-box identificades en els promotors dels gens *nrd.* Un estudi transcripcional global (*microarray*) ens ha permès determinar una desregulació sobre altres gens diferents de *nrd* degut a la mutació isogènica en *nrdR*. Finalment, en aquest capítol es va analitzar la virulència de la soca Δ*nrdR* i la soca salvatge PAO1 en el model animal d'infecció de *Drosophila melanogaster*.

Per una altra banda, també es van analitzar altres factors de transcripció involucrats en la regulació dels gens de les tres classes de RNR de *P. aeruginosa*. A partir d'un estudi bioinformàtic dels promotors de les diferents RNRs es van identificar possibles caixes d'unió de diferents factors

de transcripció (anaeròbics, *quorum sensing*, virulència, etc). Específicament, un dels capítols del treball descriu l'estudi del paper del factor de transcripció AlgR sobre la regulació de les tres classes de RNR. S'ha vist que AlgR és capaç de regular l'expressió de la RNR classe Ia i II de *P. aeruginosa*. Hem demostrat la interacció directa de l'AlgR sobre les regions promotores de la RNR de classe Ia i II mitjançant EMSA, així com la seva regulació transcripcional depenent si el factor de transcripció està fosforilat o no. Finalment, hem identificat que l'AlgR regula les RNRs en funció dels senyals d'estrès oxidatiu.

Aquests resultats s'han dividit en 2 capítols corresponents als objectius 1 i 2.

Article 1

Function of the *Pseudomonas aeruginosa* NrdR Transcription Factor: Global Transcriptomic Analysis and Its Role on Ribonucleotide Reductase Gene Expression.

Anna Crespo, Lucas Pedraz i Eduard Torrents.

Referència:

Crespo A, *Pedraz L, Torrents E (2015) Function of the Pseudomonas aeruginosa NrdR Transcription Factor: Global Transcriptomic Analysis and Its Role on Ribonucleotide Reductase Gene Expression. PLoS ONE 10(4): e0123571. doi:10.1371/journal.pone.012357*

Article 2

***Pseudomonas aeruginosa* AlgR regulates ribonucleotide reduction and links it to oxidative stress signals.**

Anna Crespo, Lucas Pedraz, Marc Van Der Hofstadt, Gabriel Gomila i Eduard Torrents.

Submitted.

Els resultats obtinguts, corresponents als objectius 3, 4 i 5, es resumeixen a continuació:

P. aeruginosa és un dels principals bacteris implicats en la formació de biofilm, capaç de produir infeccions cròniques, com ara les causades en pacients de la malaltia pulmonar obstructiva crònica (MPOC) o de fibrosi quística (FQ). Durant el procés d'infecció o de formació de biofilm, els nivells de dNTP són essencials per permetre la replicació del seu ADN i, per tant, claus en el procés de divisió cel·lular de *P. aeruginosa*. Així doncs, en aquesta part del treball s'han estudiat les condicions d'expressió dels gens de cadascuna de les diferents classes de RNR durant la formació de biofilm i durant el procés d'infecció.

També, hem estudiat soques amb delecions cromosòmiques de les RNRs de classe II i III per estudiar la importància d'aquestes durant la formació de biofilm. S'ha vist que totes dues classes de RNR són necessàries per un biofilm format, estructurat i madur. A més, s'ha estudiat l'expressió transcripcional dels gens de les tres classes de RNR mitjançant fusions transcripcionals i s'ha analitzat els nivells de mRNA en el procés de formació de biofilm. S'ha observat que en el biofilm hi tenen un paper essencial els gens anaeròbics, ja que existeix un gradient d'oxigen al llarg de la verticalitat d'un biofilm, sent anaeròbic en les parts més internes del biofilm [201], permetent l'activitat de les RNRs de classe II i III. Hem identificat els factors transcripcionals anaeròbics (Anr, Dnr i NarL) com activadors de l'expressió dels gens de les RNRs en un biofilm.

Per una altra banda, s'ha realitzat un estudi del paper de les RNRs durant el procés d'infecció de *P. aeruginosa*, ja que estudis previs han demostrat la importància de les RNRs de classe II i III durant el procés d'infecció en *D. melanogaster* [110]. En aquest capítol, s'ha estudiat l'expressió de les tres classes de RNR en soques salvatges (*wild-type*) de col·lecció (PAO1 o PA14) i en soques d'aïllaments clínics de *P. aeruginosa* provinents de pacients de FQ o d'infeccions agudes. S'ha tingut en compte la morfologia cel·lular de les diferents soques, ja que en alguns estudis s'ha vist que la soca de *P. aeruginosa* PAO1 no és capaç de dividir-se correctament en condicions anaeròbiques [177]. Els resultats demostren que no hi ha suficient expressió de la RNR de classe III en condicions anaeròbiques en una soca de *P. aeruginosa* PAO1 (laboratori), trobant-se significativament induïda en les soques d'origen clínic i la soca de *P. aeruginosa* PA14. S'ha identificat una mutació en el promotor de *nrdD* que altera la seva expressió a *P. aeruginosa* PAO1. A més, s'han infectat peixos zebra per tal de determinar la virulència de les soques que expressen diferencialment la RNR de classe III. Els resultats mostren més virulència en les soques que expressen més la RNR de classe III a diferència de la soca de *P. aeruginosa* PAO1. Per tant, la soca de *P. aeruginosa* PAO1 no és la millor soca de laboratori per l'estudi de la virulència de *P. aeruginosa.*

Per últim, s'ha estudiat el paper de la vitamina B_{12} en l'expressió o l'activació de les RNRs en diferents soques de *P. aeruginosa*, així com també, s'ha esbrinat les condicions de síntesi d'aquesta, ja que determinarà l'activitat de la RNR de classe II en qualsevol condició (infecció o biofilm).

Aquests resultats s'han dividit en 3 capítols corresponents als objectius 3, 4 i 5.

Article 3

***Pseudomonas aeruginosa* Exhibits Deficient Biofilm Formation in the Absence of Class II and III Ribonucleotide Reductases Due to Hindered Anaerobic Growth.**

Anna Crespo, Lucas Pedraz, Josep Astola i Eduard Torrents.

Referència:

Crespo A, *Pedraz L, Astola J and Torrents E (2016). Pseudomonas aeruginosa Exhibits Deficient Biofilm Formation in the Absence of Class II and III Ribonucleotide Reductases Due to Hindered Anaerobic Growth. Front. Microbiol. 7:688. doi: 10.3389/fmicb.2016.00688*

Article 4

The deficient anaerobic growth and infection in the *Pseudomonas aeruginosa* PAO1 strain are due to a single point mutation in the class III ribonucleotide reductase promoter

Anna Crespo, Joan Gavaldà i Eduard Torrents.

Submitted.

Article 5

Aerobic vitamin B_{12} biosynthesis is essential for *Pseudomonas aeruginosa* class II ribonucleotide reductase activity during planktonic and biofilm growth

Anna Crespo, Núria Blanco-Cabra i Eduard Torrents

Manuscrit en preparació.

Article 1

Function of the Pseudomonas aeruginosa NrdR Transcription Factor: Global Transcriptomic Analysis and Its Role on Ribonucleotide Reductase Gene Expression

PLOS ONE

RESEARCH ARTICLE

Function of the *Pseudomonas aeruginosa* NrdR Transcription Factor: Global Transcriptomic Analysis and Its Role on Ribonucleotide Reductase Gene Expression

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Abstract

Ribonucleotide reductases (RNRs) are a family of sophisticated enzymes responsible for the synthesis of the deoxyribonucleotides (dNTPs), the building blocks for DNA synthesis and repair. Although any living cell must contain one RNR activity to continue living, bacteria have the capacity to encode different RNR classes in the same genome, allowing them to adapt to different environments and growing conditions. *Pseudomonas aeruginosa* is well known for its adaptability and surprisingly encodes all three known RNR classes (Ia, II and III). There must be a complex transcriptional regulation network behind this RNR activity, dictating which RNR class will be expressed according to specific growing conditions. In this work, we aim to uncover the role of the transcriptional regulator NrdR in *P. aeruginosa*. We demonstrate that NrdR regulates all three RNR classes, being involved in differential control depending on whether the growth conditions are aerobic or anaerobic. Moreover, we also identify for the first time that NrdR is not only involved in controlling RNR expression but also regulates topoisomerase I (*topA*) transcription. Finally, to obtain the entire picture of NrdR regulon, we performed a global transcriptomic analysis comparing the transcription profile of wild-type and *nrdR* mutant strains. The results provide many new data about the regulatory network that controls *P. aeruginosa* RNR transcription, bringing us a step closer to the understanding of this complex system.

Introduction

The opportunistic pathogen *Pseudomonas aeruginosa* has the ability to grow under a variety of environmental conditions; it can be free-living in soil and water, as well as growing in human and plant host-associated environments. It is responsible for severe nosocomial infections in immunocompromised patients and, in particular, causes chronic lung infections in patients suffering from the inherited disease cystic fibrosis [1]. The genome of *P. aeruginosa* is relatively

Competing Interests: The authors have declared that no competing interests exist.

large (6.3 Mb), and contains a large number of genes to perform different metabolic activities, which might contribute to the environmental adaptability of this bacterium [2].

One such example is the enzyme ribonucleotide reductase (RNR), a key enzyme that catalyzes the reduction of all four ribonucleotides to their corresponding deoxyribonucleotides, providing the necessary precursors for DNA synthesis and repair in all organisms. All known RNRs can be divided into three classes (I, II and III) based on their structural differences, metallocofactor requirements, and mechanisms used for radical generation [3–6]. Class I RNRs require oxygen to produce a tyrosyl radical using a diferric iron or dimanganese iron center, and thereby functions only under aerobic conditions. Based on sequence identity, the metal cofactor center and allosteric properties, class I RNRs are subdivided into classes Ia, Ib and Ic, encoded, respectively, by *nrdAB*, *nrdHIEF* and the *nrdAB* genes. Class II enzymes require S-adenosylcobalamine (AdoCob) for radical generation and do not depend on oxygen. Members of class III RNR carry a stable but oxygen-sensitive glycyl residue plus an iron-sulfur center that catalyzes the reduction of S-adenosylmethionine to generate this radical. This class can only function under anaerobic conditions.

P. aeruginosa is one of the few microorganisms that encodes the three-different RNR classes (Class Ia, II and III) in its genome, which are apparently redundant, but reflect its need to adapt its metabolism to grow under specific conditions or during infection [7,8].

Relatively little is known about how bacteria control RNR activity at the gene level, and particularly in *P. aeruginosa*, in which it is totally unknown which transcriptional factors regulate the expression of the three RNR classes. The original study conducted by an Israeli group identified a novel transcriptional regulator in *Streptomyces coelicolor* termed NrdR, which controlled the expression of both class I and II RNR gene clusters. It was shown for the first time that in streptomycetes *nrdR* gene is linked to and co-transcribed with *nrdJ*. In *S. coelicolor*, a deletion of this gene produces a transcriptional derepression of the *nrd* genes [9,10]. Later, Rodionov and Gelfand described a bacterial regulatory system through a bioinformatics approach, with the identification of a highly conserved 16 bp palindromic signal, named NrdR-box, upstream of most operons encoding the ribonucleotide reductases [11]. Subsequently, our group described an analogous situation in *Escherichia coli*, with an NrdR homolog that was shown to regulate all three *nrd* systems (class Ia, Ib and III) and binding to the predicted NrdR binding sites. Remarkably, class Ib was highly derepressed (more than 150 times) in the *nrdR* mutant compared with the wild-type strain [12].

NrdR proteins are composed of 140–200 amino-acids, and present two differentiated domains: a zinc ribbon DNA-binding domain and an ATP-cone domain similar to that present in the N-terminal part (the allosteric activity site) of many RNRs. It seems that when the NrdR ATP-domain binds dATP instead of ATP, it changes its conformation and binds to its cognate DNA recognition sequences to repress RNR gene expression [10,13]. A recent study has unveiled a more complex control behind the NrdR nucleotide binding activity [14].

In this study, we uncovered the role of NrdR on the transcriptional regulation of the different ribonucleotide reductase and *topA* genes in *P. aeruginosa*. This is the first report in which the role of NrdR was analyzed in *P. aeruginosa* whose genome encodes all three different RNR classes. We also studied the global expression profile of *P. aeruginosa* when the *nrdR* gene was mutated, and the role of this transcription factor as a global regulator.

Materials and Methods

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids are listed in Table 1. *Escherichia coli* and *Pseudomonas aeruginosa* cells were routinely grown in Luria-Bertani broth (LB) at 37°C. When necessary,

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmids	Description	Source
Plasmids		
pGEM-T easy	A/T cloning vector, Amp^R	Promega
pUCP20T	Broad-host-range vector, Amp^R	[32]
pBBR1MCS-5	High-copy number cloning vector, Gm^R	[33]
pETS130-GFP	Broad host range, promoterless GFP, Gm^R	[7]
pETS134	pETS130 derivative carrying *nrdA* promoter, Gm^R	[7]
pETS136	pETS130 derivative carrying *nrdD* promoter, Gm^R	[7]
pETS161	pETS130 derivative carrying *nrdR* promoter, Gm^R	This work
pETS176	pUCP20T derivative carrying *nrdR* gene, Amp^R	This work
pETS177	pETS130 derivative carrying *topA* promoter, Gm^R	This work
pETS178	pETS130 derivative carrying NrdR box mutated in *topA* promoter, Gm^R	This work
pETS180	pETS130 derivative carrying *nrdJ* promoter, Gm^R	This work
pETS181	pETS130 derivative carrying NarL box1.1 mutation (box NarL1) in *nrdR* promoter, Gm^R	This work
pETS182	pETS130 derivative carrying NarL box1.2 mutation (box NarL1) in *nrdR* promoter, Gm^R	This work
pETS183	pETS130 derivative carrying NarL box1.3 mutation (box NarL1) in *nrdR* promoter, Gm^R	This work
pETS184	pETS130 derivative carrying NarL box2.1 mutation (box NarL2) in *nrdR* promoter, Gm^R	This work
pETS185	pETS130 derivative carrying NarL box2.2 mutation (box NarL2) in *nrdR* promoter, Gm^R	This work
pETS186	pETS130 derivative carrying NarL box2.3 mutation (box NarL2) in *nrdR* promoter, Gm^R	This work
pETS187	pETS130 derivative carrying NarL box1 and box2 mutation in *nrdR* promoter, Gm^R	This work
pETS188	pETS130 derivative carrying NrdR box2 mutated in *nrdA* promoter, Gm^R	This work
pETS189	pETS130 derivative carrying NrdR box2 mutated in *nrdJ* promoter, Gm^R	This work
pETS190	pETS130 derivative carrying NrdR box2 mutated in *nrdD* promoter, Gm^R	This work
Strains		
E. coli		
DH5α	*recA1 endA1 hsdR17 supE44 thi-1 relA1 Δ(lacZYA-argF)U169 deoR Φ80dlacZM15*	Laboratory stock
P. aeruginosa		
PAO1	Wild-type (ATCC 15692 / CECT 4122)- Spanish Type Culture Collection	Lab strain
PW7549	*P. aeruginosa* PAO1 *narL*::*ISlacZ*/hah, Tc^R	[20]
PW7855	*P. aeruginosa* PAO1 *nrdR*::*ISlacZ*/hah, Tc^R	[20]

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antibiotics were added at the following concentrations: for *E. coli*, 10 μg/ml gentamicin and 50 μg/ml ampicillin; for *P. aeruginosa*, 150 μg/ml gentamicin, 300 μg/ml carbenicillin and 50 μg/ml tetracycline. Liquid cultures were shaken on a horizontal shaker at 200 rpm. Anaerobic growth was performed in LB medium containing 10 g/l KNO_3 in screw-cap tubes (Hungate Tubes) that were filled to the top with N_2.

Strains and plasmids construction

Recombinant DNA manipulations were carried out according to published protocols [15]. Plasmid DNA was prepared using the QIAprep miniprep kit (Qiagen) and was transformed into *P. aeruginosa* cells by electroporation as previously described [16] using a Gene Pulser Xcell™ electroporator (Bio-Rad). Digestions with restriction enzymes were performed according to the manufacturer's instructions (Fermentas). Ligations were performed with T4 DNA ligase (Fermentas, Thermo Scientific), except as otherwise stated. DNA fragments were amplified by PCR using High-Fidelity PCR enzyme mix (Fermentas) using chromosomal DNA as a template.

When necessary, specific restriction site sequences were incorporated at the 5' ends of the primers to facilitate the cloning of the fragments in the appropriate vector. Plasmids pETS161, pETS177 and pETS180 were constructed as follows: First, the *nrdR* (277 bp), *topA* (348 bp) and *nrdJ* (419 bp) promoter regions were amplified from *P. aeruginosa* PAO1 genomic DNA using the primer pair P*nrdRBHI*-up/P*nrdRClaI*-lw; PtopA-BamHI-up/PtopA-ClaI-low; and PnrdJ-BamHI new-up/PnrdJSmaI-new-low, respectively (S1 Table). The resulting DNA fragment and the pETS130-GFP plasmid were both digested with the corresponding restriction enzymes, and ligation was performed. Complementation plasmid (pETS176) was constructed by cloning the *nrdR* gene, under the control of its native promoter, into plasmid pUCP20T using the primer pair P*nrdRBamHI*-up/NrdRHindIII-low.

Site-directed mutagenesis of the putative NarL and NrdR binding sites

The two NarL binding boxes (NarL1 and NarL2) in the *nrdR* promoter region were mutated using PCR-based site-directed mutagenesis using the following primer pairs: mutNarL1up/mutNarL1low; mutNarL1.2 up/mutNarL1.2 low; mutNarL1.3 up/mutNarL1.3 low; mutNarL R-dir/mutNarL-rev; mutNarL2.2 up/mutNarL2.2 low; and mutNarL2.3 up/mutNarL2.3 low, to generate pETS181, pETS182, pETS183, pETS184, pETS185, pETS186 and pETS187, respectively.

The putative NrdR box2 in the promoter regions of *nrdAB*, *nrdJ*, *nrdDG* and *topA* was mutagenized using the following primer pairs: AmR2-up/AmR2-low; JmR2-up/JmR2-low; DmR2-up/DmR2-low; and TmR-up/TmpR-low, to generate pETS188, pETS189, pETS190 and pETS178, respectively. The resulting amplicons were cloned into the pGEM-T easy vector, according to the manufacturer's instructions, and then, after digestion with the corresponding restriction enzymes, to pETS130-GFP. Each mutation was verified by DNA sequencing.

Green fluorescent protein gene reporter assay

Bacterial cultures were grown to the corresponding A_{550}, and three independent 1-ml samples of each culture were collected. Cells were pelleted, and fixed with 1 ml of freshly prepared phosphate buffered saline (PBS) solution containing 2% formaldehyde and stored in the dark at 4°C. Fluorescence was measured in 96-well plates on an Infinite 200 Pro fluorescence microplate reader (Tecan). Three measurements were performed for each independent sample.

DPA assay

For total cellular dNTP quantification we used the diphenylamine assay (DPA) following the described procedures [17,18]. Briefly, DPA reagent (Sigma-Aldrich) was dissolved in a 2:1 acetic acid–sulfuric acid mixture. The solution was incubated at 37°C for 4 h, and all measurements were performed at 595 nm. Bacterial cell extracts from *P. aeruginosa* wild-type cells grown to an A_{550} of 0.5 and normalized by equal protein content were analyzed using the DPA assay. Three independent experiments were performed.

Supercoiling assay

pUCP20T plasmid was transformed into PAO1 wild-type and *nrdR* mutant strains by electroporation, to corroborate differences in supercoiling activity. Strains were grown aerobically at 37°C to mid-logarithmic and stationary phases (A_{600} of 0.5 and 2, respectively) in LB containing 300 μg/ml of carbenicillin. Plasmid DNA was purified via a previously described protocol [19]. Briefly, a 16 h gel electrophoresis at 50 V was performed in 1.2% agarose gels containing 5 mg/L of chloroquine, to separate 0.5 μg of plasmid. After washing for 3 h in water, to remove

chloroquine, the gels were stained with ethidium bromide and visualized on an ultraviolet transilluminator.

RNA extraction, reverse transcription and real-time PCR

Total RNA from *P. aeruginosa* PAO1 was isolated with an RNeasy Mini Kit (Qiagen) and RNAprotect Bacteria Reagent (Qiagen), according the manufacturer's instructions. DNase I (Turbo DNA-free, Applied Biosystems) was used to remove DNA contamination. Reverse transcription PCR (RT-PCR) was performed with 1 μg of RNA in a total 20-μl reaction volume, using the SuperScript III First-Strand Synthesis System for RT-PCR (Applied Biosystems), and PCR amplification of the cDNA was performed with High-Fidelity PCR enzyme mix (Fermentas). Primers used in this study are listed in S1 Table. The first-strand cDNA synthesis step was conducted at 55ºC for 1 h, and the cycling conditions for PCR were performed as follows: 3-min denaturation period at 94ºC; 20 cycles for 1 min at 94ºC, 45 s at 51ºC, and 1 min per kb of DNA template at 72ºC; and final 7-min extension at 72ºC.

Real-Time PCR measurements were carried out using TaqMan primers and probes (S1 Table), and detection was performed using and ABI Step One Plus detection system from Applied Biosystems as described previously [12]. The *gapA* sequence was used as an internal standard since their expression is constitutive during *P. aeruginosa* growth.

Microarray analysis

The *P. aeruginosa* strains were grown aerobically and anaerobically until the mid-logarithmic growth phase. Total bacterial RNA was isolated as previously stated from each of three independent cultures. Eight micrograms of purified RNA were used for a GeneChip genome array analysis. The GeneChip probes (Affymetrix) were prepared according to Affymetrix's instructions. RNA integrity, target hybridization, washing, staining and scanning steps were performed by the Functional Genomics Core facility at the Institute for Research in Biomedicine (Barcelona, Spain). Data analysis was initially performed with the Microarray suite software and then imported into Microsoft Excel for further statistical analysis. Only those genes that had a mean signal $\log_2$-ratio of >1.5 (up-regulated transcripts) and <1.5 (down-regulated transcripts) were kept in the final list of genes. Microarray data are available in the Array express database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-3006.

Fly infection assays

All experiments used healthy 3–4 days old adult *Drosophila melanogaster* Oregon^R flies, maintained at 25ºC in vials with standard corn-meal agar medium. A suspension of *P. aeruginosa* cells in PBS, adjusted at $A_{550}\approx0.1$, was injected using a capillary glass with a microinjector (TriTEch Research, CA) as previously described [7]. Survival curves were plotted using Kaplan-Meier analysis and differences of survival rates were analyzed by the log-rank test (GraphPad Prism 6.0, GraphPad Software, La Jolla California USA).

Results

NrdR expression pattern in *P. aeruginosa*

The *P. aeruginosa* transcriptional regulator homolog of the *E. coli nrdR* gene is the PA4057 gene (Fig 1A). The translation of the PA4057 gene, here denoted as *nrdR*, is expected to produce a 154 amino-acid protein, with a predicted molecular weight of 17.9 kDa. A search in the Conserved Domain Database revealed two major domains: a zinc-finger (3–34 aa) and an ATP-cone domain (49–139 aa), at the N-terminus and C-terminus, respectively, showing a

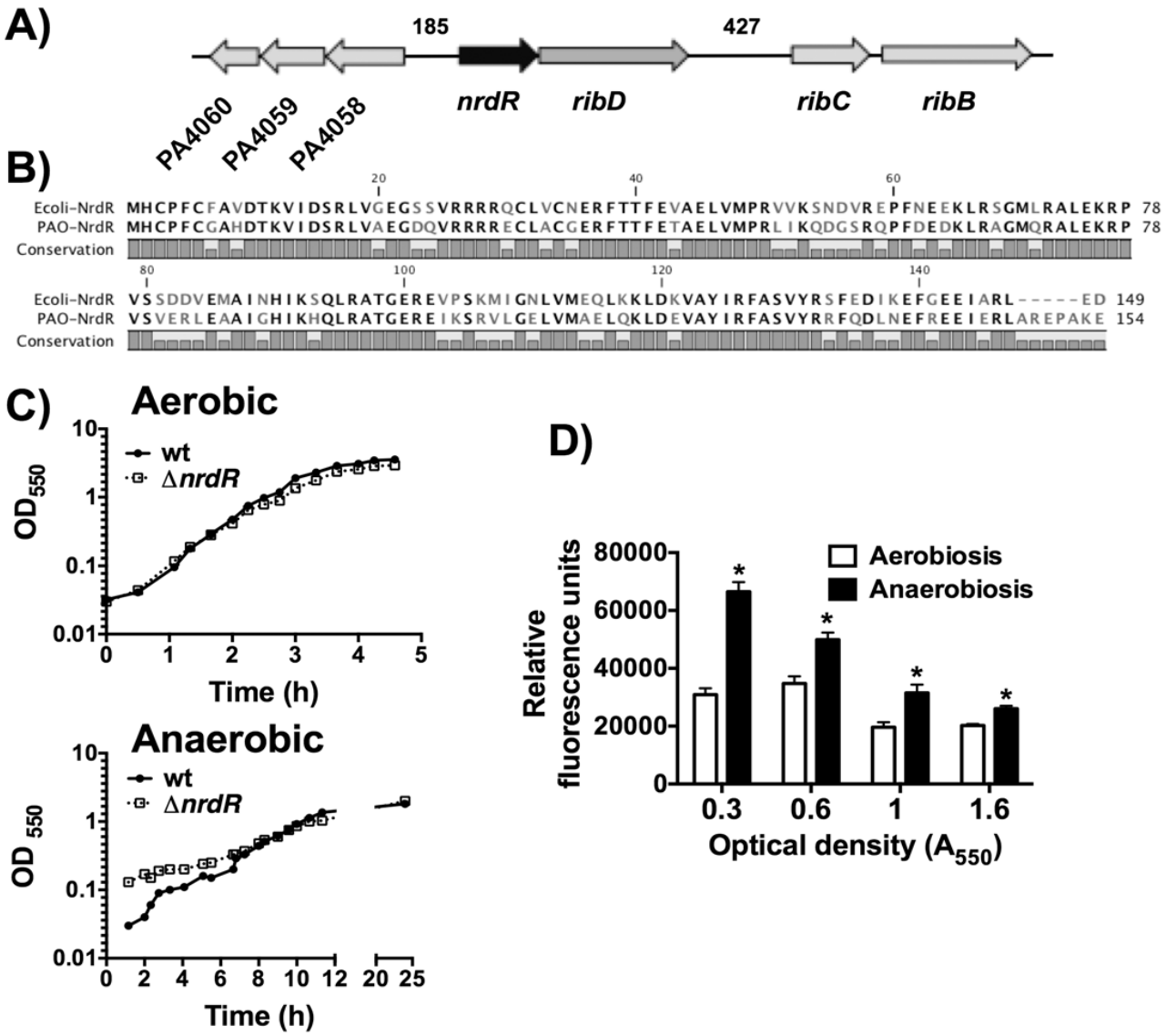

Fig 1. *nrdR* operon organization and expression. A) Gene organization scheme of the *nrdR-rib*D operon. **B)** Sequence alignment (Clustal W) of *P. aeruginosa* (PAO-NrdR; Uniprot Q9HWX1) and *Escherichia coli* (Ecoli-NrdR; Uniprot P0A8D0) NrdR proteins. **C)** Aerobic and anaerobic growth curve of *P. aeruginosa* strains PAO1 (wild-type) and PW7855 (Δ*nrdR*). **D)** Fluorescence (GFP) was measured in both strains harboring pETS161 (P*nrdR*-GFP) at different points of growth, at 37ºC in LB medium. The fluorescence was normalized dividing by the optical density (A_{550}), and it is given in relative fluorescence units. Each experiment was repeated three times, and the results are the mean ± standard deviation. *: Significantly different compared with wild-type strain in an unpaired *t*-test (*P*<0.05).

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structure similar to all NrdR proteins [13]. In a 149 amino-acid overlap, *P. aeruginosa* NrdR showed 70% identity and 82% similarity with *E. coli* NrdR (see alignment in Fig 1B).

Contrary to the observation in the initial IS insertion *nrdR* mutant in *E. coli* [12], a *P. aeruginosa nrdR* mutant showed a similar growth curve to that of the wild-type strain, under both aerobic and anaerobic conditions (Fig 1C). The insertion sequence element disrupting the

nrdR gene (strain PW7855, from now on Δ*nrdR*) contains an internal promoter that allows the expression of downstream genes, as indicated by the authors [20].

As shown in Fig 1D, the transcriptional fusion of the *nrdR* promoter to the green fluorescent protein (GFP) (see materials and methods) revealed an increased *nrdR* expression in exponential phase and a decrease in stationary phase, under both aerobic and anaerobic growing conditions. Clearly, the NrdR protein is expressed at higher levels during the exponential growth phase, particularly under anaerobic conditions.

NarL is responsible for the anaerobic expression of the *nrdR* gene

We investigated the molecular mechanism that carries out the transcriptional activation of the *nrdR* gene under anaerobic conditions. An initial examination of the *nrdR* promoter region, using the Virtual Footprint tool from the PRODORIC database [21], revealed two heptameric NarL-binding sites located at 18 and 40 bp upstream of the translation start codon, denoted here as NarL1 (CTACCAT) and NarL2 (TACGCCT) boxes (Fig 2). To confirm the bioinformatical prediction, the two putative heptameric NarL-binding sites (NarL1 and NarL2 boxes) were mutated.

Three mutations were performed in each box, focusing on the most important nucleotides according to the published *P. aeruginosa* NarL consensus binding sequence ($TAC^{C}/_{T}N^{A}/_{C}T$) [22]. Therefore, plasmids harboring the different mutations in NarL1 and NarL2 boxes were made (pETS181 to pETS187, see Table 1). A decrease of the promoter expression under anaerobic conditions was observed, compared with the wild-type promoter (pETS161) (Fig 2, S1 Fig). The activities obtained when mutating NarL1 and NarL2 boxes were similar to those obtained for the wild-type promoter region (pETS161) in the *narL* knockout strain (PW7549; from now on Δ*narL*) (Fig 2). These results confirm a direct activation of the *nrdR* expression via binding of NarL.

NrdR regulates the expression of the three different ribonucleotide reductase classes

To study whether the NrdR protein regulates the expression of the different *nrd* genes, we measured the expression of the different *nrd* promoters in *P. aeruginosa* wild-type and a Δ*nrdR* mutant strain (PW7855), using plasmids carrying a transcriptional fusion of each RNR promoter region and the *gfp* reporter gene (see materials and methods, and [7]).

Under aerobic conditions (Fig 3A–3C), all three *nrd* genes (*nrdA*, *nrdJa* and *nrdD*) showed an evident increase in their expression (from 3 to 6-fold) in the *nrdR* mutant, compared with the wild-type strain, indicating that NrdR acts as its repressor. The maximal difference appeared in the transcription of the class II RNR (*nrdJa*). Note that the aerobic transcription of the *nrdJa* and *nrdD* genes was approximately 8–10 times lower compared with the *nrdA* gene and highly expressed under anaerobic conditions in the wild-type strain, compared to aerobic conditions.

A completely different expression pattern was observed under anaerobic conditions (Fig 3D–3F). Expression of the *nrdA* gene slightly increased in the *nrdR* mutant (1.3-fold, Fig 3D). Expression of *nrdJa* is down-regulated in the *nrdR* mutant, and no change in *nrdD* expression was observed compared with the wild-type strain (Fig 3E and 3F). Under all conditions, complementation with the *nrdR* gene cloned into plasmid pUCP20T (pETS176) returned the expression to the wild-type level.

One of the two putative NrdR boxes that were identified in all RNRs promoters (S3 Fig) was mutated by PCR-based site-directed mutagenesis. Plasmids harboring the mutant promoter

Constructions	Sequence	Strains	RFU ±SD	Ratio
	NarL box1 **NarL box2**			
pETS161	cgcggatgcg**ctaccat**gcggcttccttcagc**tacgcct**gtatcagcacc**ATG**	wt	495664 ±15181	100%
pETS161	cgcggatgcg**ctaccat**gcggcttccttcagc**tacgcct**gtatcagcacc**ATG**	Δ*narL*	354540 ±8914*	71%
	NarL box1 mutations			
pETS181	cgcggatgcg**cCCAATt**gcggcttccttcagc**tacgcct**gtatcagcacc**ATG**	wt	327658 ±6400*	66%
pETS182	cgcggatgcg**ctGGcat**gcggcttccttcagc**tacgcct**gtatcagcacc**ATG**	wt	243025 ±3033*	49%
pETS183	cgcggatgcg**ctGccat**gcggcttccttcagc**tacgcct**gtatcagcacc**ATG**	wt	309904 ±4057*	62%
	NarL box2 mutations			
pETS184	cgcggatgcg**ctaccat**gcggcttccttcagc**ATAATTA**gtatcagcacc**ATG**	wt	279610 ±6003*	56%
pETS185	cgcggatgcg**ctaccat**gcggcttccttcagc**tGGgcct**gtatcagcacc**ATG**	wt	306321 ±2615*	61%
pETS186	cgcggatgcg**ctaccat**gcggcttccttcagc**tGcgcct**gtatcagcacc**ATG**	wt	317780 ±7775*	64%
	NarL box1 and box2 mutations			
pETS187	cgcggatgcg**cCCAATt**gcggcttccttcagc**ATAATTA**gtatcagcacc**ATG**	wt	336341 ±2008*	67%

Fig 2. NarL-dependent expression of *nrdR*. A) Representation of the *P. aeruginosa* PAO1 *nrdR* promoter region sequence, indicating the different mutated NarL binding sites. Black boxes indicate the putative NarL recognition sites, and mutated sequences are shown in upper case and in bold letters. The transcription start site is indicated in bold. The RFU column shows the relative fluorescence intensity presented by the *P. aeruginosa* wild-type *nrdR* promoter fusion (pETS161), compared with their mutated NarL boxes (pETS181, pETS182 and pETS183 for NarL box1, pETS184, pETS185 and pETS186 for NarL box2, and pEST187 harboring the double mutation). The expression of wild-type *nrdR* promoter under a Δ*narL* mutant background is also stated. The ratio column shows a comparison of all the conditions with the expression of a wild-type promoter under a wild-type background. Strains were grown anaerobically until the mid-logarithmic phase. Values represent the mean of three independent experiments. Transcriptional start codon is shown in bold. Three independent experiments were performed and the mean±standard deviation is shown). *: Significantly different compared with wild-type promoter region (pETS161) in an unpaired *t*-test ($P<0.05$).

doi:10.1371/journal.pone.0123571.g002

confirmed our previous results, hence indicating the functionality of the NrdR boxes (S2 and S3 Figs).

To correlate the transcriptional data with mRNA quantity, we measured the levels of mRNA for each RNR class in wild-type cells and Δ*nrdR* mutant strain by real-time PCR (Fig 3G and 3H) at mid-logarithmic phase (A_{550} = 0.6). Aerobically, (Fig 3G) all RNR genes were highly expressed (from 13 to 56 times) in the *nrdR* mutant compared with the wild-type. By contrast, anaerobically (Fig 3H), the *nrdA* gene slightly increased its expression (2.1 times), and no effect was observed on the transcription of the *nrdJa* and *nrdD* genes in the *nrdR* mutant compared with the wild-type strain, corroborating our transcriptional fusion expression results.

As expected, when we inactivated the *nrdR* gene, the dNTP pool levels observed were 25% higher compared with the wild-type strain (Fig 3I), suggesting that we eliminated the NrdR repressor, and, therefore, increased global RNR activity under all conditions.

Finally, we aimed to address the effect of the NrdR regulation at a physiological level by changing the levels of dNTPs as seen by other authors [23,24]. Hydroxyurea is a known radical

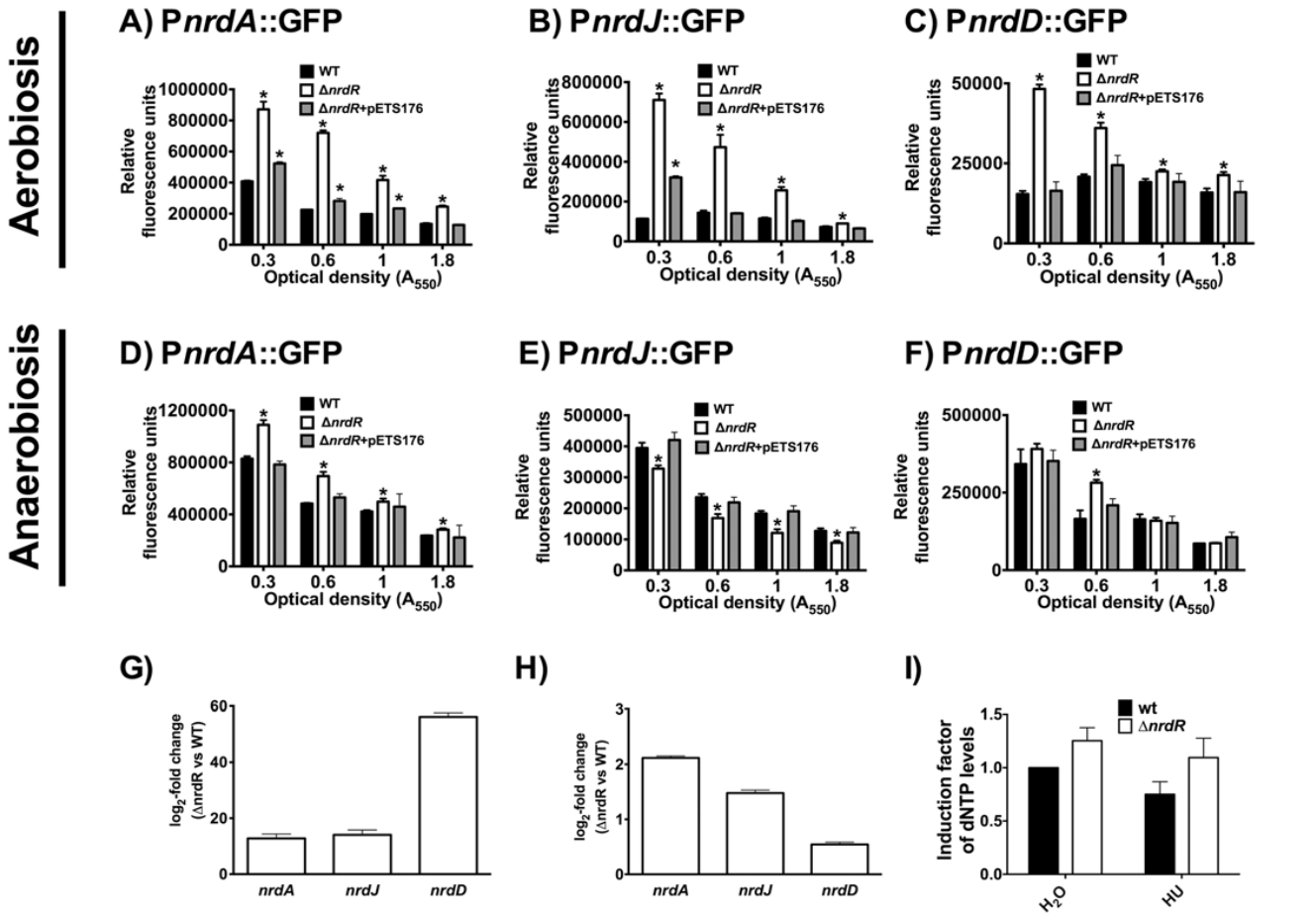

Fig 3. NrdR differentially regulates ribonucleotide reductase genes in aerobiosis or anaerobiosis. Aerobic expression studies are shown in **A-C** and **G**, and anaerobic expression studies in **D-F** and **H**. *P. aeruginosa* wild-type strain (black bars), Δ*nrdR* mutant strain (white bars) and the deficiency-complemented *nrdR* strain (Δ*nrdR*+pETS176) (gray bars) bearing the promoter fusions P*nrdA-gfp* (panel **A** and **D**), P*nrdJ-gfp* (panel **B** and **E**) and P*nrdD-gfp* (panel **C** and **F**), were grown as indicated in the material and methods. GFP fluorescence is expressed as arbitrary units subtracting the reads of the control plasmid pETS130. **G**) and **H**) Quantitative RT-PCR analysis of genes encoding three different classes of RNR. qRT-PCR was conducted on cDNA synthesized from wild-type, compared with Δ*nrdR* cells, both grown aerobically (A_{550} = 0.6) (**G**) and anaerobically (A_{550} = 0.6) (**H**). The means of three independent experiments are displayed, and the error bars represent the positive standard deviation **I)** dNTPs pool level of aerobic *P. aeruginosa* wild-type and *nrdR* mutant cells treated with 10 mM hydroxyurea (HU), measured by DPA assay. DNA contents were normalized with those of wild-type strain. Three independent experiments were performed and the mean ± standard deviation is shown. *, Significantly different compared with the wild-type strain in an unpaired *t*-test (P<0.05).

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scavenger that inhibits class Ia RNR catalytic activity, thus reducing the amounts of dNTPs. When 10 mM hydroxyurea were added to the medium during aerobic growth, the dNTP amounts were 25% lower. This reduction could be restored in an *nrdR* mutant strain (enhancing class II RNR activity) which returns to wild-type dNTPs levels (Fig 3I).

NrdR represses *topA* expression

All genes that have been described as transcriptionally regulated by NrdR were ribonucleotide reductase encoding genes. Rodionov and Gelfand [11] and recent databases (RegPrecise; http://regprecise.lbl.gov/RegPrecise/index.jsp) highlighted the possible implication of NrdR in

the regulation of the *P. aeruginosa* DNA topoisomerase I gene *topA* (PA3011), identifying a single putative NrdR box in its promoter region (see S3 Fig).

Expression of the *topA* gene during exponential growth under aerobic or anaerobic conditions was repressed in the *nrdR* mutant (2–3 times) compared with the wild-type strain, suggesting that NrdR acts as a *topA* activator during the exponential growth phase (Fig 4).

Complementation with an extra *nrdR* gene (pETS176) returned the *topA* expression level to the wild-type levels. When the NrdR binding box was mutated in the promoter *topA* region (pETS178) the expression levels were similar to the levels found in the Δ*nrdR* strain, therefore corroborating the functionality of the unique NrdR-binding region on the *topA* promoter region (Fig 4, S3 Fig).

The degree of supercoiling in bacterial DNA is determined by the balance between DNA-relaxing activity and DNA-supercoiling activity, regulated by the opposing actions of topoisomerase I encoded by the *topA* gene and DNA gyrase, respectively [19]. The prokaryotic topoisomerase I is only capable of relaxing negatively supercoiled DNA. To phenotypically corroborate the *topA* down-regulation in the *P. aeruginosa* Δ*nrdR* strain, we analyzed the DNA topology of pUCP20T in an electrophoresis assay in an agarose gel with chloroquine. pUCP20T

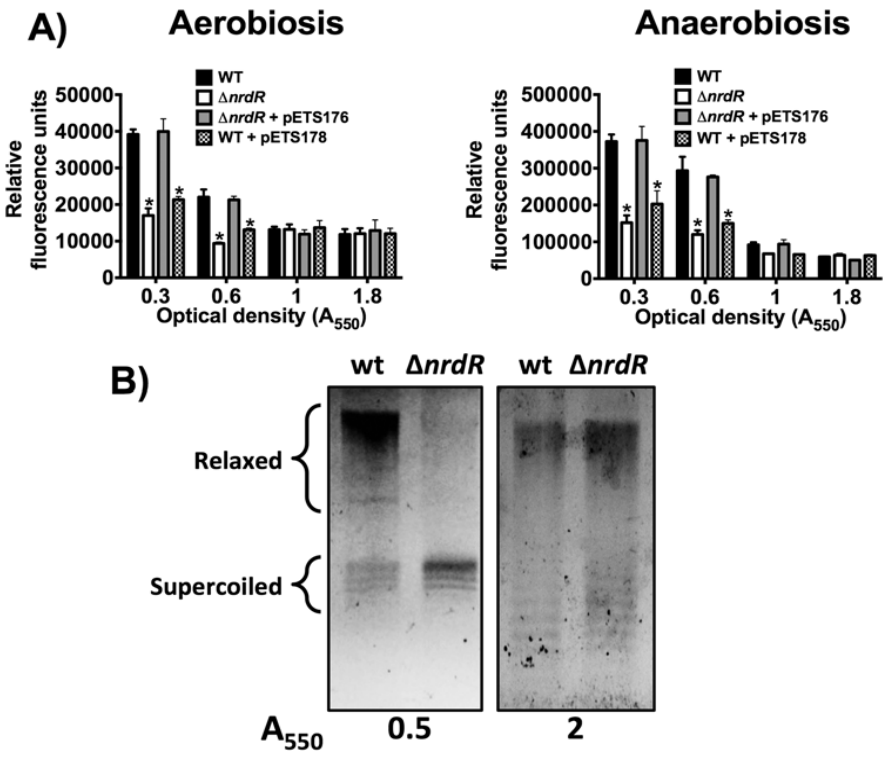

Fig 4. *topA* expression is activated aerobically and anaerobically by NrdR. A) GFP fluorescence was measured in *P. aeruginosa* strains PAO1 (wild-type) and PW7855 (Δ*nrdR*) harboring plasmid pETS177 (P*topA*::GFP). The *nrdR* cloned into plasmid pUCP20T (pETS176) was used to complement *nrdR* deficiency in strain PW7855. Plasmid pETS178 harbors a *topA* promoter with a mutation in the NrdR box. The fluorescence was normalized with the absorbance at 550 nm (A_{550}) and it is given in relative fluorescent units. The bars represent the mean of three independent experiments ± standard deviation. **B)** A gel electrophoresis assay, in an agarose gel containing chloroquine, of plasmid DNA isolated from *P. aeruginosa* wild-type and Δ*nrdR* strains, at mid-logarithmic and stationary phases. The direction of migration was from top to bottom. *, Significantly different compared with the wild-type strain in an unpaired *t*-test ($P<0.05$).

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plasmid extracted from the Δ*nrdR* mutant strain showed more negative supercoiled DNA compared with the wild-type strain (Fig 4B) when measured during exponential growing phase. According to the gene reporter assay, no difference in supercoiled DNA levels appears during stationary growing phase. This result reflects a possible change in DNA topology in the *P. aeruginosa* Δ*nrdR* strain, compared with the wild-type strain.

Global gene expression profiling of the *P. aeruginosa nrdR* mutant strain compared with the wild-type strain

We previously showed that NrdR directly regulates the three *P. aeruginosa* RNR classes and topoisomerase I (*topA*), all of which are involved in bacterial DNA replication. To determine the global transcriptional changes produced by a mutation in *nrdR*, we initiated a gene profiling experiment using the Affymetrix *P. aeruginosa* GeneChip microarray platform.

RNA was isolated from a *P. aeruginosa* PAO1 wild-type strain and a Δ*nrdR* mutant strain, both grown aerobically and anaerobically in LB medium to mid-logarithmic growth phase. Labeled RNA was hybridized to Affymetrix *P. aeruginosa* GeneChips and gene expression levels between Δ*nrdR* mutant and wild-type strains were compared.

Results showed altered transcription levels in only a few genes, comparing the Δ*nrdR* mutant strain to the wild-type strain. Aerobically only 47 genes (0.8%) were significantly deregulated, with 31 genes up-regulated (0.5%) and 16 genes down-regulated (0.3%). Anaerobically, 111 genes were differentially regulated, with 26 genes up-regulated (0.45%) and 85 genes down-regulated (1.45%). Only few genes were expressed or repressed more than 3 $\log_2$ fold change (Fig 5B). To corroborate our array a selection of some deregulated genes was measured by quantitative PCR (S5 Fig).

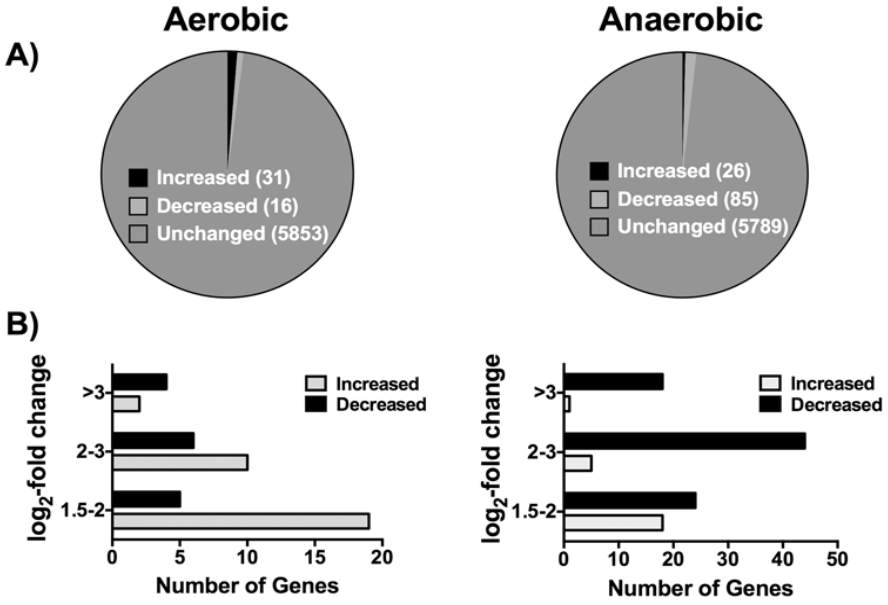

Fig 5. Summary of the effects of the *nrdR* mutation on *P. aeruginosa* gene expression under aerobic and anaerobic conditions. A) Distribution of the different genes (up-regulated, down-regulated and unchanged) in gene expression (>1.5 Log_2 fold change). The number of gens in each category is indicated. **B)** Distribution of genes whose expression was either increased or decreased in a Δ*nrdR* mutant strain, grouped according to fold-changes in expression levels.

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Under aerobic conditions, among the most up-regulated genes we found the fimbrial subunit *cupA1* (log_2-fold change of 4.13), several hypothetical proteins (PA4139, PA1383 and PA2223 with log_2-fold changes of 3.78, 2.42 and 2.37, respectively) and antibiotic resistance related genes, such as the entire *mexAB-orpM* operon (log_2-fold change from 2.13 to 1.43) (Table 2 and S2 Table). In addition the RNR genes were found to be up-regulated, as expected (log_2-fold changes from 2.41 and 1.96). The highest repression under this condition was found in several hypothetical proteins (PA3283, PA3281, PA0565 with log_2-fold changes of -4.57,

Table 2. Global transcriptomic analysis of a Δ*nrdR* mutant strain compared with the *P. aeruginosa* PAO1 wild-type strain.

ID	*Gene*	*Operon arrangement*	Log2 Fold-Change	*Gene Product*
Aerobic				
PA2128	*cupA1*	*cupA12345*	4.13	Fimbrial subunit CupA1
PA1383			2.42	Hypothetical protein
PA4139			3.78	Hypothetical protein
PA5497	*nrdJa*	*nrdJab*	2.41	Class II (cobalamin-dependent) ribonucleotide-diphosphate reductase subunit, NrdJa
PA1718	*pscE*	*pscBCDEFGHIJKL*	2.32	Type III export protein PscE
PA1156	*nrdA*	*nrdAB*	2.24	Ribonucleoside reductase, large chain
PA0992	*cupC1*	*cupC123*	2.19	Fimbrial subunit CupC1
PA0425	*mexA*	*mexAB-oprM*	2.13	Resistance-Nodulation-Cell Division (RND) multidrug efflux membrane fusion protein
PA0424	*mexR*		2.06	Multidrug resistance operon repressor MexR
PA1693	*pscR*	*PA1697-pscOPQRSTU*	2.00	Translocation protein in type III secretion
PA1155	*nrdB*	*nrdAB*	1.96	Ribonucleoside reductase, small chain
PA0426	*mexB*	*mexAB-oprM*	1.93	Resistance-Nodulation-Cell Division (RND) multidrug efflux transporter MexB
PA4086	*cupB1*	*cupB123456*	1.89	Probable fimbrial subunit CupB1
PA1723	*pscJ*	*pscBCDEFGHIJKL*	1.63	Type III export protein PscJ
PA0958	*oprD*		1.61	Basic amino acid, basic peptide and imipenem outer membrane porin OprD precursor
PA0427	*oprM*	*mexAB-oprM*	1.43	Major intrinsic multiple antibiotic resistance efflux outer membrane protein Opr
PA2491	*mexS*		-2.17	Hypothetical protein (MexEF-OprN regulator)
PA0998	*pqsC*	*pqsABCDE*	-2.83	Homologous to beta-keto-acyl-acyl-carrier protein synthase
Anaerobic				
PA1718	*pscE*	*pscBCDEFGHIJKL*	2.23	Type III export protein PscE
PA0958	*oprD*		1.76	Basic amino acid, basic peptide and imipenem outer membrane porin OprD precursor
PA3616		*recA-PA3616*	-1.61	Hypothetical protein
PA3008		*lexA-PA3008*	-1.81	Hypothetical protein
PA3007	*lexA*	*lexA-PA3008*	-1.88	Repressor protein LexA
PA3617	*recA*	*recA-PA3616*	-1.96	RecA protein
PA0807	*ampDh3*		-2.44	Beta-lactamase expression regulator AmpD
PA2485		*PA2485-PA2486*	-2.66	Hypothetical protein
PA4763	*recN*		-2.71	DNA repair protein RecN
PA2486	*ptrC*	*PA2485-PA2486*	-2.94	Hypothetical protein (T3SS regulator)
PA2494	*mexF*	*mexEF-oprN*	-3.01	Resistance-Nodulation-Cell Division (RND) multidrug efflux transporter MexF
PA2484			-3.55	Hypothetical protein

Selected differentially regulated genes, under both aerobic and anaerobic conditions. Complete list of all the genes (>1.5-fold) is available in S2 and S3 Table.

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-3.73, -3.06) and also several genes involved in antibiotic resistance, such as the entire *mexEF-oprN* operon (log_2-fold change from -3.19 to -1.13).

Under anaerobic conditions, despite the hypothetical proteins (PA5507 and PA5509, with log_2-fold changes of 3.21 and 2.99), *mexS* and *pyoS5* were the more strongly repressed genes (log_2-fold changes of -4.69 and -3.9) (Table 2 and S3 Table).

Classifying the transcriptionally altered genes in metabolic categories [2] (S4 Fig), the categories in which more genes were included were antibiotic resistance, antibiotic susceptibility and small molecules transportation. By contrast, under anaerobic conditions, the main metabolic category with altered transcription was small molecules transportation.

NrdR is not essential during *P. aeruginosa* infection

We have previously shown that the *nrdJ* and the *nrdD* genes of *P. aeruginosa* are important during infection of *Drosophila melanogaster* [7]. As the NrdR regulates the expression of the different *nrd* genes, both aerobically and anaerobically, we wondered whether a mutant for this transcriptional regulatory protein is important during bacterial infections.

Injections of the same number of cells of a wild-type strain and a Δ*nrdR* mutant strain showed exactly the same killing behavior in flies (Fig 6), showing a 50% death rate 25 h post-infection. Therefore, this situation does not alter the virulence capacity of PAO to infect flies despite presenting an up-regulation of all *nrd* genes in the Δ*nrdR* mutant strain.

Discussion

Ribonucleotide reductases are key enzymes for all living cells, as they are responsible for the dNTP supply that is essential for DNA synthesis and repair. Eukaryotic cells encode only one type of RNR, class Ia, which is responsible for providing the different dNTPs under all conditions. Surprisingly, prokaryotic cells, which can be considered *a priori* as less complex organisms, have the capacity to encode different RNR classes in the same genome [25,26]. The

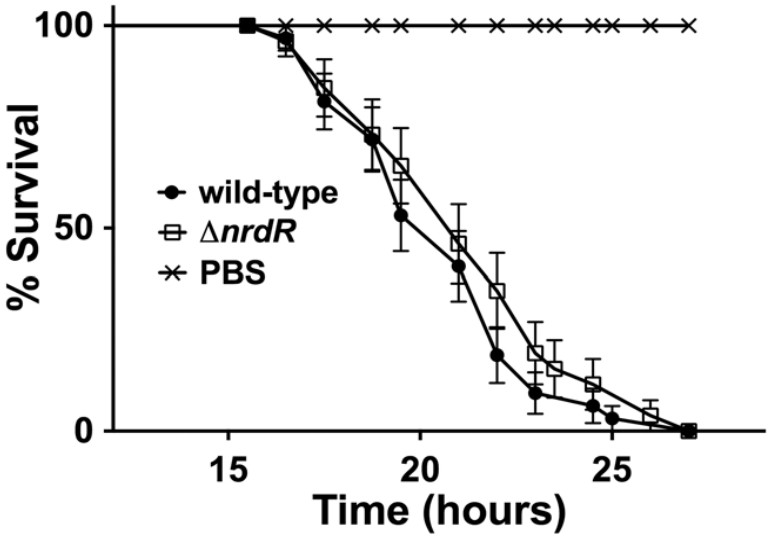

Fig 6. The *nrdR* mutant of *P. aeruginosa* does not alter the kinetics of *D. melanogaster* killing. Control flies were injected with PBS. Fly survival was monitored for 48 h. Approximately 100 flies were used for each experiment.

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presence of different paralogous genes performing the same enzymatic activity is astonishing, leading us to question why prokaryotes encode different RNR classes. Addressing this question is crucial for the understanding of the transcriptional regulation of each RNR class.

The NrdR factor was first identified in *S. coelicolor* [10], and proposed, by phylogenetic profiling, as a potential transcriptional regulator of different RNR genes [11], which was later confirmed in *E. coli* [12,13] and mycobacteria [27]. In our study, we aimed to gain insight into the role of this transcriptional factor in *P. aeruginosa*; this is the first attempt to study NrdR-related regulation in an organism in which all three RNR classes are encoded [7,28]. The *nrdR* gene also presents a unique genomic context in this bacterium: a polycistronic transcript encoding for *nrdR* and *ribD* genes can be detected (unpublished data), as an evidence of a *nrdR-ribD* operon (Fig 1A), instead of the longer operon that is present in other γ-Proteobacteria (*nrdR-ribD-ribH-nusB*) [11,12].

We showed that NrdR is transcribed under both aerobic and anaerobic conditions, but increases substantially during anaerobic growth, and especially in the exponential growth phase (Fig 1). This increase can be explained through the transcriptional activation by NarL, a transcription factor that is strongly related to anaerobic growth [29], and according to the presented results (Figs 1 and 2, S1 Fig) activates *nrdR* transcription by binding and interacting with two NarL boxes located at the *nrdR* promoter region.

As expected, NrdR regulates all three RNR classes, but surprisingly, it acts differently during aerobic or anaerobic growth. Aerobically, NrdR acts as a repressor of all RNR genes (Fig 3), although maximum repression is exerted on class II and class III RNRs, while class Ia repression is less strict, conforming fully with the hypothesis that class Ia supports aerobic growth in this bacterium [3,7,8,28,30]. By contrast, NrdR does not repress class II and class III expression under anaerobic conditions, showing only a slight repression of class Ia RNR: as class II and III RNRs support the *P. aeruginosa* anaerobic growth [7]; this also fits with our model (Fig 7). The results of the gene reporter assay were confirmed by qRT-PCR (Fig 3), therefore providing strong evidence supporting our model.

Two NrdR boxes have been identified in each RNR gene promoter (S2 and S3 Figs). Although we could not obtain pure soluble NrdR to perform DNA gel shift assays (all attempts to overproduce this protein lead to the formation of inclusion bodies), the results of the gene reporter assay with a mutation in the identified NrdR boxes agree with our previous results [12];

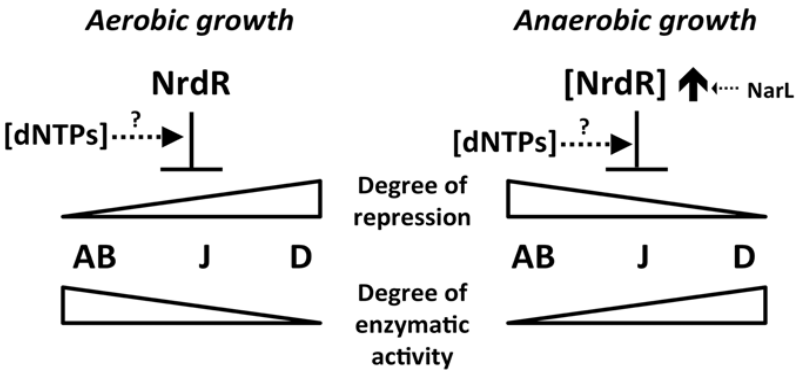

Fig 7. Model of NrdR-related control on RNR gene expression. The degree of repression on each RNR class expression, under aerobic or anaerobic conditions, is opposite to the enzymatic activity of these classes under each condition. Considering the presence of an ATP-cone domain in NrdR, dNTPs level alterations could also be affecting the results.

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in all cases we have mutated the NrdR-box2 (because NrdR-box1 overlaps with the -10 promoter sequence), and we have obtained similar expression levels compared with the Δ*nrdR* mutant strain demonstrating the physiological role of these DNA regions to bind a functional NrdR.

To this point we can assume that we have strong evidence to support our working model (Fig 7); RNR activity in *P. aeruginosa* is controlled by the NrdR factor, which acts by binding in the NrdR boxes in all RNR gene promoters (it is believed that it can act by forming a dimer to bind the two characteristic boxes), and repressing RNR activity according to the needs of the bacterium: high repression of class II and III RNRs under aerobic conditions, and repression of class I only under anaerobic conditions.

As the NrdR protein harbors an ATP-cone domain that is able to bind nucleotides, it seems logical to assume that NrdR activity is modulated by differential nucleotide binding [13,14] so that high dNTP levels (indicating high RNR activity) might activate NrdR-related repression. To do so, its ATP-cone is likely to be fully occupied (to its allosterically controlled limit) with ATP in usual situations, but competent to bind dATP and act as a dNTP pool sensor [14]; the binding of the nucleotide should control the oligomeric state of NrdR by a conformational change in the zinc-finger domain, and thereby modulate its interaction with the NrdR boxes. To evaluate this control we compared RNR transcription in wild-type and Δ*nrdR* mutant strains while treating with hydroxyurea (decreasing dATP levels, and hence modulating the bound nucleotide) but we did not obtain significant results (data not shown), most likely because treatment with hydroxyurea only affected class Ia RNR activity (and not class II and class III RNRs), and therefore the dNTP pool slightly decreased (Fig 3) and class II was fully active [7]. In other studies in *S. typhimurium* and *Chlamydia sp* [23,24], hydroxyurea treatment completely abolished the dNTP supply, making this type of study far easier. In our model the role of the alteration of the dNTP pool in the fine-tuning transcriptional regulation of the different RNR genes by NrdR is still inconclusive. Despite the lack of data in our working model, which was still not completely set, we hypothesized that NrdR could be responding to alteration of the dNTP pool, inhibiting RNR gene transcription when necessary. In contrast to anaerobic conditions when only class Ia RNR is affected by NrdR, during aerobic growth NrdR is able to down-regulate all RNR gene transcription, so this response to increased dNTPs may be its main role under this condition. This model is summarized in Fig 7.

Moreover, we also identified for the first time that NrdR is not only involved in RNR activity regulation but also regulates *topA* expression, a gene encoding for *P. aeruginosa* topoisomerase I. The *topA* promoter region presents only a single NrdR putative binding site (S2 Fig), suggesting different NrdR binding and regulation on this gene compared to RNR genes. In agreement with this hypothesis, we have shown that NrdR up-regulates *topA* transcription, instead of repressing it (Fig 4). As with RNR activity, we confirmed this effect at a physiological level: as TopA relaxes negative supercoiled DNA, a high level of negative supercoiled DNA appears in a Δ*nrdR* mutant strain, during exponential growing phase, without NrdR-related topA activation (Fig 4B).

There were no more promoters harboring putative NrdR boxes, but, according to our global transcriptomic analysis results, in a Δ*nrdR* mutant strain, a small but significant group of 47 genes (log_2-fold change>1.5) was deregulated during aerobic conditions, and 111 genes (log_2-fold change>1.5) presented similar behavior during anaerobic conditions (Fig 6, S2 and S3 Tables). Among those genes we can identify some related to the SOS system, antibiotic resistance, transport of small molecules, etc (S4 Fig). This deregulation does not lead to a loss of infectivity (Fig 6). Given the absence of putative NrdR-boxes on the promoter regions of the deregulated genes detected in the array, we believe the change in the expression of these genes to be due to indirect effects. We propose that TopA down-regulation in the absence of NrdR may alter gene

transcription by changing DNA topology and causing the accumulation of cleavage complexes. For instance, it has been described that SOS system can be deregulated by TopA depletion during antibiotic treatment [31]. In addition, some of the deregulated genes appear to also show altered transcription in a Δ*topA* mutant strain, according to a previous transcriptomic assay by the Lawrence G. Rahme group (unpublished data, Gene Expression Omnibus GSE24038).

The difference observed between the two groups of deregulated genes under aerobic and anaerobic conditions provides further evidence of the differential behavior of NrdR in *P. aeruginosa* as we have proposed, although the NarL-related activation and the dNTP-binding modulation may not be the only systems acting on this regulation.

In summary, this study has provided evidence of control of the three RNR operons and the *topA* gene by NrdR in *P. aeruginosa*, which is a differential control sensitive to oxygenation conditions and the growth phase. This control clearly plays an important role in the coordination of the expression of the different RNRs, dictating which RNR is expressed under certain growing conditions. By studying this and other factors controlling RNR activity we will be nearer to an explanation of the apparent redundancy among the three RNR classes, and to an understanding of how this bacterium uses all three classes to survive under different environmental conditions.

Supporting Information

S1 Fig. NarL-dependent expression of *nrdR*. Fluorescence intensity measurements of *P. aeruginosa nrdR* promoter fusions compared with their mutagenized NarL boxes (box1 and box2, three different mutations in each one), expressed in relative fluorescence units. The experiment was performed in a wild-type *P. aeruginosa* background (pETS161 (wt), pETS181 (box1), pETS182 (box1.2), pETS183 (box1.3), pETS184 (box2), pETS185 (box2.2), pETS186 (box2.3) and pEST187 (box1 and 2)) and in a Δ*narL* background (only wt promoter, pETS161). Strains were grown anaerobically until the mid-logarithmic phase. Values represent the mean of three independent experiments. *: Significantly different compared with wild-type promoter region (pETS161) in an unpaired *t*-test ($P<0.05$).
(TIFF)

S2 Fig. NrdR binding sites in the three *P. aeruginosa* PAO1 RNRs and *topA* promoter regions. An overview of the entire operon is shown, with open rectangular frames indicating the two NrdR boxes in RNR promoters and the one in the *topA* promoter. The detailed sequence of the area surrounding the boxes is displayed below; the predicted NrdR binding sites are indicated by the nucleotides in bold and black boxes. The position of the NrdR boxes is given relative to the translation start codon of the first gene of the *nrd* operon, as previously described (Rodionov DA and Gelfand MS (2005) Identification of a bacterial regulatory system for ribonucleotide reductases by phylogenetic profiling. Trends in Genetics 21:385–389).
(TIF)

S3 Fig. Site-directed mutagenesis of the predicted NrdR box in RNR promoters and *topA* promoter. Representation of RNR and *topA* promoters' region sequence of *P. aeruginosa* strain PAO1 indicating the NrdR binding sites. Black boxes indicate NrdR recognition sites, and the NrdR box2 mutated residues are shown in upper case and in bold letters. Fluorescence measurements of *P. aeruginosa* RNR promoter fusions (pETS134, pETS180 and pETS136) and P*topA* (pETS177) compared with their mutagenized NrdR mutated box2 (pETS188, pETS189, pETS190 and pETS178, respectively) were measured in relative fluorescence units (RFUs) in a wild-type *P. aeruginosa* background and in a Δ*nrdR* background. Strains were grown aerobically and anaerobically until the mid-logarithmic phase. Values represent the mean of three

independent experiments. *: Significantly different compared with wild-type promoter region (pETS161) in an unpaired *t*-test ($P<0.05$).
(TIF)

S4 Fig. Distribution of deregulated genes in the global transcriptional analysis according to assigned metabolic classes [2].
(TIFF)

S5 Fig. Transcriptional analysis of deregulated genes on global transcriptional analysis of a Δ*nrdR* mutant strain. Total RNA was reverse transcribed with gene-specific primers as described in Materials and Methods. The analysis demonstrates the specificity of global transcriptional analysis in the absence of *nrdR*. *gapA* was used as internal standard.
(TIF)

S1 Table. Primers and probes used in this study.
(PDF)

S2 Table. Global transcriptomic analysis of a Δ*nrdR* mutant strain compared with *P. aeruginosa* PAO1 wild-type strain grown aerobically.
(PDF)

S3 Table. Global transcriptomic analysis of a Δ*nrdR* mutant strain compared with *P. aeruginosa* PAO1 wild-type strain grown anaerobically.
(PDF)

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Author Contributions

Conceived and designed the experiments: AC LP ET. Performed the experiments: AC LP ET. Analyzed the data: AC LP ET. Contributed reagents/materials/analysis tools: AC LP ET. Wrote the paper: AC LP ET.

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Supplementary Material

Figure S1: NarL-dependent expression of *nrdR*. Fluorescence intensity measurements of *P. aeruginosa nrdR* promoter fusions compared with their mutagenized NarL boxes (box1 and box2, three different mutations in each one), expressed in relative fluorescence units. The experiment was performed in a wild-type *P. aeruginosa* background (pETS161 (wt), pETS181 (box1), pETS182 (box1.2), pETS183 (box1.3), pETS184 (box2), pETS185 (box2.2), pETS186 (box2.3) and pEST187 (box1 and 2)) and in a Δ*narL* background (only wt promoter, pETS161). Strains were grown anaerobically until the mid-logarithmic phase. Values represent the mean of three independent experiments. *: Significantly different compared with wild-type promoter region (pETS161) in an unpaired *t*-test ($P<0.05$).

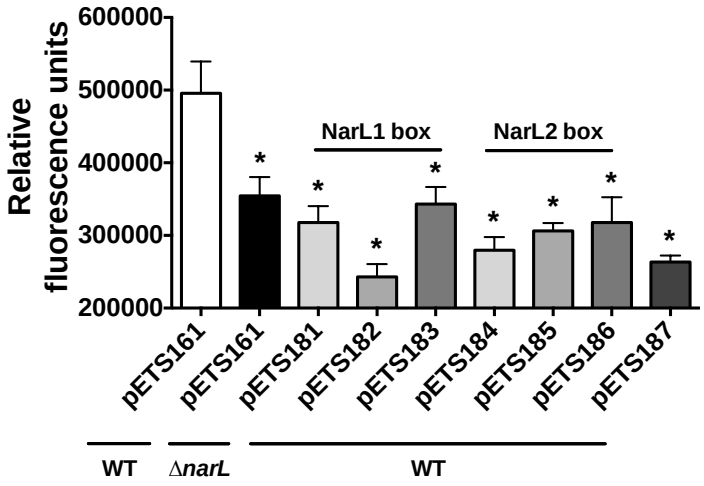

Figure S2: NrdR binding sites in the three *P. aeruginosa* PAO1 RNRs and *topA* promoter regions. An overview of the entire operon is shown, with open rectangular frames indicating the two NrdR boxes in RNR promoters and the one in the *topA* promoter. The detailed sequence of the area surrounding the boxes is displayed below; the predicted NrdR binding sites are indicated by the nucleotides in bold and black boxes. The position of the NrdR boxes is given relative to the translation start codon of the first gene of the *nrd* operon, as previously described (Rodionov DA and Gelfand MS (2005) Identification of a bacterial regulatory system for ribonucleotide reductases by phylogenetic profiling. Trends in Genetics 21:385-389).

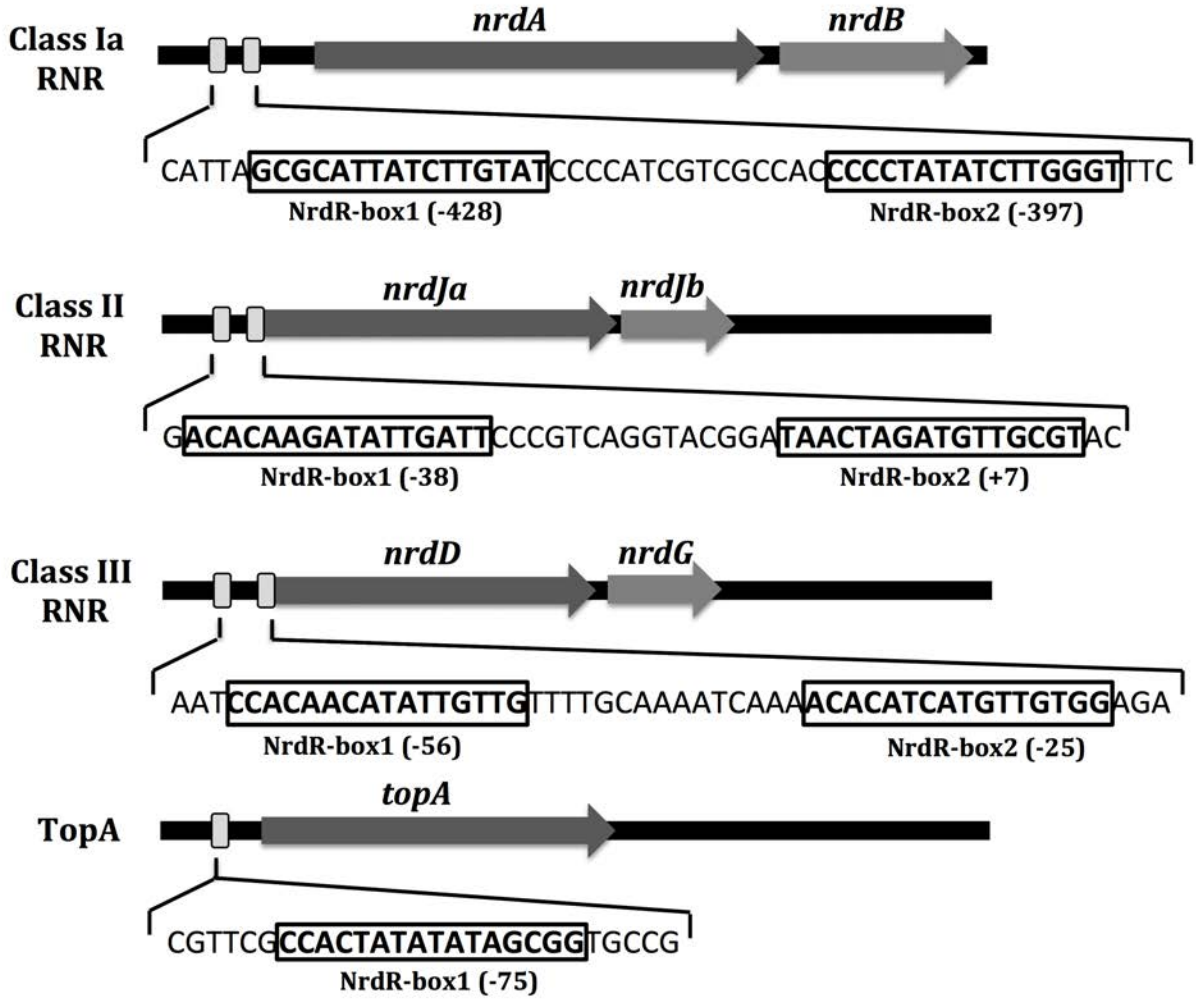

Figure S3: Site-directed mutagenesis of the predicted NrdR box in RNR promoters and *topA* promoter. Representation of RNR and *topA* promoters' region sequence of *P. aeruginosa* strain PAO1 indicating the NrdR binding sites. Black boxes indicate NrdR recognition sites, and the NrdR box2 mutated residues are shown in upper case and in bold letters. Fluorescence measurements of *P. aeruginosa* RNR promoter fusions (pETS134, pETS180 and pETS136) and P*topA* (pETS177) compared with their mutagenized NrdR mutated box2 (pETS188, pETS189, pETS190 and pETS178, respectively) were measured in relative fluorescence units (RFUs) in a wild-type *P. aeruginosa* background and in a Δ*nrdR* background. Strains were grown aerobically and anaerobically until the mid-logarithmic phase. Values represent the mean of three independent experiments. *: Significantly different compared with wild-type promoter region (pETS161) in an unpaired *t*-test ($P<0.05$).

			Aerobic		Anaerobic	
Constructions	**Sequence**	**Strains**	**RFU ±SD**	**Ratio**	**RFU ±SD**	**Ratio**
	NrdR box1 NrdR box2					
	P*nrdA*					
pETS134	attagcgcattatcttgtatccccatcgtcgccaccccctatatcttgggttttc	wt	408141.2 ±2973	100%	113428.8 ±36175	100%
pETS134	attagcgcattatcttgtatccccatcgtcgccaccccctatatcttgggttttc	Δ*nrdR*	871571.3 ±27307*	213.5%	710367 ±72724*	626.2%
pETS188	attagcgcattatcttgtatccccatcgtcgccacGAcctatGCcCCAggttttc	wt	603210.4 ±15645*	147.8%	521431.1 ±26777*	459.7%
	P*nrdJ*					
pETS180	cctgacacaagatattgattcccgtcaggtacggataactagatgttgcgtac	wt	146666.7 ±3013	100%	398534.6 ±50965	100%
pETS180	cctgacacaagatattgattcccgtcaggtacggataactagatgttgcgtac	Δ*nrdR*	530250 ±55841*	361.5%	430670 ±22796*	108%
pETS189	cctgacacaagatattgattcccgtcaggtacggataGAGGgatgGCTcgtac	wt	257274.3 ±8930*	175.4%	378124 ±24946*	94.8%
	P*nrdD*					
pETS136	aatccacaacatattgttgttttgcaaaatcaaaacacatcatgttgtggaga	wt	15385.3 ±1701	100%	359914.2 ±82360	100%
pETS136	aatccacaacatattgttgttttgcaaaatcaaaacacatcatgttgtggaga	Δ*nrdR*	48245 ±2385*	313.5%	280989.6 ±29531*	78%
pETS190	aatccacaacatattgttgttttgcaaaatcaaaacaTGGcCGgCtgtggaga	wt	40334.17 ±2788*	262.1%	276954.7 ±34865*	76.9%
	P*topA*					
pETS177	cgttcgccactatatatagcggtgccgcgctagtcgctccctcctttatattc	wt	39156.4 ±2383	100%	372360.9 ±38648	100%
pETS177	cgttcgccactatatatagcggtgccgcgctagtcgctccctcctttatattc	Δ*nrdR*	17022.5 ±3172*	43.5%	152059.7 ±34076*	40.8%
pETS178	cgttcgcAGTtatGGaCaTcggtgccgcgctagtcgctccctcctttatattc	wt	21336.3 ±1507*	54.5%	202601.3 ±62273*	54.40%

Figure S4: Distribution of deregulated genes in the global transcriptional analysis according to assigned metabolic classes.

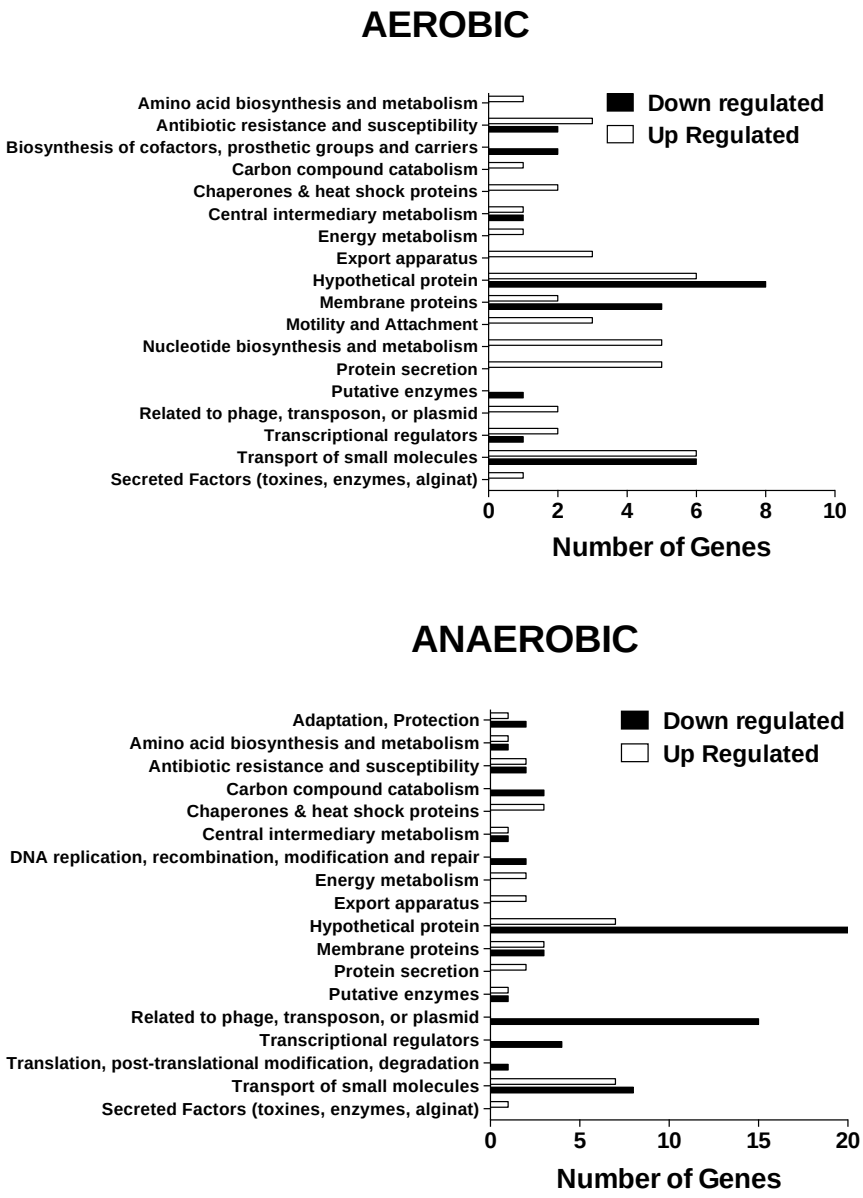

Figure S5: Transcriptional analysis of deregulated genes on global transcriptional analysis of a Δ*nrdR* mutant strain. Total RNA was reverse transcribed with gene-specific primers as described in Materials and Methods. The analysis demonstrates the specificity of global transcriptional analysis in the absence of *nrdR*. *gapA* was used as internal standard.

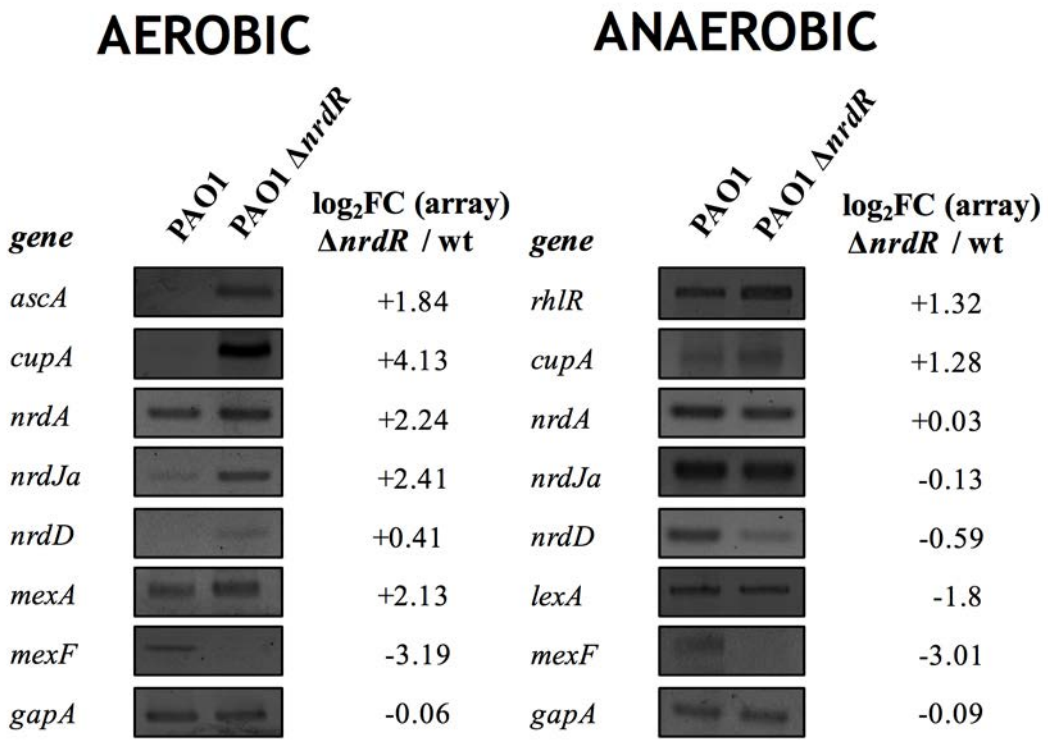

Table S1. Primers and probes used in this study.

Name	Sequence (5'→3')	Application
PnrdRBHI-up	AGGATCCAGGAGAAGGACGGCCAGCAG	Promoter-probe cloning
PnrdRClaI-lw	AAAATCGATATGGTGTCATGGGCACCGCAG	Promoter-probe cloning
NrdRHindIII-low	AAGCTTCATGTACAGCGGGT	Cloning
pBBR1-up	CATCGCAGTCGGCCTATTGG	Check-Cloning
pBBR1-lw	CACTTTATGCTTCCGGCTCG	Check-Cloning
M13-dir	GTTTTCCCAGTCACGAC	Check-Cloning
M13-rev	CAGGAAACAGCTATGAC	Check-Cloning
pUCP20T-up	CCTCTTCGCTATTACGCCAG	Cloning
pUCP20T-low	TCCGGCTCGTATGTTGTGTG	Cloning
PtopA BamHI-up	GGATCCGAAGAGGCGCTGGTGATCTA	Cloning
PtopA ClaI-low	ATCGATGTACTGGCTGCCCAGGTACT	Cloning
PnrdA-up	AGGATCCGAATTCTTGCTCCACACAGCCTC	Cloning/RT-PCR
PnrdA-low	ACCCGGGTTCTCGCGTGTGGTGTCG	Cloning/RT-PCR
PnrdJ BamHI new-up	GGATCCCGCGCCCAGCTGAAGGCC	Cloning/RT-PCR
PnrdJ SmaI new-low	AACCCGGGGACTGCGTTGCGTCTGTC	Cloning/RT-PCR
PnrdD-up	AGGATCCGAATTCGCCCGCCTCGCCCAGG	Cloning/RT-PCR
PnrdD new-low	AATCGATCAGGGTGGCCGGCCAGGTAG	Cloning/RT-PCR
AmR2-up	CAACGACCTATGCCCCAGGTT	Mutation of NrdRbox2 in P*nrdA*
AmR2-low	AACCTGGGGCATAGGTCGTTG	Mutation of NrdRbox2 in P*nrdA*
JmR2-up	GGATAGAGGGATGGCTCGTAC	Mutation of NrdRbox2 in P*nrdJ*
JmR2-low	GTACGAGCCATCCCTCTATCC	Mutation of NrdRbox2 in P*nrdJ*
DmR2-up	AAACATGGCCGGCTGTGGAG	Mutation of NrdRbox2 in P*nrdD*
DmR2-low	CTCCACAGCCGGCCATGTTT	Mutation of NrdRbox2 in P*nrdD*
TmR up	CAGTTATGGACATCGGTGCC	Mutation of NrdRbox2 in P*topA*
TmR low	GGCACCGATGTCCATAACTG	Mutation of NrdRbox2 in P*topA*
nrdATaqM-up	CCCTTCCTGAAAGTGGTCAA	qRT-PCR
nrdATaqM2-low	TGTTCATGTCGTGGGTACG	qRT-PCR
nrdJTaqM-up	CGGGTCAACGAACTGAACA	qRT-PCR
nrdJTaqM2-low	GTAAACACCCGCACCACTTC	qRT-PCR
nrdDTaqM-up	CCGAGATGGACCTGATCAAC	qRT-PCR
nrdDTaqM2-low	CCGAGTTGAGGAAGTTCTGG	qRT-PCR
nrdRTaqM-up	GTTCGACGAGGACAAGCTG	RT-PCR
nrdRTaqM2-low	ATGTAGGCGACTTCGTCGAG	RT-PCR
gapTaqM-up	GAGTGCACGGGGCTCTTC	qRT-PCR
gapTaqM-low	GAGGTTCTGGTCGTTGGT	qRT-PCR
mexA-up	GCCATGCGTGTACTGGTTCC	RT-PCR
mexA-low	GCTCTGGTAGTCGGCCTCGT	RT-PCR
mexF-up	CGAACTACGCGGTGCTCAAC	RT-PCR
mexF-low	GCGCGGATGATGATGTTCTC	RT-PCR
cupA-up	GTGATCCTCGACAGCGTACC	RT-PCR
cupA-low	GTCGTGCTGGTGCTGGTG	RT-PCR
RhlRNdeI-up	ACATATGAGGAATGACGGAGGCT	RT-PCR
RhlR-low	ATCAGATGAGACCCAGCGC	RT-PCR
acsAa up	TGGTACGACGACCTGATGAA	RT-PCR
acsAa low	CCTCGAACAGAATGGTGGTG	RT-PCR
PAlexA- up	ACATATGCAGAAGCTGACGCC	RT-PCR
PAlexA-lw	ACTCGAGTCAGCGCCGGATCACG	RT-PCR
nrdA-FAM	CTGGCACCTGGACATC	qRT-PCR probe
nrdJ-FAM	TCGGCTCGGTCAACCT	qRT-PCR probe
nrdD-FAM	CCCGACCTACAACATC	qRT-PCR probe
gap-FAM	CCTGCACCACCAACTG	qRT-PCR probe
mutNarL1 up	CGCGGATGCGCCCAATTGCGGCTTCCTTCA	Mutation of NarLbox1 in P*nrdR*
mut NarL1 low	TGAAGGAAGCCGCAATTGGGCGCATCCGCG	Mutation of NarLbox1 in P*nrdR*
mutNarL1.2 up	CGCGGATGCGCTGGCATGCGGCTTCCTTCA	Mutation of NarLbox1 in P*nrdR*
mutNarL1.2 low	TGAAGGAAGCCGCATGCCAGCGCATCCGCG	Mutation of NarLbox1 in P*nrdR*
mutNarL1.3 up	CGCGGATGCGCTGCCATGCGGCTTCCTTCA	Mutation of NarLbox1 in P*nrdR*
mutNarL1.3 low	TGAAGGAAGCCGCATGGCAGCGCATCCGCG	Mutation of NarLbox2 in P*nrdR*

mutNarL R dir	TTCCTTCAGCATAATTAGTATCAGCACCA	Mutation of NarLbox2 in P*nrdR*
mutNarL R rev	TGGTGCTGATACTAATTATGCTGAAGGAA	Mutation of NarLbox2 in P*nrdR*
mut NarL2.2 up	TTCCTTCAGCTGGGCCTGTATCAGCACCA	Mutation of NarLbox2 in P*nrdR*
mut NarL2.2 low	TGGTGCTGATACAGGCCCAGCTGAAGGAA	Mutation of NarLbox2 in P*nrdR*
mut Narl2.3 up	TTCCTTCAGCTGCGCCTGTATCAGCACCA	Mutation of NarLbox2 in P*nrdR*
mutNarL 2.3 low	TGGTGCTGATACAGGCGCAGCTGAAGGAA	Mutation of NarLbox2 in P*nrdR*

Table S2: Global transcriptomic analysis of a Δ*nrdR* mutant strain compared with *P. aeruginosa* PAO1 wild-type strain grown aerobically. List of all differentially regulated genes identified (more than 1.5-fold change in expression).

ID	*Gene*	*Operon arrangement*	Log2 Fold Change	*Gene Product*
PA2128	*cupA1*	*cupA12345*	4.13	Fimbrial subunit CupA1
PA4139			3.78	Hypothetical protein
PA1383			2.42	Hypothetical protein
PA5497	*nrdJa*	*nrdJab*	2.41	Class II (cobalamin-dependent) ribonucleotide-diphosphate reductase subunit, NrdJa
PA2223			2.37	Hypothetical protein
PA1718	*pscE*	*pscBCDEFGHIJKL*	2.32	Type III export protein PscE
PA1156	*nrdA*	*nrdAB*	2.24	Ribonucleoside reductase, large chain
PA0992	*cupC1*	*cupC123*	2.19	Fimbrial subunit CupC1
PA0425	*mexA*	*mexAB-oprM*	2.13	Resistance-Nodulation-Cell Division (RND) multidrug efflux membrane fusion protein
PA3188			2.11	Hypothetical protein
PA0424	*mexR*		2.06	Multidrug resistance operon repressor MexR
PA1693	*pscR*	*PA1697-pscOPQRSTU*	2	Translocation protein in type III secretion
PA3144			1.99	Hypothetical protein
PA2322			1.98	Hypothetical protein
PA3842			1.96	Hypothetical protein
PA1155	*nrdB*	*nrdAB*	1.96	Ribonucleotide reductase, small chain
PA0865	*hpd*		1.94	4-hydroxyphenylpyruvate dioxygenase
PA0426	*mexB*	*mexAB-oprM*	1.93	Resistance-Nodulation-Cell Division (RND) multidrug efflux transporter MexB
PA4086	*cupB1*	*cupB123456*	1.89	Probable fimbrial subunit CupB1
PA0887	*acsA*		1.84	Acetyl-coenzyme A synthetase
PA0978			1.78	Hypothetical protein
PA5169			1.77	Hypothetical protein
PA1333			1.65	Hypothetical protein
PA1723	*pscJ*	*pscBCDEFGHIJKL*	1.63	Type III export protein PscJ
PA0958	*oprD*		1.61	Basic amino acid, basic peptide and imipenem outer membrane porin OprD precursor
PA3720			1.61	Hypothetical protein
PA1386			1.54	Hypothetical protein
PA5491			1.53	Hypothetical protein
PA0717			1.51	Hypothetical protein
PA0427	*oprM*	*mexAB-oprM*	1.43	Major intrinsic multiple antibiotic resistance efflux outer membrane protein Opr
PA2813			-1.58	Hypothetical protein
PA4881			-1.59	Hypothetical protein
PA0567			-1.64	Hypothetical protein
PA2812			-1.68	Hypothetical protein
PA2811			-1.75	Hypothetical protein
PA0281	*cysW*		-2.05	Sulfate transport protein CysW
PA2491	*mexS*		-2.18	Hypothetical protein
PA3931			-2.21	Hypothetical protein
PA3229			-2.37	Hypothetical protein
PA4691			-2.55	Hypothetical protein
PA0998	*pqsC*	*pqsABCDE*	-2.83	Homologous to beta-keto-acyl-acyl-carrier protein synthase
PA0565			-3.06	Hypothetical protein
PA2494	*mexF*		-3.19	Resistance-Nodulation-Cell Division (RND) multidrug efflux transporter MexF
PA3281			-3.73	Hypothetical protein
PA3283			-4.57	Hypothetical protein

Table S3: Global transcriptomic analysis of a Δ*nrdR* mutant strain compared with *P. aeruginosa* PAO1 wild-type strain grown anaerobically. List of all differentially regulated genes identified (more than 1.5-fold change in expression).

ID	*Gene*	*Operon arrangement*	Log2 Fold Change	*Gene Product*
PA5507		*PA5506-PA5507-pauA7-PA5509*	3.2	Hypothetical protein
PA5509		*PA5506-PA5507-pauA7-PA5509*	2.98	Hypothetical protein
PA4058		*PA4058-PA4059-PA4060*	2.23	Hypothetical protein
PA1718	*pscE*	*exsD-pscBCDEFGHIJKL*	2.23	Type III export protein PscE
PA1984	*exaC*	*exaB-exaC*	2.13	Hypothetical protein
PA0713			2.03	Hypothetical protein
PA1556	*ccoO2*	*ccoN2-ccoO2*	1.9	Hypothetical protein
PA1073	*braD*	*braD-braE-braF-braG*	1.79	Branched-chain amino acid transport protein BraD
PA1337	*ansB*	*ansB-PA1336-PA1335*	1.77	Glutaminase-asparaginase
PA1340			1.76	Hypothetical protein
PA1555	*ccoP2*		1.75	Hypothetical protein
PA0958	*oprD*		1.7	Basic amino acid, basic peptide and imipenem outer membrane porin OprD precursor
PA1072	*braE*	*braD-braE-braF-braG*	1.69	Branched-chain amino acid transport protein BraE
PA1070	*braG*	*braD-braE-braF-braG*	1.68	Branched-chain amino acid transport protein BraG
PA5506		*PA5506-PA5507-pauA7-PA5509*	1.67	Hypothetical protein
PA1341		*PA1341-PA1340-PA1339*	1.63	Hypothetical protein
PA2436		*PA2436-PA2435-PA2434*	1.62	Hypothetical protein
PA1596	*htpG*	*htpG-PA1597*	1.57	Heat shock protein HtpG
PA3842	*spcS*	*spcS-PA3843*	1.56	Hypothetical protein
PA1339		*PA1341-PA1340-PA1339*	1.54	Hypothetical protein
PA4587	*ccpR*		1.54	Cytochrome c551 peroxidase precursor
PA1338	*ggt*		1.53	Gamma-glutamyltranspeptidase precursor
PA1571			1.53	Hypothetical protein
PA0026	*plcB*	*plcB-PA0027-PA0028*	-1.5	Phospholipase C, PlcB
PA5107	*blc*	*fbp-PA5109-PA5108-blc*	-1.5	Outer membrane lipoprotein Blc
PA0612	*ptrB*	*ptrB-PA0613*	-1.54	Transcriptional regulators
PA0909		*PA0908-PA0909*	-1.54	Membrane proteins
PA0462		*PA0462-creB-creC*	-1.55	Hypothetical, unclassified, unknown
PA3720		*PA3720-armR*	-1.55	Hypothetical, unclassified, unknow
PA5526			-1.55	Hypothetical, unclassified, unknown
PA4817		*PA4816-PA4817*	-1.56	Hypothetical, unclassified, unknown
PA5217		*PA5218-PA5217-PA5216*	-1.58	Transport of small molecules
PA0490		*PA0491-PA0490*	-1.59	Hypothetical, unclassified, unknown
PA5212			-1.6	Hypothetical, unclassified, unknown
PA3616		*recA-PA3616*	-1.61	Hypothetical, unclassified, unknown
PA0284			-1.68	Hypothetical, unclassified, unknown
PA0327			-1.69	Hypothetical, unclassified, unknown
PA4515		*PA4515-PA4516*	-1.72	Hypothetical, unclassified, unknown
PA3413		*PA3413-PA3414*	-1.73	Hypothetical, unclassified, unknown
PA0529			-1.79	Hypothetical, unclassified, unknown
PA3008		*lexA-PA3008*	-1.81	Hypothetical, unclassified, unknown
PA0130	*bauC*	*bauA-bauB-bauC*	-1.83	Carbon compound catabolism
PA3007	*lexA*	*lexA-PA3008*	-1.88	Repressor protein LexA
PA0922			-1.89	Hypothetical, unclassified, unknown
PA3268			-1.91	Membrane proteins
PA0132	*bauA*	*bauA-bauB-bauC*	-1.92	Amino acid biosynthesis and metabolism
PA0132	*recA*	*recA-PA3616*	-1.96	RecA protein
PA0131	*bauB*	*bauA-bauB-bauC*	-2.01	Carbon compound catabolism
PA0283	*sbp*		-2.01	Sulfate-binding protein precursor
PA0204		*PA0206-PA0205-PA0204-PA0203*	-2.17	Membrane proteins/Transport of small molecules
PA0622		*PA0622-PA0623-PA0624*	-2.18	Related to phage, transposon, or plasmid
PA2813		*PA2813-PA2812-PA2811*	-2.24	Central intermediary metabolism
PA0911			-2.32	Hypothetical, unclassified, unknown
PA4195		*PA4195-PA4194-PA4193-PA4192-PA4191*	-2.33	Transport of small molecules
PA3445			-2.34	Hypothetical, unclassified, unknown
PA0621		*PA0617-PA0618-PA0619-PA0620-PA0621*	-2.4	Related to phage, transposon, or plasmid
PA5525		*PA5524-PA5525*	-2.42	Transcriptional regulators
PA0641			-2.44	Related to phage, transposon, or plasmid

PA0807	*ampDh3*		-2.44	Antibiotic resistance and susceptibility
PA3866			-2.45	Secreted Factors (toxins, enzymes, alginate)
PA0646		*PA0646-PA0647-PA0648*	-2.48	Hypothetical, unclassified, unknown
PA3931			-2.48	Hypothetical, unclassified, unknown
PA0610	*prtN*		-2.49	Transcriptional regulator PrtN
PA0633		*PA0633-PA0634-PA0635*	-2.52	Related to phage, transposon, or plasmid
PA0203		*PA0206-PA0205-PA0204-PA0203*	-2.59	Transport of small molecules
PA1150	*pys2*		-2.62	Pyocin S2
PA0642			-2.63	Related to phage, transposon, or plasmid
PA0648		*PA0646-PA0647-PA0648*	-2.63	Related to phage, transposon, or plasmid
PA0615		*PA0614-PA0615-PA0616*	-2.67	Hypothetical, unclassified, unknown
PA0647		*PA0646-PA0647-PA0648*	-2.68	Related to phage, transposon, or plasmid
PA0623		*PA0622-PA0623-PA0624*	-2.71	Related to phage, transposon, or plasmid
PA4763	*recN*		-2.71	DNA repair protein RecN
PA0644		*PA0643-PA0644-PA0645*	-2.72	Related to phage, transposon, or plasmid
PA0910			-2.72	Hypothetical, unclassified, unknown
PA0616		*PA0614-PA0615-PA0616*	-2.73	Related to phage, transposon, or plasmid
PA0619		*PA0617-PA0618-PA0619-PA0620-PA0621*	-2.74	Related to phage, transposon, or plasmid
PA2485		*PA2485-PA2486*	-2.77	Hypothetical, unclassified, unknown
PA0626		*PA0625-PA0626-PA0627*	-2.78	Related to phage, transposon, or plasmid
PA0620		*PA0617-PA0618-PA0619-PA0620-PA0621*	-2.79	Related to phage, transposon, or plasmid
PA0625		*PA0625-PA0626-PA0627*	-2.84	Related to phage, transposon, or plasmid
PA0320		*PA0319-PA0320*	-2.85	Hypothetical, unclassified, unknown
PA0613		*ptrB-PA0613*	-2.87	Hypothetical, unclassified, unknown
PA0629		*PA0628-PA0629-PA0630-PA0631-PA0632*	-2.87	Related to phage, transposon, or plasmid
PA0639		*PA0636-PA0637-PA0638-PA0639-PA0640*	-2.93	Related to phage, transposon, or plasmid
PA2486		*PA2485-PA2486*	-2.94	Hypothetical, unclassified, unknown
PA3446			-2.95	Hypothetical, unclassified, unknown
PA0624		*PA0622-PA0623-PA0624*	-2.96	Related to phage, transposon, or plasmid
PA0637		*PA0636-PA0637-PA0638-PA0639-PA0640*	-2.96	Related to phage, transposon, or plasmid
PA3938		*PA3938-PA3937-PA3936*	-2.97	Transport of small molecules
PA2494	*mexF*	*mexE-mexF-oprN*	-3.01	Resistance-Nodulation-Cell Division (RND) multidrug efflux transporter MexF
PA0632		*PA0628-PA0629-PA0630-PA0631-PA0632*	-3.06	Related to phage, transposon, or plasmid
PA0635		*PA0633-PA0634-PA0635*	-3.13	Related to phage, transposon, or plasmid
PA0645		*PA0643-PA0644-PA0645*	-3.13	Related to phage, transposon, or plasmid
PA0627		*PA0625-PA0626-PA0627*	-3.15	Related to phage, transposon, or plasmid
PA0638		*PA0636-PA0637-PA0638-PA0639-PA0640*	-3.16	Related to phage, transposon, or plasmid
PA0640		*PA0636-PA0637-PA0638-PA0639-PA0640*	-3.17	Related to phage, transposon, or plasmid
PA0628		*PA0628-PA0629-PA0630-PA0631-PA0632*	-3.21	Related to phage, transposon, or plasmid
PA0634		*PA0633-PA0634-PA0635*	-3.21	Related to phage, transposon, or plasmid
PA0643		*PA0643-PA0644-PA0645*	-3.21	Related to phage, transposon, or plasmid
PA0617		*PA0617-PA0618-PA0619-PA0620-PA0621*	-3.33	Related to phage, transposon, or plasmid
PA0636		*PA0636-PA0637-PA0638-PA0639-PA0640*	-3.41	Related to phage, transposon, or plasmid
PA0618		*PA0617-PA0618-PA0619-PA0620-PA0621*	-3.46	Related to phage, transposon, or plasmid
PA3229			-3.51	Hypothetical, unclassified, unknown
PA2484			-3.55	Hypothetical, unclassified, unknown
PA4881			-3.77	Hypothetical, unclassified, unknown
PA0985	*pyoS5*		-3.9	Membrane proteins/Secreted Factors (toxins, enzymes, alginate)
PA2491	*mexS*		-4.69	Negative regulation of secondary metabolite biosynthetic process

Article 2

Pseudomonas aeruginosa AlgR regulates ribonucleotide reduction and links it to oxidative stress signals.

Pseudomonas aeruginosa AlgR regulates ribonucleotide reduction and links it to oxidative stress signals

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Running title: AlgZR regulation of ribonucleotide reductases

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Abstract

Ribonucleotide reductases (RNR) are key enzymes for the metabolism of all living cells. RNR mediate the synthesis of deoxyribonucleotides, DNA precursors, for DNA synthesis and repair. Three forms of RNR exist, classes I, II and III, each accounting for a different activation mechanism and allowing biosynthesis to occur under different atmospheric oxygen conditions. The perilous opportunistic pathogen *Pseudomonas aeruginosa* is one of the few known organisms that encodes all three classes, allowing it to adapt to different environments and form its characteristic biofilms. Here, we examine the relationship between the regulation of RNR activity and the AlgZR two-component system, one of the global regulators behind alginate synthesis and the mucoid phenotype. Through bioinformatics, we identify the AlgR binding locations in the class I and class II RNR promoter regions, which we characterize functionally through EMSA and physically through AFM imaging. Gene reporter assays are used to determine that both classes are regulated by AlgR *in vivo* and to uncover the mechanism through which the AlgZR system controls the RNR network under different environmental conditions and physiological states. Finally, we demonstrate that AlgZR-mediated regulation is the link that causes the very well-described induction of ribonucleotide reductase activity through oxidative stress.

Importance

Pseudomonas aeruginosa is one of the most important pathogens causing chronic pulmonary infections in patients suffering from cystic fibrosis (CF) or other risk factors. In CF, it causes an initial asymptomatic infection that can evolve into a “mucoid” form, which is the main cause of mortality in CF patients. *P. aeruginosa* is also well-known for its antibiotic resistance and adaptability; therefore, we need to find new ways to combat its chronic infections. One promising strategy for new antimicrobial therapies is targeting ribonucleotide reductases (RNR), the enzymes that form new precursors for DNA synthesis and repair, as they are essential for bacterial life. Here, we show that the two-component system AlgZR, which is crucial for the bacterial conversion to the mucoid form, also controls the RNR network, changing how these enzymes work in mucoid and non-mucoid biofilms and allowing them to respond to oxidative stress.

Introduction

Pseudomonas aeruginosa is a ubiquitous environmental gram-negative bacterium, but it can also be a dangerous and adaptable opportunistic pathogen. In particular, it is known to cause severe chronic lung infections in immunocompromised patients and other at-risk groups. In cystic fibrosis (CF) patients, this infection is associated with a poor prognosis, leading to severely impaired lung function and an increased risk of respiratory failure, and is the main cause of morbidity and mortality (1). *P. aeruginosa* initially colonizes the CF lung in a non-mucoid form (characterized by non-detectable alginate production and causing an asymptomatic infection). However, at later stages of lung colonization, *P. aeruginosa* switches its phenotype and mucoid alginate-overproducer variants emerge, leading to rapid pulmonary deterioration (2,3).

Alginate production protects *P. aeruginosa* from phagocytosis, antibiotic penetration, and desiccation (4,5), but it is also an energy-intensive process and is therefore closely regulated and activated only when a chronic infection reaches a critical point. This involves a wide number of enzymes and precursor substrates; of special importance is the *algD* (and consecutive genes) operon, encoding the main enzymes for alginate production, and the *algC* gene from the *algC-argB* operon, a multifunctional enzyme required for several pathways including alginate biosynthesis and LPS production (6). These genes are controlled by products of the *algU/mucABCD* operon; their transcription is directed by the alternative sigma factor AlgU (sigma E), which is commonly sequestered by the anti-sigma factor MucA. It has been reported that several types of cellular stress can induce proteolytic degradation of MucA and transiently activate alginate synthesis (7), but the constitutive mucoid phenotype is generated through the selection of mutations in the regulatory genes, usually in *mucA* (8,9).

Apart from the *algD* and *algC* operons, the AlgU sigma factor regulates the production of the AlgZR two-component system, encoded by the *fimS*(*algZ*)-*algR* operon (10,11). This system regulates both alginate biosynthetic operons, but it has also been reported to regulate many aspects of bacterial physiology and fitness. FimS is the membrane kinase that can detect an unknown environmental signal and accordingly modulate AlgR phosphorylation. AlgR is the transcriptional factor that regulates several aspects of anaerobic metabolism, type IV pili, rhamnolipid biosynthesis, type III secretion, cyanide and nucleotide synthesis (12,13), and it has recently been reported to bind with high affinity to 157 loci in the *P. aeruginosa* genome (12). Many of the functions regulated by the AlgZR system are important for biofilm formation and chronic infection (14). Among these, several observations have linked this system with the *P. aeruginosa* ribonucleotide reductases (RNR) network (6,12).

Ribonucleotide reductases are the enzymes responsible for reducing the ribonucleotides (NTP) to the corresponding deoxyribonucleotides (dNTP), thereby forming the building blocks for DNA synthesis and repair (15). There are three known RNR classes (I, II and III), and all use a free-radical-based catalysis; however, they rely on different metallo-cofactors for the initiation of the radical reduction step, and each one exhibits a different behavior towards oxygen. Class I RNR can be enzymatically active only under aerobic conditions, class II RNR is oxygen-independent and requires vitamin B_{12} for enzyme activation, and class III RNR requires strict anaerobic conditions to be active. While almost all eukaryotic organisms encode exclusively class Ia RNR, prokaryotes are known to encode more than one, in all possible combinations (16). Surprisingly, *P. aeruginosa* encodes all three RNR classes: class Ia (*nrdAB*), class II (*nrdJab*) and class III (*nrdDG*) (17). Their different requirements and relationships with oxygen give them different roles throughout the *Pseudomonas* life cycle and in the biofilm structure (15,18,19). The RNR activity is known to be extensively regulated at both the transcriptional and post-translational levels; it is delicately modulated to keep a balanced nucleotide pool and globally regulated according to the life cycle, stress situations and environmental conditions. However, much remains unknown about which factors allow bacteria to activate the different classes under different circumstances.

Several years ago, one of the genes found in a transcriptomics experiment to be regulated by AlgR in *P. aeruginosa* was the *nrdJ* gene (PA5497) (6), which encodes a class II ribonucleotide reductase that plays a crucial role during biofilm formation and infection. In addition, a recent study that aimed to identify AlgZR-regulated genes using ChIP-seq showed a specific region for AlgR binding (AlgR-box) in a short DNA fragment within the intergenic region between the class I RNR operon first gene (P*nrdA*; PA1156) and the PA1157 gene (12). All of these observations point to the existence of a relationship between the AlgZR system and the RNR network.

In this study, we aimed to uncover this relationship. We demonstrate that AlgR regulates both RNR classes I and II in a differential way, depending on its phosphorylation state. We explore how this differential regulation allows bacteria to adapt to different situations when living in a free form, during colonization of surfaces and in mucoid or non-mucoid biofilms. Finally, we unravel for the first time the molecular mechanisms behind the well-known activation of ribonucleotide reductase activity that occurs under oxidative stress.

Results

Ribonucleotide reduction is regulated by AlgR in *P. aeruginosa*. In light of the different observations linking AlgZR regulation with ribonucleotide reduction, we initially used plasmids carrying a transcriptional fusion of the *nrdA* (pETS134), *nrdJ* (pETS180), *nrdD* (pETS136) and PA1157 (pETS206) promoters to the green fluorescent protein (GFP). To investigate the AlgR binding spot found in ChIP-seq studies (12), we aimed to clarify whether AlgR regulates the expression of class I RNR (*nrdA*, PA1156) or the adjacent PA1157 gene; additionally, we also explored regulation of class II and III RNR (*nrdJa*, PA5497 and *nrdD*, PA1920, respectively).
As shown in Fig. 1, comparing the expression of the wild-type *P. aeruginosa* PAO1 strain with its isogenic *algR* mutant strain (Δ*algR*; PW9855), there is small but significant positive regulation by AlgR on the class I operon (*nrdAB*) and class II operon (*nrdJab*). Complementation with a *fimS-algR* construct (pETS203) restored the expression of both promoters to that of the wild-type cells or increased it further. The PA1157 promoter was not affected by either the absence or overexpression of the AlgR regulator. Thus, AlgR binding in the PA1156-PA1157 intergenic region does not regulate the PA1157 gene as previously reported (12) but instead regulates the adjacent *nrdAB* genes. No change was found on P*nrdD* promoter expression (pETS136). The regulation of P*algD* promoter expression by AlgR (pETS205, our positive control) that we observed here is consistent with what has been extensively reported (3,14,20), acting as a clear activator of its transcription, although it is typically almost fully inactive in the non-mucoid phenotype.

The AlgZR phosphorylation switch modulates RNR regulation. It has been shown that a conserved substitution of the D54 residue of AlgR to an asparagine (AlgR D54N) abolishes its *in vitro* and *in vivo* phosphorylation by the FimS(AlgZ) kinase in response to environmental signals (21). Hence, we used the wild-type AlgR (pUCP-AlgR) and its variant AlgRD54N (pUCP-D54N) to determine the influence of phosphorylation in regulating *nrdA* and *nrdJ* transcription (Fig. 1). In liquid culture experiments, both pETS203 (wild-type AlgR protein) and pETS204 (AlgR D54N) were able to restore AlgR deficiency similarly to wild-type *nrdJ* transcription levels, both in the exponential and stationary growth phases and in aerobic or anaerobic environments. No clear global effect of AlgR phosphorylation on class II RNR regulation could be identified under these conditions. However, the transcription of class Ia RNR (*nrdA*) was clearly affected by the phosphorylation state of AlgR, demonstrating a much higher increase in expression with phosphorylatable AlgR under aerobic and anaerobic growth conditions.

In the case of *algD* regulation, AlgR D54N clearly increases its expression compared to wild-type AlgR complementation, as AlgR does not need to be phosphorylated to positively regulate *algD* transcription (14,22).

AlgR binds to the *nrdA* and *nrdJ* promoter regions through specific AlgR-binding boxes. In order to precisely localize the AlgR binding sites in the class Ia and II RNR promoter regions, a thorough bioinformatics search was conducted. First, to characterize the AlgR-box consensus sequence, we used MEME (MEME suite), starting from three different sources of information (see Materials and Methods), to obtain three count matrices characteristic of the AlgR binding site (Fig. S1). A FIMO search (MEME suite) was later conducted with all three matrices. Using positive and negative control probes (see Materials and Methods), we concluded that a 1e-4 p-value threshold showed no false positives and identified strong AlgR binding sites in all situations. On the other hand, a 1e-3 p-value threshold identified all boxes with all sets but also showed up to 5 nonspecific hits in the negative control. Using the three count matrices on a FIMO search of promoters P*nrdA*, P*nrdJ* and P*nrdD*, applying the 1e-4 p-value threshold, a single binding site was identified on P*nrdA* and P*nrdJ*, while no hits were retrieved from P*nrdD*. As further results showed that P*nrdJ* included more than one binding spot (see Fig. S2), a more flexible search was conducted for this probe, in which all hits obtained from searching with all three matrices while applying a 1e-3 p-value threshold were considered. To characterize the AlgR-DNA binding activity and experimentally demonstrate AlgR binding to the identified putative AlgR boxes, we performed Electrophoretic Mobility Shift Assays (EMSA). Initially, large DNA probes spanning all the putative promoter regions for class I (P*nrdA*), class II (P*nrdJ*) and class III (P*nrdD*) were analyzed, with the corresponding positive control (a band of the P*algD* promoter including the two strong binding sites) and negative control. We identified one binding site in the P*nrdA* promoter and two clear binding sites in the P*nrdJ* promoter, while no evidence of an AlgR-DNA interaction was found for P*nrdD* (Fig. 2A).

To localize more precisely the AlgR binding locations in the aforementioned promoters, we segmented them into smaller DNA probes (Fig. S2). As seen in Fig. 2A, we established that binding activity was localized to one spot in probe P*nrdA* short 1 for class I regulation and in one spot in P*nrdJ* short 1 and another in P*nrdJ* short 2 for class II regulation. We then proceeded with putative AlgR box mutagenesis, determining as definitive binding spots the ones now labeled as P*nrdA* box 1, P*nrdJ* box 1 and P*nrdJ* box 2 (Fig. 2A), whose mutagenesis fully abolished DNA shifts. The other boxes we identified in P*nrdJ* short 2 are considered artifacts of

the bioinformatic search. P*nrdA* box 1 colocalizes with the DNA fragment enriched by AlgR-precipitation in ChIP-seq (9).

Therefore, the identified boxes confirmed the presence of the previously described AlgR binding site in the intragenic region between the *nrdA* and PA1157 genes (12) and also included previously unreported putative binding sites in the class II RNR promoter region. EMSA experiments under stricter conditions were used to explore possible differences in *nrd* binding affinity between AlgR-WT and AlgR-D54N, but no clear effect was observed (Fig. S3).

The specific *in vivo* effect of the described boxes was first assessed under liquid culture conditions by using promoter-GFP fusions in gene reporter assays (Fig. 2B). In P*nrdA* class I RNR, we determined that the identified AlgR box is fully responsible for the AlgR regulation of this promoter, as mutation of this box clearly resembles the effect of mutating the *algR* gene. The effect of the boxes identified in P*nrdJ* class II RNR is more complex; even though it was demonstrated that mutating box 1 abolished AlgR binding in the immediate region, this mutation had no significant effect on P*nrdJ* expression in liquid cultures. Mutating box 2 or both boxes reduced the P*nrdJ* expression, but not to the levels seen in an Δ*algR* mutant strain. The complex effect of P*nrdJ* AlgR boxes is further studied below, under different conditions.

Finally, when comparing their sequences with the previously known sequence, we realized that P*nrdA* box 1 is more similar to those described as “strong binders”, while P*nrdJ* boxes are both similar to the so-called “weak-binders” (Fig. S4) (14). Specifically, there is one cytosine in position 7 present in all strong binders that is absent in all weak binders. A comparative EMSA with a wide array of small concentrations of protein (Fig. S4) shows that, as expected, binding in P*nrdA* and P*algD* requires smaller concentrations of protein and results in sharper, more stable bands, while binding in P*nrdJ* requires higher concentrations for full occupation of both boxes and forms blurrier bands, indicative of a more unstable complex.

AlgR binding on RNR promoters causes DNA bending and stiffening. It has been reported that AlgR regulation, usually performed through binding hundreds of base pairs upstream from the basal promoter, often implies DNA bending (14). In the regulation of the promoter of the *algD* operon, the best-known AlgR regulatory process, a wide DNA loop is formed integrating the actions of the three AlgR binding spots and the histone-like protein IHF (14,23).

To study the physical effect of AlgR binding on RNR promoters, we observed previously formed *nrd*-AlgR complexes, compared to free DNA probes, using Atomic Force Microscopy (AFM) (Fig. 3). AlgR binding can be clearly observed on both P*nrdA* and P*nrdJ* probes, although several series of images showed that the P*nrdA* complex was easier to obtain and more stable.

One single binding spot is observed in the P*nrdA* promoter, while some of the P*nrdJ*-AlgR complexes show up to two binding events. To explore the nature of these bindings, they were compared to the very well-known binding of the LexA repressor to the damage-inducible DNA polymerase IV (*dinB)* promoter region (24). We can clearly see, despite the artifact introduced by the natural positioning of the DNA probes on the mica surface, that AlgR binding on the P*nrdA* and P*nrdJ* promoters colocalizes with a strong DNA bending event, while no bending is observed due to LexA binding on P*dinB* (Fig. 3A). This effect is also clearly seen in the P*algD* probe, although it contains only the binding sites RB1 / RB2, and IHF is not present in the reaction.

Moreover, we determined the apparent length of the DNA fragments in the AFM images (Fig. 3B), clearly observing that although it was already reduced when the DNA was in the protein binding buffer rather than in water prior to drying, it was reduced to a greater extent when AlgR protein was bound, suggesting a clear DNA stiffening event. This effect is quite apparent in P*nrdA* and P*algD* probes and is also detectable in P*nrdJ,* whereas no evidence of stiffening is found due to LexA-P*dinB* binding.

AlgR regulation during surface colonization reveals a complex mechanism behind RNR transcription fine tuning. The AlgZR system is required for fimbrial biogenesis (11) and rhamnolipid formation (25), both activities of the utmost importance for surface colonization and colony and biofilm formation (26,27). We therefore considered it necessary to explore the AlgR regulation of *nrd* genes during surface growth. In our surface colonization experiments, different strains harboring promoter::*gfp* fusion plasmids were grown on agar plates for 36 h, and fluorescence was determined at 3 hour intervals during all growth. This model is also useful for exploring the AlgR action on *nrd* genes in the mucoid phenotype, using the PAOMA (Δ*mucA*) strain, which forms very characteristic mucoid colonies.

We first analyzed the regulation of the P*algD* promoter (Fig. 4A). The basal level of P*algD* expression in a non-mucoid phenotype is very low, although it can be seen that the Δ*algR* deletion reduces its expression and that it can be complemented by the non-phosphorylatable AlgR D54N protein, whereas the wild-type protein does not complement (or even slightly inhibits) P*algD* transcription. In the Δ*mucA* strain, the great increase in non-phosphorylated AlgR levels causes a very significant increase in P*algD* transcription (>6000 RFU). All results agree with our previous observations and published data (3,14,20), serving as a control for this technique.

For the class I RNR P*nrdA* promoter (Fig. 4B), the results confirm what was observed in the liquid cultures, although they are more evident under these conditions: mutating the *algR* gene causes a clear reduction in *nrdAB* transcription, and mutating the AlgR-box in the promoter mimics this effect. Complementation with AlgR WT protein over-activates the promoter whereas D54N is not able to fully complement the mutation, demonstrating that AlgR phosphorylation is required for class I RNR induction.

The results are more complex for the class II RNR P*nrdJ* promoter (Fig. 4C). As previously described, mutating the *algR* gene causes a reduction in P*nrdJ* transcription, which can be complemented by introducing additional copies of *algR* (pUCP-AlgR). The overexpression of wild-type AlgR protein complements the mutation, while, under these conditions, AlgR D54N overexpression causes an inhibition of the operon transcription. This first evidence that accumulation of AlgR can, under these conditions, inhibit P*nrdJ* transcription, is supported by the fact that, unlike for P*nrdA*, the activity of the promoter is severely reduced in the mucoid phenotype (Δ*mucA*). Additionally, mutation of both AlgR boxes in the P*nrdJ* promoter causes not a reduction, but a clear increase in the transcription of the promoter, demonstrating the presence of a more complicated underlying mechanism.

To explore the independent action of the AlgR boxes, we performed several colony formation experiments with single-box mutants (Fig. 4D). Both boxes display very different behaviors. Mutating box 1 increases the expression of the promoter, therefore suggesting that AlgR is inhibiting P*nrdJ* transcription by binding to box 1. Eliminating box 1 can switch the effect of the Δ*mucA* background from a large reduction to a large increase in transcription, demonstrating that the previously observed inhibition of class II RNR transcription in the mucoid phenotype happens through AlgR binding in the AlgR box 1. The effect of mutating box 2 is not detectable in a wild-type background, but its mutation switches the effect of the AlgR-overexpression strain from an increase to a dramatic decrease in P*nrdJ* transcription. Box 2 is therefore believed to be implicated in P*nrdJ* activation in response to AlgR phosphorylation in competition with the action of box 1 and involved in P*nrdJ* inhibition in the mucoid phenotype. The implications of this dual mechanism on stress conditions and the mucoid phenotype are further discussed below (see Discussion).

End-point anaerobic colony formation experiments were also performed (see Fig. S5), although no differences with aerobic experiments worth highlighting were observed.

The AlgR regulation mechanism is reproduced in mucoid and non-mucoid biofilms. Our group has recently demonstrated the importance of class II RNR (*nrdJab*) during *P. aeruginosa*

biofilm formation and its transcriptional activation by anaerobic regulators under this condition (19). However, the main regulators involved in modulating RNR transcription in the biofilm are still unknown. The AlgZR system has been extensively associated with different aspects of biofilm formation, and here we have demonstrated that it regulates the *nrd* genes, which are also differentially regulated in the mucoid phenotype. Therefore, we decided to explore the modulation of class I and II RNR expression by AlgZR in mucoid and non-mucoid biofilms. In Fig. 5, we determined the *nrdA* and *nrdJ* expression, together with *algD* expression as a control, during biofilm formation. Measurements were taken at different time intervals during growth (from 3h to 72h). The un-complemented PW9855 (Δ*algR* mutant) strain could not be used, at it presents severely impaired biofilm formation capabilities.

As in our previous experiments in colonies, AlgR functions in the biofilm as an activator of P*algD* transcription, where it is more responsive to non-phosphorylatable AlgR D54N and shows a high induction in the mucoid PAOMA (Δ*mucA*) strain (Fig. 5A).

Class I RNR transcription (Fig. 5B) is clearly induced in the mucoid biofilm at a very early stage in its formation. P*nrdA* induction occurs only due to phosphorylated AlgR overexpression and not with its non-phosphorylatable counterpart AlgR D54N (although the effect of AlgR overexpression does not go beyond complementing the mutation). The AlgZR regulation appears to be responsible for the effect in the mucoid biofilm, as mutating AlgR box 1 eliminates this induction, as well as in the non-mucoid variant.

Finally, the complex regulation of class II RNR is also reproduced in biofilm formation conditions (Fig. 5C and 5D). The changes in transcription can be more easily detected in mature biofilms (72h), while younger biofilms show almost no evidence of regulation. In a mature mucoid biofilm, there is a clear reduction in P*nrdJ* expression, which can be restored with the mutation of AlgR box 1. Mutating this box causes a general increase in class II transcription, while mutating box 2 causes a reduction. The double mutation causes an opposite effect in both the regular mature biofilm and in the mucoid biofilm. The differential action of AlgR box 1 and AlgR box 2 is therefore demonstrated, and it is related to both AlgR-mediated induction of class II RNR in non-mucoid biofilms and AlgR-mediated repression of class II RNR in the mucoid biofilm.

Ribonucleotide reductase induction under oxidative stress acts through AlgR regulation. There are several reports which describe that RNR activity is strongly activated under oxidative stress conditions by increasing *nrd* gene transcription through an unknown molecular mechanism

(15,28,29). Here, we explore the ability of AlgR to sense oxidative stress and accordingly regulate RNR gene expression.

As hypothesized, class I (*nrdA*) and II (*nrdJ*) RNR respond to oxidative stress (induced through hydrogen peroxide treatment) by significantly increasing their transcription (Fig. 6). Surprisingly, this response to oxidative stress is totally abolished if the *algR* gene is inactivated (Δ*algR* mutant strain). Introducing mutations in the identified AlgR binding regions of the *nrdA* and *nrdJ* promoter regions mimics the effect of the *algR* isogenic mutant, rendering the promoters unable to respond to oxidative stress.

These results clearly indicate, for the first time, that the well-described activation of the *nrd* genes by oxidative stress occurs through the action of the AlgZR two-component system.

Discussion

Being absolutely essential for the life of any cell, ribonucleotide reductase activity is always thoroughly regulated. In bacteria, where different RNR classes could be present and required for different situations, the activation and inactivation of the several classes adds another layer of complexity.

Several pieces of this regulatory puzzle are already known. In *P. aeruginosa*, apart from class Ia *nrdAB* (whose transcription has been mainly studied in *E. coli*), class II is known to be especially important in biofilms and positively regulated by the anaerobic system Anr/Dnr, although we have proposed the action of other biofilm-related factors (19). Class III is highly activated under strictly anaerobic conditions by still unknown regulators. Moreover, the global regulator NrdR, which negatively regulates all RNR expression in almost all bacterial species (15), is also present in the *P. aeruginosa* network (30). However, despite all known information, there are yet many missing pieces. RNR activity is modulated under oxidative stress conditions (28,29) and specific environmental conditions through unknown factors. The data linking the AlgZR two-component system to the RNR network (6,12) could reveal another piece of this complex regulation.

For our bioinformatics analysis, we took advantage of published data regarding DNA sequences that bind AlgR (12), but we realized that these data accounted only for high-affinity binders. Therefore, we also used several published sequences (14), including weak binders, to render a more relaxed search pattern (Fig. S1). We identified putative binding sites in the P*nrdA* and P*nrdJ* promoters (Fig. S2) that were experimentally demonstrated (Fig. 2A); the absence of sites in P*nrdD* suggests that class III RNR is not regulated by AlgZR. Contrary to the well-known *algD* promoter, which contains 3 AlgR-boxes (RB1, RB2, and RB3) (3,31), one unique AlgR-

box was identified in the P*nrdA* promoter, and two were identified in the P*nrdJ* promoter. Although members of the AgrA family such as AlgR usually bind to direct repeats of their binding sequence (14), it is known that other genes regulated by AlgR contain different numbers of boxes in their promoters (*fimU*, 2 boxes; *hcnA*, 1 box; *rhlA*, 1 box; and *rhlI*, 1 box). The distance from these boxes to their predicted transcription start sites (from 100 bp to 300 bp; Fig. S2) suggests that DNA bending will be necessary to interact with the transcription machinery. A deeper analysis of their sequences also reveals that the box in the P*nrdA* promoter is that of a strong binder, while P*nrdJ* boxes resemble that of known weak binders (Fig. S4B). The cytosine (C) in position 7 is present only in strong binding sequences; this difference can be used to conduct new bioinformatics searches specifically geared towards AlgR weak-binding sequences.
Consequently, the results of the AFM imaging (Fig. 3) of the DNA-protein complexes confirmed that binding of AlgR to the RNR promoters causes bending of the DNA (together with a clear stiffening effect); this explains how binding spots that are so far away from the transcription start site can interfere with transcription. Although AlgR-mediated bending has been proposed many times, to our knowledge, this is the first time that it has been experimentally demonstrated. The so-formed loops suggest interactions with other factors, such as the Anr/Dnr system, which also regulates class II RNR.
In studying the AlgR *in vivo* regulation of the RNR network, we used different models of growth to analyze its effects under different metabolic conditions: aerobic and anaerobic liquid cultures, a model for aerobic and anaerobic surface colonization, and a model for biofilm formation, which was initially aerobic and naturally formed anaerobic areas during growth. The P*algD* promoter was used as a control, demonstrating that all methods are suitable for studying the effects of the AlgZR system; in all models it is acting as an activator of P*algD*, whose transcription is more reactive to non-phosphorylated AlgR. As expected, under some circumstances its basal expression is not sufficient to observe an effect when mutating the gene, and regulation becomes apparent only when over-activating it. Using these methods, we identified a clear control by the AlgZR system of class I and class II RNR, while class III RNR demonstrated no evidence of regulation, not even under anaerobic conditions (data not shown). This shows that the bioinformatics search identified binding sites only where regulation was later demonstrated.
For class I RNR regulation, we determined that AlgR is activating P*nrdA* transcription (Fig. 1). The identified box, which correlates with the DNA fragment recovered in prior ChIP-seq experiments (12), despite facing PA1157, is regulating the *nrdAB* operon. Other boxes with the same orientation have been described, such as the RB3 site on *algD* (10). The functionality of

the box can be demonstrated *in vivo* (Fig. 2B), and the effects of mutating the box or the gene are reproducible and even more evident during surface colonization (Fig. 4B) or biofilm formation (Fig. 5B). We used the AlgR D54N mutant to determine the involvement of AlgR phosphorylation in *nrd* regulation (11,14), determining that phosphorylatable AlgR is a better inducer of class I RNR expression in all models, whereas AlgR D54N is not so powerful or even causes an opposite effect in biofilms and colonies. It is known that several stress conditions, such as oxidative stress, induce *nrd* transcription (28,29) and can also activate the two-component AlgZR system, inducing genes for cell attachment and biofilm formation (6,32). We hypothesized that FimS, the kinase of the system, could respond to stress or stress-derived signals to activate the phosphorylation of AlgR. This would give significance to P*nrdA* control by AlgR, which would be activating it in response to stress conditions. Additionally, our surface colonization and static biofilm models (Fig. 4B and Fig. 5B) determined that class I RNR is induced in the mucoid phenotype and that this happens at least partially through AlgZR control.

Concerning class II RNR regulation, initial experiments suggested that it was also activated by AlgZR (Fig. 1); however, the mutation of the identified boxes quickly suggested a more complex mechanism (Fig. 2B). Surprisingly, although mutating the gene causes a reduction in P*nrdJ* expression, mutating both boxes in a wild-type background caused an increase in biofilm and colony formation (Fig. 4C, Fig. 5C). A more detailed mutagenesis of the boxes (Fig. 4D, Fig 5C-D) revealed that mutating box 2 causes a slight reduction of class II expression, while mutating box 1 causes a clear induction of biofilms or colonies, an effect that was not seen in liquid cultures. The simplest explanation is that the positive regulation by AlgZR occurs through binding to AlgR box 2, whereas box 1 is responsible for inhibition under some circumstances. By overexpressing AlgR and AlgR D54N, we realized that the wild-type AlgR protein is able to complement the mutation, whereas AlgR D54N causes a clear inhibition in colonies and biofilms (Fig. 4C-D, Fig. 5C). However, mutating AlgR box 2 can immediately switch the effect of AlgR overexpression from an induction to a strong repression (Fig. 4D). We therefore deduce that under some circumstances the AlgR protein can bind to box 1 to inhibit class II RNR. In the mucoid phenotype, we can see a surprisingly strong reduction of class II expression in both colonies and biofilms (Fig. 4C, Fig 5D). Correlating these data with the class I RNR regulation, we propose that box 2 could be responsible for increasing P*nrdJ* expression under some stress conditions. Meanwhile, box 1 could be inhibiting class II RNR expression in the mucoid phenotype, likely in favor of class III RNR activity, but further experiments will be needed to determine this.

In light of the differences observed with AlgR phosphorylation, which must be dependent on an external signal, we tested the effect of oxidative stress; this condition is reported to dramatically induce RNR transcription through unknown mechanisms (25, 26). Surprisingly, we demonstrated that despite being in the exponential phase, where AlgR regulation is not normally very prominent, eliminating the AlgR system caused the RNR network to be insensitive to stress (Fig. 6). This is, to our knowledge, the first description of a molecular link between oxidative stress conditions and RNR expression.

Based on these results, we suggest a model for *nrd* regulation by the AlgZR system (Fig. 7). In this model, on the one hand, class I and class II RNR are being activated by AlgR under planktonic or early colonies/biofilms, responding to AlgR phosphorylation under stress; on the other hand, in the mucoid biofilm the great accumulation of AlgR would cause an inhibition of class II RNR through binding of box 1. Examining this throughout all life cycles, when planktonic cells suffer the presence of a stress condition (oxidative stress, antibiotic treatment, etc.) it activates AlgR (13,14,33). Under these conditions, the *algR* gene is expressed from promoters ZT1 (further activated by Vfr) and ZT2 (constitutive) as *fimS-algR* (33), a combination that favors phosphorylation. AlgR will induce the *nrdAB* and *nrdJ* genes, the operon of *fimUpilVWXY1Y2E* (Type IV pili) for the adhesion to surfaces (34), and the *rhlAB* for quorum sensing cell communication and microcolony formation (25). In the absence of these genes, a biofilm is not formed and cells are more sensitive to stress conditions or antibiotic treatment (13,26). When *P. aeruginosa* is attached to the surface, cells grow as microcolonies, which can slowly evolve to a biofilm phenotype. In biofilm conditions, a further induction of the RT1 and RT2 promoters starts to appear, controlled by AlgU and RpoS, and *algR* is therefore expressed as *algR-hemCD* (avoiding the *fimS* gene). Then, AlgR can initiate the induction of genes such as the *algD* and *algC* operons for the synthesis of alginate (33) and *hemC* and *hemD* for the synthesis of the heme group, as well as allowing the synthesis of uroporphyrinogen III, a precursor of vitamin B_{12} (35) which is the essential cofactor for NrdJ activity. NrdJ is also induced by the Dnr transcriptional factor (19). When biofilm becomes mature, fully anaerobic conditions appear (36,37), inducing both *nrdJab* and *nrdDG* (19). It has been reported that many genes regulated by AlgR are also regulated by anaerobic transcriptional factors such as Anr or Dnr *(arcDABC, ccoP2, hcnB, oprG, hemN or nrdJ)* (6, 14, 19, 38-42). We observed a significant decrease when inhibiting AlgR and Dnr binding and a gradual reduction of P*nrdJ* expression when mutating both systems (Fig. S7). If selected mutations degenerate the biofilm into a mucoid phenotype, the full release of AlgU from MucA causes a dramatic induction of RT1 and RT2 promoters in P*algR*, which decreases the expression of NrdJ by activating P*nrdJ* AlgR-box

1 and favors class III RNR, which is better-suited for full anaerobiosis and does not require vitamin B_{12}.

There are several pieces of information that remain unknown. Several results, like the fact that mutating the *algR* gene has a different effect than mutating both AlgR-boxes on P*nrdJ*, show us that there are more regulation events that have not yet been described, perhaps even an indirect action of AlgR on RNR activity, and the differences observed between surface colonization and biofilm formation conditions suggest interactions with other regulation systems. However, we believe that these new results bring us closer to understanding the regulation of complex RNR networks such as that of *P. aeruginosa*, as well as how it adapts to environmental conditions and evolves throughout its life cycle. The link between oxidative stress, the AlgZR system, and RNR regulation provides, for the first time, a molecular explanation for this effect; further experiments will be conducted to evaluate whether these results extend to other bacterial species.

Material and methods

Bacterial strains, plasmids and growth conditions. Different *Pseudomonas aeruginosa* and *Escherichia coli* strains were used, as listed in Table S1. Bacteria were routinely grown in LB medium (Scharlab, Spain) at 37˚C; when needed, antibiotics were added at the following concentrations: 100 μg ml^{-1} gentamicin, 40 μg ml^{-1} tetracycline, or 500 μg ml^{-1} carbenicillin for *P. aeruginosa* and 30 μg ml^{-1} chloramphenicol, 10 μg ml^{-1} gentamicin, 50 μg ml^{-1} ampicillin and 50 μg ml^{-1} kanamycin for *E. coli*. Anaerobic growth was performed in LB medium containing 10 g/l KNO_3 in screw-cap tubes (Hungate Tubes) that were filled to the top with N2.

DNA manipulation and plasmid construction. Molecular biology enzymes and kits were purchased from Fermentas (ThermoFisher) and used according to the manufacturer's instructions. DNA amplification was performed by PCR using DreamTaq MasterMix (2X) or High-Fidelity PCR Enzyme Mix (Fermentas, ThermoFisher) following the manufacturer's instructions, with the primers listed in Table S2. All other manipulations were performed using standard procedures (43). DNA was transferred into *P. aeruginosa* cells via electroporation using a Gene Pulser XCell™ electroporator (Bio-Rad) as previously described (30). The absence of mutations introduced during cloning was verified via DNA sequencing.

An AlgR transcriptional factor overproducer was built by cloning the *algR* gene (PA5261) into the pET28a overexpression system (Novagen) downstream of the T7 RNA polymerase promoter. The *algR* gene was amplified from *P. aeruginosa* PAO1 by PCR using the primers AlgR-up and AlgR-low and High-Fidelity PCR Enzyme Mix. The fragment amplified (747 bp) was cloned

into the pGEM-T easy vector and transformed into *E. coli* DH5α. After plasmid isolation using GeneJET Plasmid Miniprep Kit, the plasmid was digested with *Nde*I and *Not*I restriction enzymes and ligated with T4 DNA ligase into the pET28a vector, obtaining plasmid pETS201. Finally, pETS201 was transformed into the Rosetta(DE3) strain for AlgR overproduction and purification.

To produce the AlgRD54N mutant overproducer, the *alg*R gene was specifically mutated by PCR-based site-directed mutagenesis as previously described (19) using primer pair 1 (see Table S3). The mutant gene obtained was cloned into the pGEM-T easy vector, transformed into *E. coli* DH5α cells and verified by DNA sequencing. *Nde*I and *Not*I restriction enzymes were used for fragment digestion and cloning into the pET28a vector using T4 DNA ligase. Finally, pETS202 was transferred into the Rosetta(DE3) strain for AlgRD54N overproduction and purification.

Complementation vectors for providing extra copies of AlgR and AlgRD54N were constructed by cloning the corresponding genes under the control of their own promoter regions into the pUCP20T vector. First, a band containing the *algR* gene, the neighboring gene *fimS,* and their promoter region was amplified (2286 bp) using primer pair 2 and cloned into pUCP20T, generating pETS203. The *algR* was site-specifically mutagenized as previously described (19) to generate *algR*D54N using mutagenic primer pair 1 and outer primer pair 2 and cloned into pUCP20T to generate pETS204.

To construct the *algD, algR* and PA1157 transcriptional GFP fusions, 900 bp, 483 bp and 769 bp long fragments encompassing the *algD, algR,* and PA1157 promoter regions were amplified by PCR using primer pairs 3-5; the obtained DNA fragments were cloned into pGEM-T easy and transformed into *E. coli* DH5α cells. *BamHI* and *SmaI* restriction enzymes were used for fragment digestion and cloning into pETS130-GFP, to generate pETS205, pETS206 and pETS207 plasmids for *algD*, *PA1157* and *algR* promoter regions, respectively.

For AlgR-box mutagenesis in the studied promoters, PCR-based site-directed mutagenesis was used as previously described (19), using outer primer pairs 6 and 7 for the *nrdAB* and *nrdJab* promoter regions, respectively; mutagenic internal primer pairs 8-10 were used. Two regions of the P*nrdJ* promoter mistakenly identified as AlgR-boxes as artifacts in the bioinformatic search were also mutated using mutagenic primer pairs 11 and 12. For all the positively identified AlgR boxes, the mutant DNA fragments were later cloned into the pGEM-T easy vector and transformed into *E. coli* DH5α cells. BamHI and SmaI restriction enzymes were used for fragment digestion and cloning into pETS130-GFP, to generate pETS208 (for P*nrdA* box1),

pETS209 (for P*nrdJ* box1) and pETS210 (for P*nrdJ* box2). For the exact sequence of the mutations introduced, see Fig. 2.

Green fluorescent protein-based gene reporter assay. The different *P. aeruginosa* strains were grown in separate Erlenmeyer flasks containing 20 ml LB broth and the specified antibiotic. Flasks were incubated at 37˚C and agitated at 200 rpm. Bacterial growth was monitored by measuring optical density at 550 nm (OD_{550}). Upon reaching the desired OD_{550}, three independent 1 ml samples were taken from each analyzed strain and centrifuged for 10 minutes at 13.000 rpm; the supernatant was removed and the pellet was washed with PBS 1x containing 2% formaldehyde. Suspensions were left on ice for ten minutes before being centrifuged again, the supernatant removed and PBS 1x added. The fluorescence was then measured after diluting the sample 8 times in PBS 1x, using 96-well plates (Costar® 96-Well Black Polystyrene plate, Corning) on an Infinite 200 Pro Fluorescence Microplate Reader (Tecan).

To determine gene expression during biofilm formation, an aerobic static biofilm was grown on 96-well plates (Nunclon Delta Surface, Thermo Scientific) in LB containing 0.2 % glucose. At the desired time, the planktonic cells on the supernatant were removed, and the biofilm was washed three times with PBS 1x and then fixed with 2 % formaldehyde. GFP was measured using Infinite 200 Pro Fluorescence Microplate Reader (Tecan). Fluorescence obtained at each time point was compared with fluorescence at 3h of biofilm formation to calculate the induction factor of the gene expression.

For gene expression measurement during colony formation, 5 µl inocula at an OD_{550} of 0.05 of the corresponding *P. aeruginosa* strains were grown on 6-well plates (Nunclon Delta Surface, Thermo Scientific) containing LB with 1.5 % agar and the corresponding antibiotics. Plates were incubated at 37ºC, and GFP expression was measured at different phases of colony formation; fluorescence measurements were performed by using an Infinite 200 Pro Fluorescence Microplate Reader (Tecan).

Bioinformatic prediction of AlgR binding boxes. To identify putative AlgR binding sites on RNR promoters, a thorough bioinformatics search was conducted. As a first step, we used MEME (MEME suite, (44,45)) to generate count matrices characteristic of the AlgR binding box. As the binding sequence is small and somehow flexible (14), different sources were considered to obtain the AlgR box motif. Three sets of sequences were therefore used: set 1, to

obtain a motif characteristic of strong binders, formed by the 50 most enriched sequences in ChIP-seq after AlgR precipitation (12); set 2, to form a more flexible motif sequence, 25 randomly selected sequences from the enriched group in the same ChIP-seq experiments; and set 3, to capture the variation observed in some experimentally demonstrated boxes, confirmed by a previously published cluster of representative binding sites (14). Assuming one occurrence of the AlgR box on every sequence, a single 11-bp long motif was obtained from each set (see Fig. S1), each one defined by a count matrix.

Using the generated count matrices, we used FIMO (MEME suite, (44,46)) to search for AlgR binding spots. We calibrated the search from each count matrix by using a negative control (a 1050 bp-long probe of random DNA with a 67% GC content, to match genomic *P. aeruginosa* DNA) and a positive control (a 1050 bp probe of *algD* promoter spanning all three identified AlgR binding sites (3), from -900 to +150 bp, counted from the *algD* start codon). For the final search, DNA probes used were 900 bp long for P*nrdJ* and P*nrdD* (from -750 bp to +150 bp, counted from the corresponding start codons) and 1050 bp long for P*nrdA*, given the predicted long 5' UTR present (from -900 bp to +150 bp, counted from the *nrdA* start codon).

AlgR overexpression and purification. AlgR and AlgRD54N proteins were overexpressed in a Rosetta (DE3) *E. coli* strain transformed with pETS201 or pETS202, respectively (Table S1). Cells were grown in LB medium with 30 μg ml^{-1} kanamycin and 17 μg ml^{-1} chloramphenicol and incubated at 37ºC with vigorous shaking (250 rpm). When cultures reached an $OD_{550} \approx 0.5$, protein overexpression was induced by adding IPTG to a concentration of 1.0 mM (Isopropyl β-D-1-thiogalactopyranoside; Fermentas, Thermo Scientific); cells were cultured at 37ºC for 6 hours and later pelleted by centrifugation.

For preparing the protein extract, the pellet was suspended in 15 ml of AlgR lysis buffer per liter of original culture (50 mM Tris, pH 7.8 at 25ºC; 300 mM NaCl; 20 mM imidazole; 2 mM DTT; 10% glycerol), supplemented with 1 mM PMSF as a protease inhibitor. The resulting suspension was sonicated on ice using a 6 mm conical microtip, until clear, to generate the crude extract (CE). It was centrifuged at 15000 g for 30 minutes at 4ºC, keeping the supernatant as the soluble fraction (SF), which was frozen at -80ºC for long term storage.

AlgR and AlgRD54N were purified from their corresponding SF by Immobilized Metal Affinity Chromatography (IMAC) using a 5 ml His-Trap™ HP column (GE Healthcare) in an FPLC system (BioLogic DuoFlow System, Bio-Rad). First, the column was equilibrated with 5 column volumes (CV) of Buffer A (50 mM TrisHCl pH 7.8 at 25ºC; 300 mM NaCl; 20 mM imidazole).

Protein samples were diluted with buffer A to a concentration of less than 1 mg/ml of total protein content and then injected into the column. A washing step was then carried out with 5 CV of Buffer A, and contaminant proteins were removed with a nonspecific elution step using 5 CV of Buffer A with 50 mM imidazole. Finally, protein was recovered in a specific elution step using 5 CV of Buffer A with 400 mM imidazole. The resulting fractions were analyzed by SDS-PAGE protein electrophoresis and dialyzed against AlgR Binding buffer (20mM TrisHCl, pH 7.8 at 25ºC; 120 mM KCl; 2 mM MgCl2; 10% glycerol) and stored at -80ºC (see purity in Fig. S5). Protein concentrations were determined by the Bradford assay (Bio-Rad) with crystalline bovine serum albumin as a standard.

Electrophoretic mobility shift assays (EMSA). DNA probes for EMSA were produced for analyzing full promoter regions of the *nrdAB* and *nrdJab* operons (P*nrdA* long and P*nrdJ* long bands) or fragments of these promoters (P*nrdA* short 1 and P*nrdA* short 2, as well as P*nrdJ* short 1, P*nrdJ* short 2 and P*nrdJ* short 3; see Fig. S2). DNA probes were also generated for the *nrdDG* operon promoter region (P*nrdD* long band) and for negative control (inner region of non-related *anr* gene) and positive control, using the *algD* operon promoter region (P*algD* band). All probes were generated by amplifying the corresponding region in a first PCR reaction that uses the reverse primer to also add the arbitrary sequence 5'-CTGGGCGTCGTTTTAC-3' at the 3' end of every probe (a sequence that we call the M13 complementarity tail) and later applying a second PCR reaction using primer WellRed-M13 to label the probes; WellRed-M13 is a WellRED dye-labeled oligo (Sigma-ALDRICH) coupled to the near-infrared fluorophore D3-phosphoramidite (D3-PA). Resultant probes are hence double-stranded DNA fragments labeled with a single molecule of D3-PA. Primer pairs 13-21 were used for EMSA band generation (see Table S3). All wild-type bands were copied from *P. aeruginosa* PAO1 genomic DNA. All bands harboring mutations in putative AlgR-boxes were copied from the corresponding plasmids including mutant promoters (see the DNA manipulation and plasmid construction section).
Purified AlgR or AlgRD54N proteins were used in binding reactions for a total amount of 0, 2, 5 or 10 pmol per reaction. A fixed amount of 100 fmol of DNA was used for all bands. Binding reactions also contained BSA (0.25 μg / reaction) and salmon sperm DNA (1 μg / reaction), as well as 2x-AlgR-binding buffer, added to a final 1x concentration of 20 mM TrisHCl (pH 7.8 at 25ºC), 100 mM KCl, 2 mM $MgCl_2$, 2 mM dithiothreitol, and 10% glycerol. Water was added to every reaction for a final volume of 20 μl. Reactions were incubated at room temperature for 20 minutes before gel electrophoresis.

Electrophoresis was performed in 5% acrylamide gels, prepared with a 37.5:1 proportion of acrylamide:bis-acrylamide and using 5% triethylene glycol as an additive for increased DNA-protein complex stability. Final images were obtained by scanning the gels using the Odyssey Imaging System (LI-COR Biosciences) working in the 700-nm channel.

Atomic force microscopy (AFM). DNA probes for AFM studies were generated by PCR from *P. aeruginosa* PAO1 genomic DNA, designed so that binding spots were closer to one of the ends, to clearly distinguish binding events. Primer pairs 22-25 were used for generating the DNA probes (see Table S3). To avoid agarose contamination, when PCR conditions were proved to result in one single amplification band, DNA probes were purified directly from PCR reactions using a GeneJET PCR Purification Kit (Fermentas, ThermoFisher). Purified DNA probes were diluted to 2-4 nM with DNA AFM buffer (10 mM HEPES, pH 7.8 at 25ºC; 5 mM $MgCl_2$; 50 mM NaCl). Ten milliliters of DNA solution was pipetted onto a freshly cleaved mica and allowed to deposit for 1 min. The mica surface was then rinsed with 200 ml of MilliQ water and dried under a nitrogen stream. For the DNA-protein complex images, protein (AlgR / LexA) was previously mixed with the DNA fragments to a molar ratio of 3:1; the complex was incubated for 20 minutes at room temperature, and 10 ml of the solution was deposited on freshly cleaved mica and allowed to deposit for 4 minutes before rinsing and drying. Topographic images were obtained with a commercial AFM system (CypherTM, Asylum Research) in conventional dynamic mode. A PPP-CONTR (Nanosensors) tip was used, with a nominal spring constant of ~0.3 N / m and tip radius of ~7 nm, scanning in ambient conditions using small oscillation amplitudes (~20 nm). Image resolution was not lower than 6 nm/pixel since this is close to the tip radius curvature. AFM image processing and determination of DNA length were carried out using WSxM 5.0 develop 7.0 (WSxM solutions).

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FIG 1 *In vivo* AlgR regulation of RNR promoters and related genes. Gene reporter assays for P*nrdA* (pETS134), P*nrdJ* (pETS180), P*nrdD* (pETS136), P_{PA1157} (pETS206) and P*algD* (pETS205) fused to GFP at exponential and stationary growth phases. Values are averages from at least three independent experiments, and error bars show positive standard deviation. Asterisks (*) indicate a statistically significant difference from the wild-type strain (*p*-value less than 0.05 in pairwise *t*-tests). Shortened names are used (see table S1).

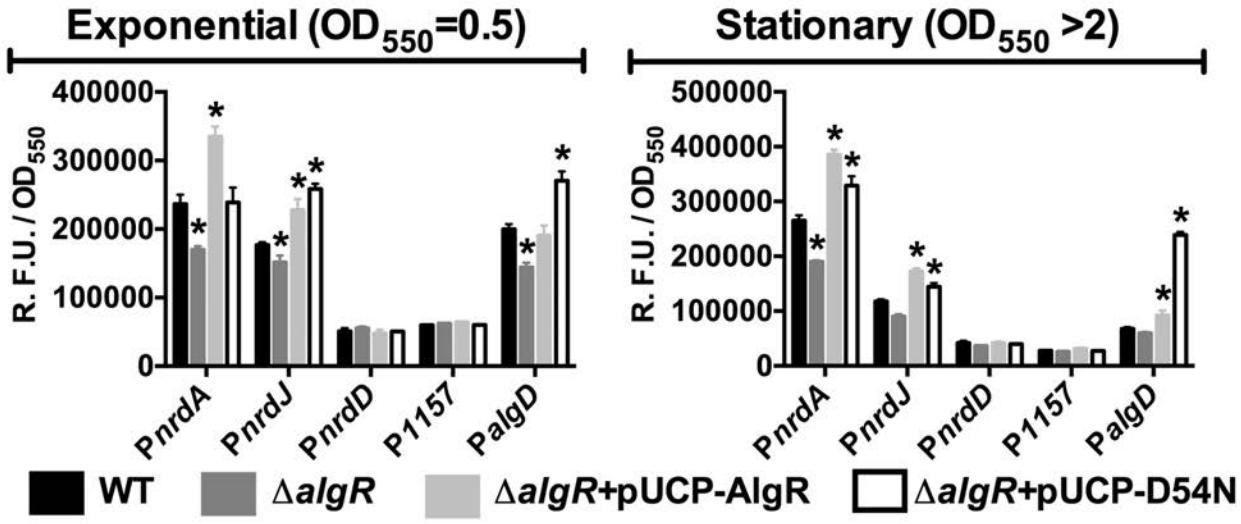


FIG 2 Functional study of the AlgR boxes in RNR promoters. **A**, EMSA experiments for DNA probes of promoters P*nrdA*, P*nrdJ* and P*nrdD*, together with positive control (P*algD*) and negative control probes. Numbers below the triangles represent pmol of AlgR. **B**, Gene reporter assay for P*nrdA* and P*nrdJ*, during the early stationary phase (OD_{550} = 2.0) and under aerobic conditions. Error bars represent positive standard deviations; the asterisk indicates a statistically significant difference from the wild-type strain (*p*-value less than 0.05 in pairwise *t*-tests). The exact mutations introduced are detailed at the right of the graphic, and a simplified consensus sequence of the AlgR box is provided for comparison. Shortened names are used (see Table S1).

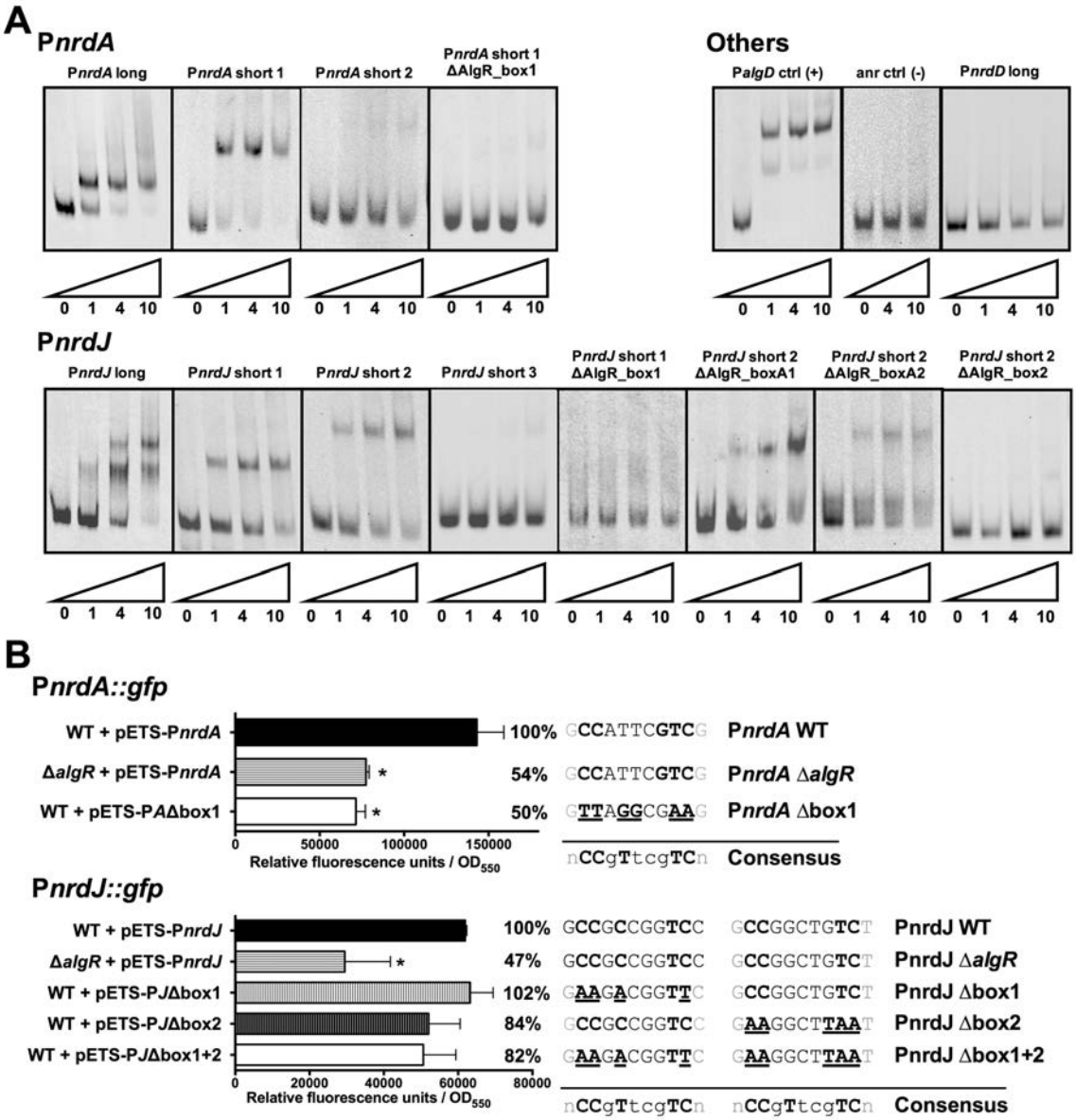


FIG 3 Atomic force microscopy images of DNA and DNA-protein complexes. A, AFM images of DNA molecules or DNA-protein complexes, taken on mica under ambient conditions, are shown for P*nrdA*, P*nrdJ*, P*algD* and P*dinB* promoters. Small images depict single DNA probes; scale bars without numbers above represent 80 nm. For the *PnrdA* and P*nrdJ* promoters, a general image at a higher scale is also shown. Colors represent the height of the structures, according to the scale at the right. **B**, comparison of the apparent length of randomly selected units of all DNA probes when, prior to drying, they were in water (images not shown) or in AlgR binding buffer and when complexed with AlgR.

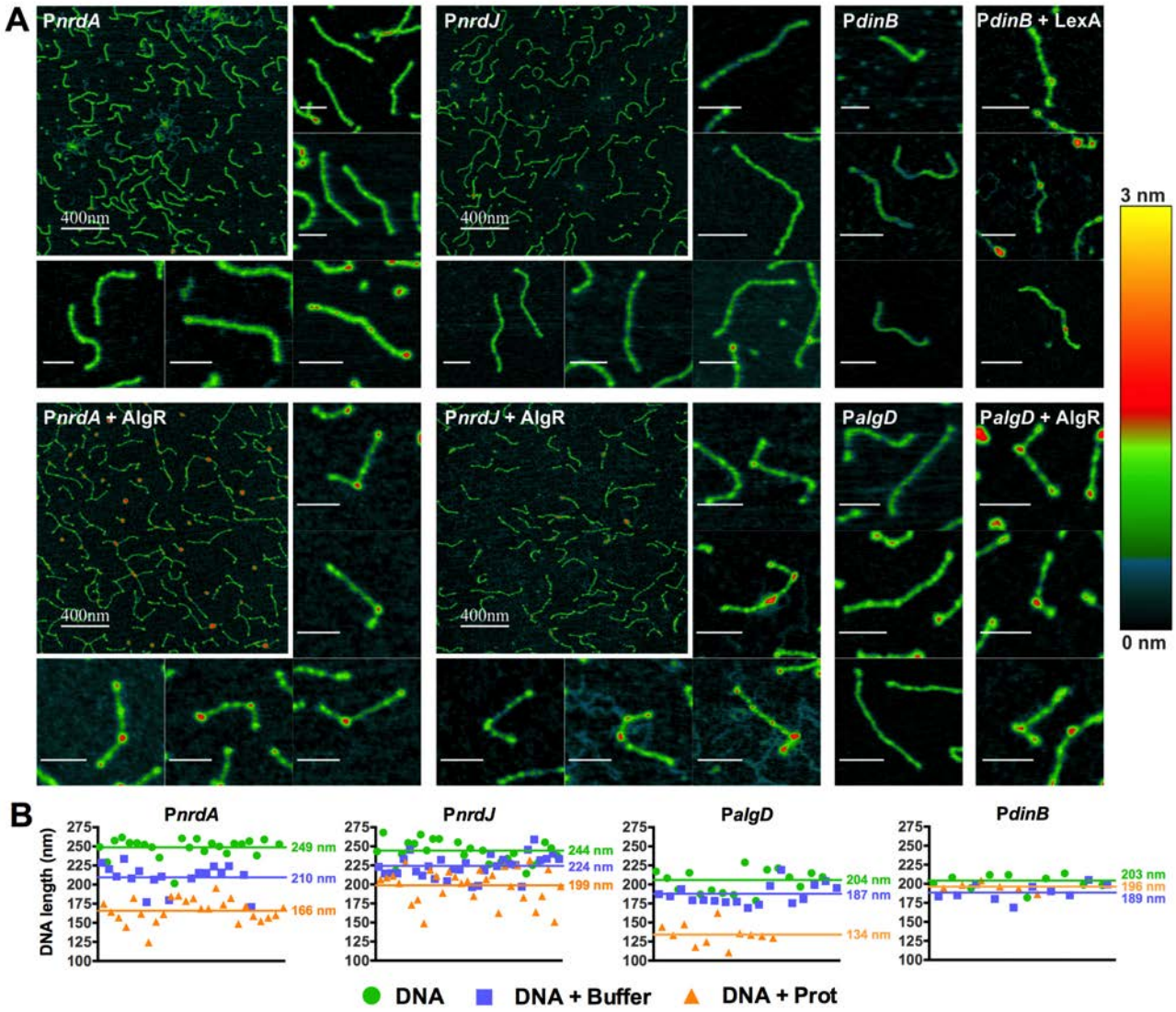


FIG 4 AlgR regulation of RNR promoters during surface colonization. GFP-based gene reporter assays for P*algD* (pETS205, **A**), P*nrdA* (pETS134, **B**) and P*nrdJ* (pETS180, **C**) promoters fused to GFP, during surface colonization. GFP fluorescence is measured at different times of incubation during colony formation and presented as relative fluorescence units. Mucoid strains (PAOMA, PAO Δ*mucA*) are included. A fourth panel (**D**) presents further experiments with P*nrdJ* AlgR boxes to study the fine regulation performed at this level. For improved readability and easier strain identification, shortened names are used (see Table S1). The key features of each strain are highlighted in bold, wild-type strains are underlined and mutant boxes are color-coded.

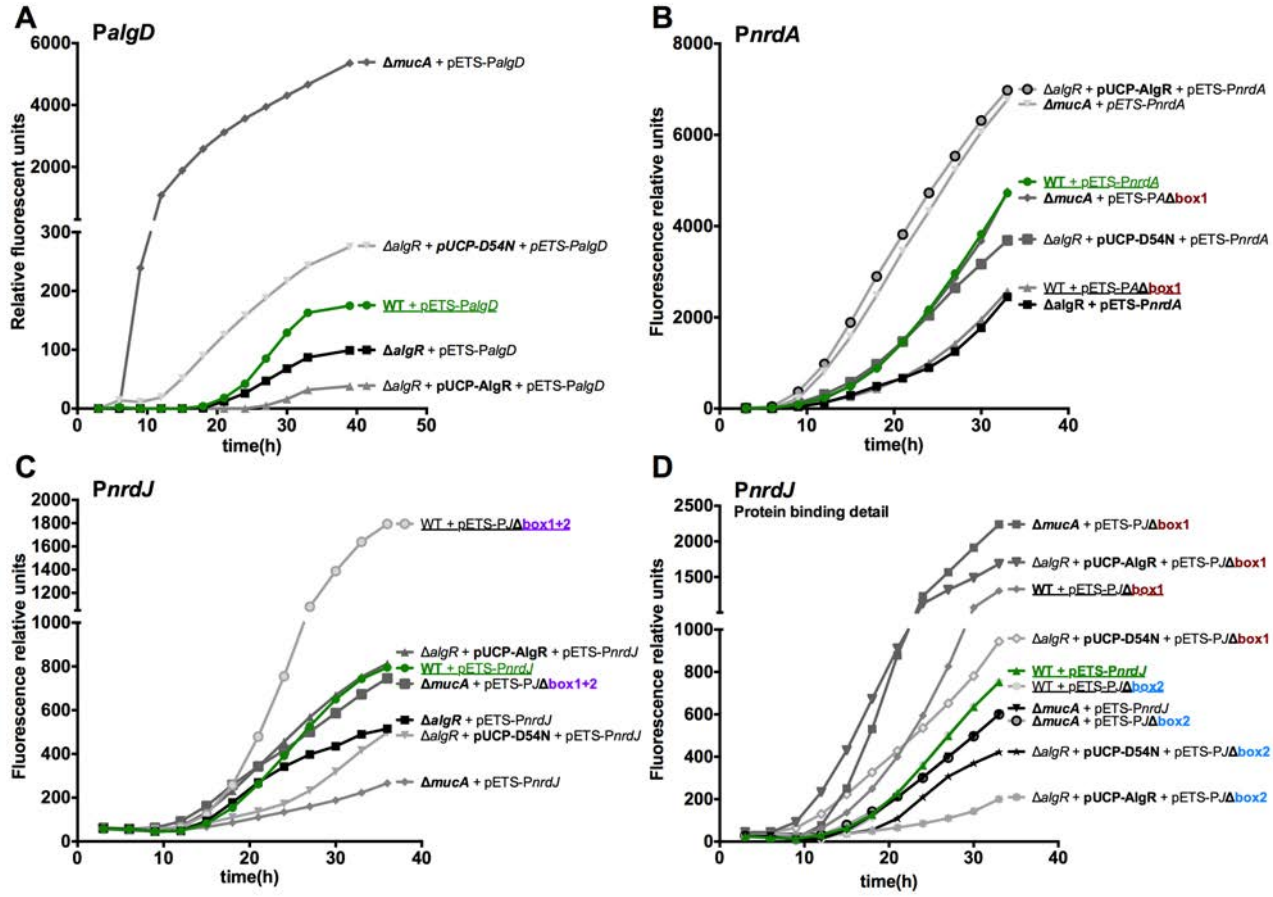


FIG 5 AlgR regulation of RNR promoters in mucoid and non-mucoid biofilms. Gene reporter assay at different time points during static biofilm formation for P*algD* (pETS205, **A**), P*nrdA* (pETS134, **B**) and P*nrdJ* (pETS180, **C** and **D**). The values shown are the means of three independent experiments in 8 wells; error bars show positive and negative standard deviation. Shortened names are used (see Table S1). For 48 h and 72 h, results are depicted as bar graphs; error bars show positive standard deviation, and the asterisk indicates a statistically significant difference from the wild-type strain (*p*-value less than 0.05 in pairwise *t*-tests).

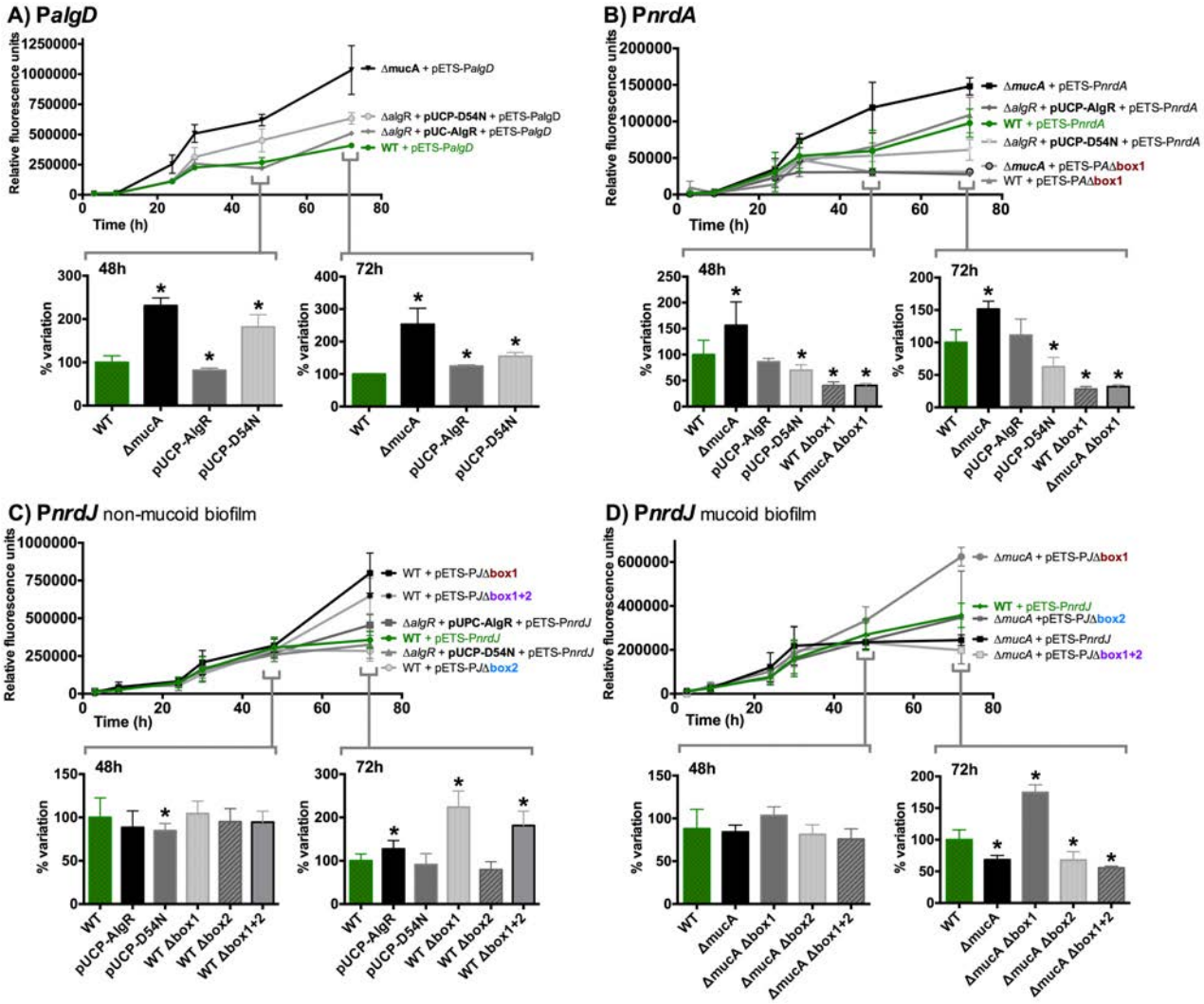


FIG 6 AlgR regulation of RNR during oxidative stress. Gene reporter assays for the P*nrdA* and P*nrdJ* promoters fused to GFP. All strains were grown to OD_{550} = 0.5 and then subjected to 30 minutes of incubation with a stressing agent (1.0 mM H_2O_2) or control (equivalent volume of water). Values are averages from three independent experiments, and error bars show positive standard deviation. Asterisks (*) indicate statistically significant difference from the untreated wild-type strain (*p*-value less than 0.05 in pairwise *t*-tests). Shortened names are used (see table S1).

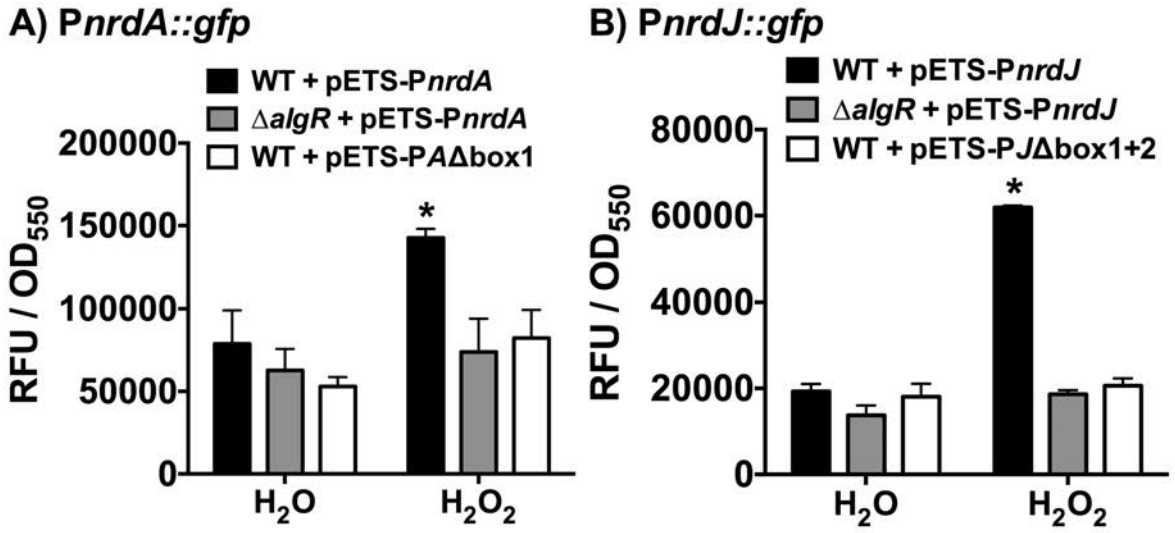
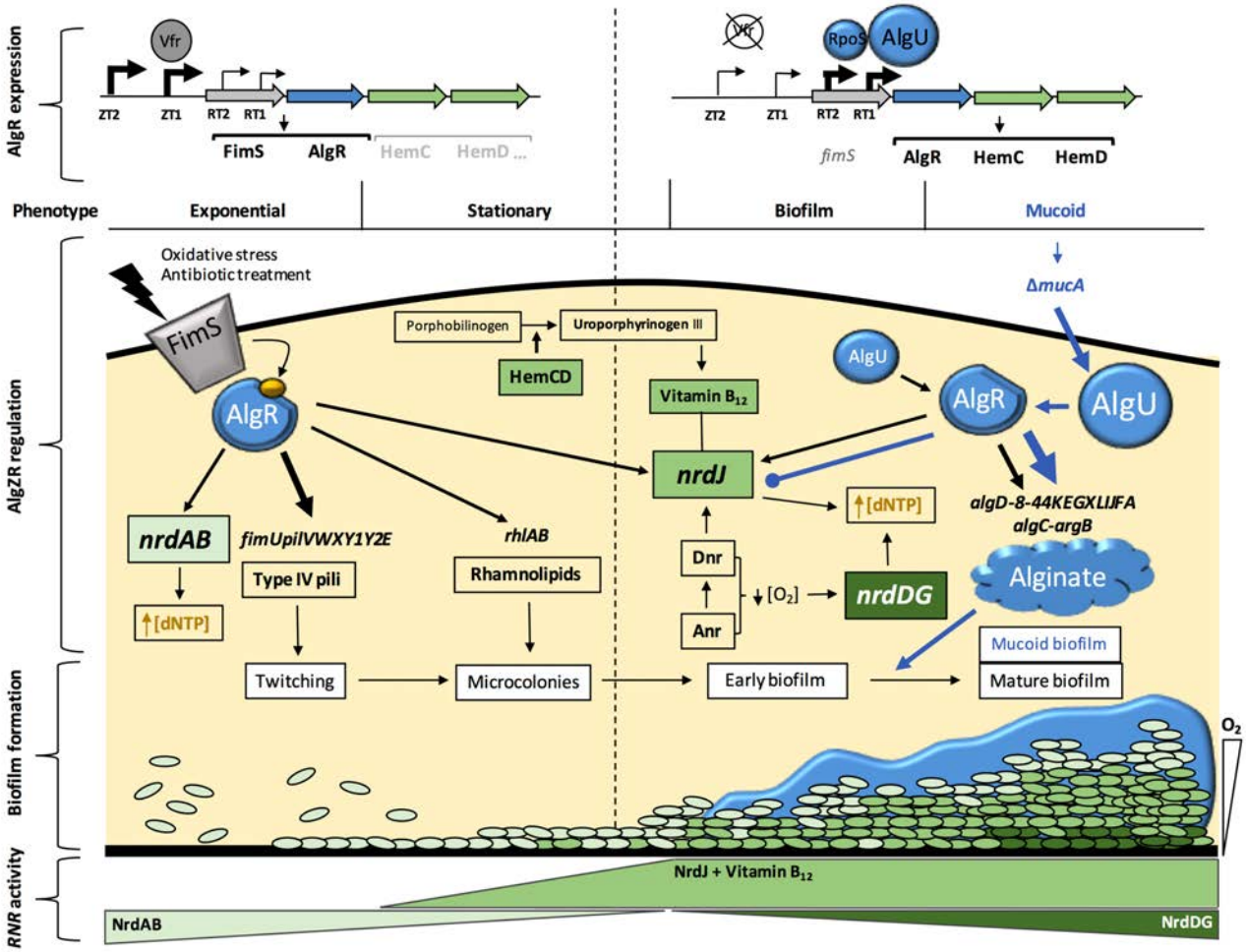


Fig 7 Model of AlgZR regulation of ribonucleotide reduction.



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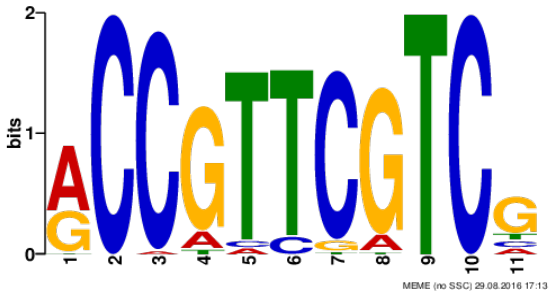
SUPPLEMENTARY MATERIAL

FIG S1 Count matrices for AlgR-box identification. Count matrices were generated by FIMO search using three different sets of sequences containing AlgR binding spots (see Materials and Methods). Matrices are adjusted for a box size of 11 bp, represented in rows, and the bases are expressed in columns in the order A–C–G–T; each matrix is accompanied by its corresponding HMM logo.

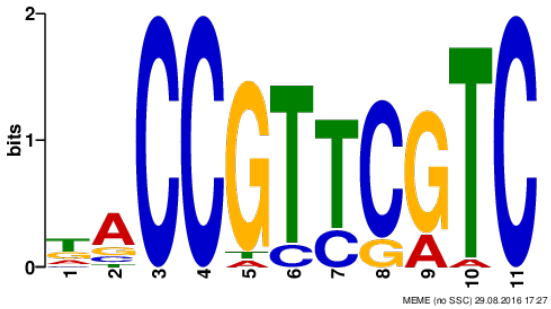
Set 1: 50 best hits in ChIP-seq

30	0	19	1
0	50	0	0
1	49	0	0
6	0	42	2
2	2	0	46
0	5	0	45
0	46	3	1
5	0	44	1
0	0	0	50
0	50	0	0
6	6	32	6



Set 2: 25 random hits in ChIP-seq

4	2	6	11
14	3	4	2
0	23	0	0
0	23	0	0
1	0	21	1
0	3	0	20
0	6	0	17
0	19	4	0
5	0	18	0
1	0	0	22
0	23	0	0



Set 3: Published sequences

3	4	3	1
0	11	0	0
0	11	0	0
0	1	10	0
0	0	0	11
0	2	1	8
0	6	3	2
1	0	8	2
0	0	2	9
0	11	0	0
1	6	3	1

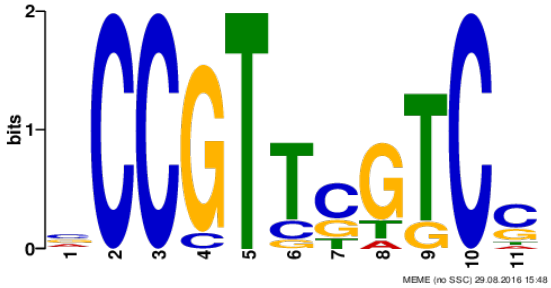


FIG S2 Representation of the DNA probes used in this study. Schematics for promoters P*nrdA* (RNR class I promoter, **A**) and P*nrdJ* (RNR class II promoter, **B**). Identified boxes are represented in green and indicated as box1 and box1 / box2 for P*nrdA and* P*nrdJ*, respectively; artifact boxes identified as false positives in the bioinformatics search are represented in red and indicated as boxA1 / boxA2 in P*nrdJ*. Genes are represented by arrows; gene *znuA* has been eliminated from the P*nrdJ* schematic for improved readability. An approximated prediction of the 5'UTR for the studied operons (BPROM) is shown as dashed lines. Locations are indicated in base pairs relative to the ATG translation start codon of the first gene of the corresponding operon. DNA probes used for EMSA studies (see Fig. 2) are indicated by solid blue lines.

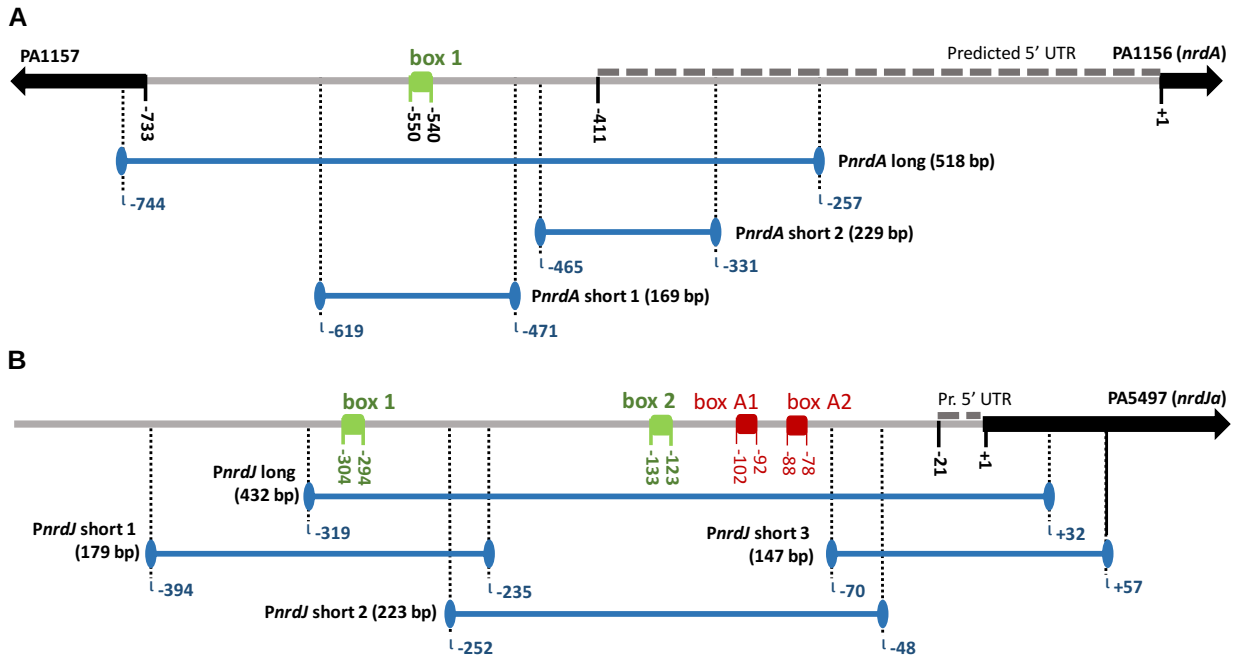


FIG S3 Effect of AlgR phosphorylation on *in vitro* DNA binding. EMSA assays for the P*nrdA* and P*nrdJ* promoter long bands, together with *anr* negative control band (NEG). The experimental conditions are harsher than those of the other EMSA assays described in Materials and Methods, reducing glycerol to 5% and removing TGE from the gels, to provide a stricter environment and highlight small differences in AlgR-DNA binding affinity. AlgR wild-type protein (AlgR WT) or AlgR D54N mutant protein was added at 0, 1, 4 or 10 pmol to promoter probes or at 0 and 10 pmol to negative control probes.

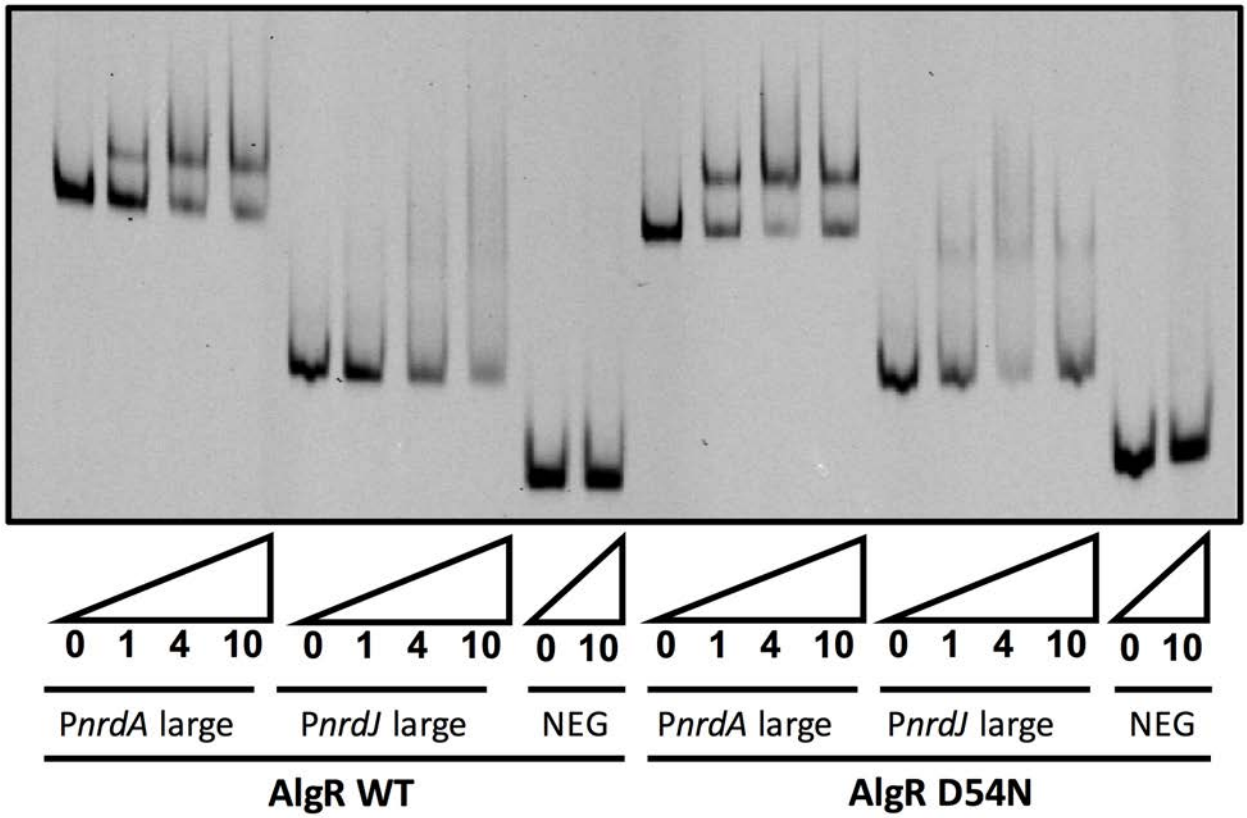
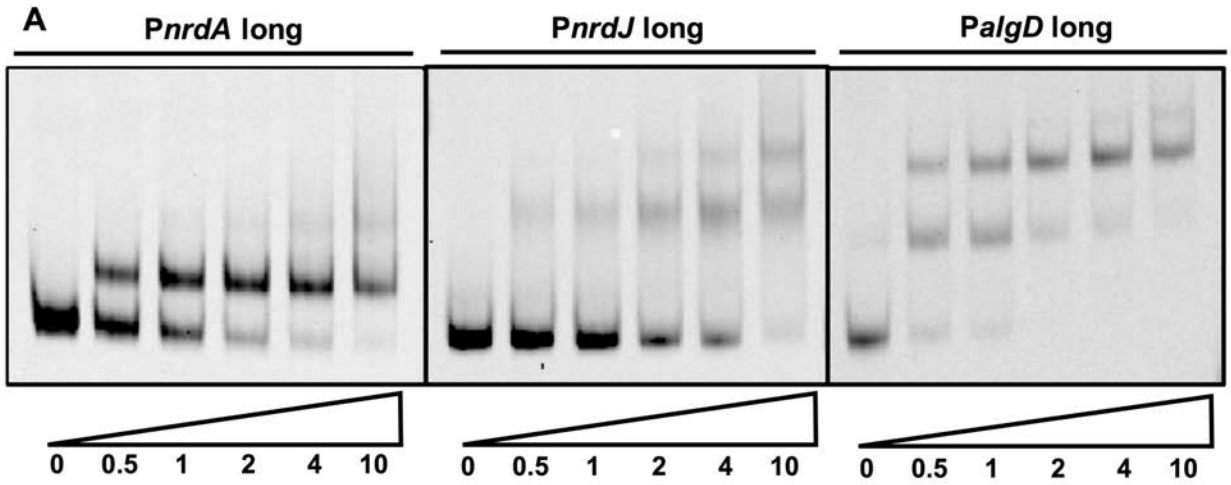


FIG S4 AlgR – DNA binding affinities. EMSA assays for P*nrdA*, P*nrdJ*, and *PalgD* promoter long bands. (**A**), A wide array of concentrations was used to illustrate different binding affinities (shown below the figures; numbers represent protein amount in pmol). Different boxes involved in bindings are shown in the table below. (**B**), Most conserved base pairs are underlined, and cytosine in position 7, which is described to distinguish weak and strong binding sites, is marked in gray.



B

Binding site	Sequence	Orientation	Strength	Source
P*nrdA* box 1	GCCATTCGTCG	3'-5' ←	Strong	This work
P*nrdJ* box 1	GCCGCCGGTCC	5'-3' →	Weak	This work
P*nrdJ* box 2	GCCGGCTGTCT	5'-3' →	Weak	This work
P*algD* RB1	ACCGTTCGTCC	5'-3' →	Strong	1, 2
P*algD* RB2	ACCGTTCGTCT	5'-3' →	Strong	1, 2
P*algD* RB3	GCCGTTTGTCC	3'-5' ←	Weak	1, 2
P*algC* ABS1	CCCGTTCGTCG	5'-3' →	Strong	3
P*algC* ABS2	TCCGTTGTTCC	5'-3' →	Weak	3
P*algC* ABS3	ACCGTGCGTCG	5'-3' →	Strong	3

(1) Kato, J. and A. M. Chakrabarty (1991). "Purification of the regulatory protein AlgR1 and its binding in the far upstream region of the algD promoter in Pseudomonas aeruginosa." Proc Natl Acad Sci U S A 88(5): 1760-1764.

(2) Mohr, C. D., et al. (1991). "AlgR, a response regulator controlling mucoidy in Pseudomonas aeruginosa, binds to the FUS sites of the algD promoter located unusually far upstream from the mRNA start site." J Bacteriol 173(16): 5136-5143.

(3) Zielinski, N. A., et al. (1991). "Characterization and regulation of the Pseudomonas aeruginosa algC gene encoding phosphomannomutase." J Biol Chem 266(15): 9754-9763.

FIG S5 AlgR regulation of RNR promoters during surface colonization under anaerobiosis. GFP-based gene reporter assay for the P*nrdA* and P*nrdJ* promoters fused to GFP during surface colonization under anaerobic conditions. GFP fluorescence is measured after 48 hours of incubation and presented as relative fluorescence units. Shortened names are used for strains and plasmids (see Table S1).

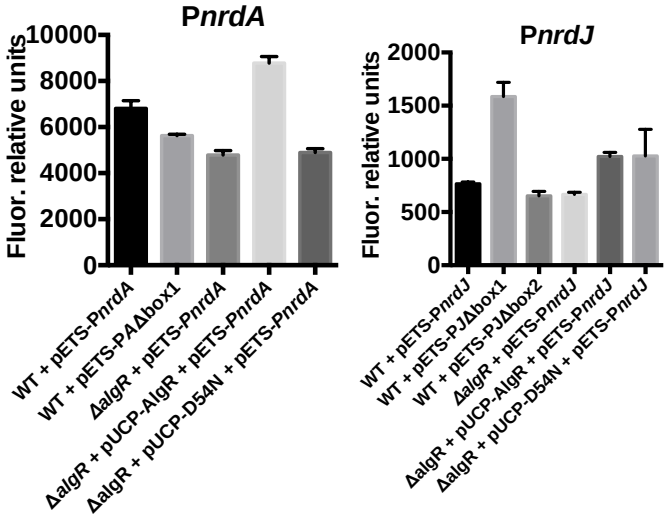


FIG S6 Protein overexpression and purification. Coomassie blue-stained gel showing SDS-PAGE analysis of AlgR wild type and AlgRD54N overexpression. MW, molecular weight marker; CE, crude extract; FT, flow through; UN, nonspecific elution step; P, protein recovered after specific elution step. Molecular weights of the standards are indicated.

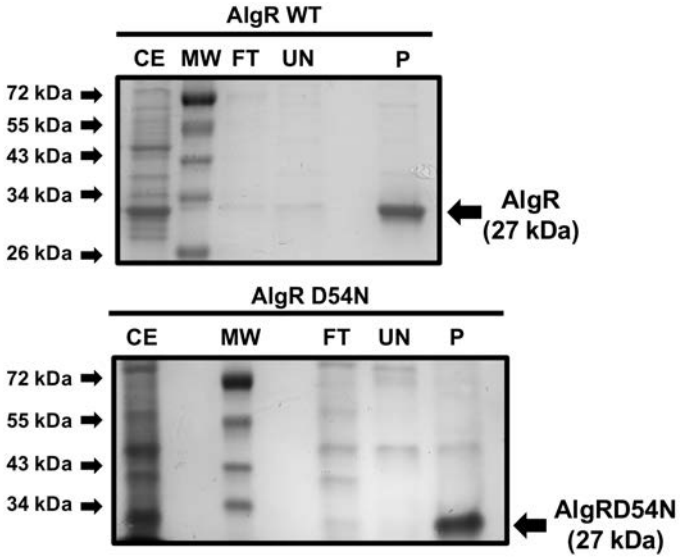


FIG S7 Cooperative regulation of AlgZR and Anr/Dnr systems on RNR class II. Gene reporter assay for the P*nrdJ* promoter fused to GFP, during anaerobic liquid cultures, grown to OD_{550} = 2.0. The cooperative action of these two systems is explored by combining a Δ*algR* background with the mutation of the Anr/Dnr box on P*nrdJ*. Values are averages from at least three independent experiments; error bars show positive standard deviation. Asterisks (*) indicate statistically significant differences from the wild-type strain harboring P*nrdJ* wild-type promoter (*p*-value less than 0.05 in pairwise T-tests). Shortened names are used (see Table S1).

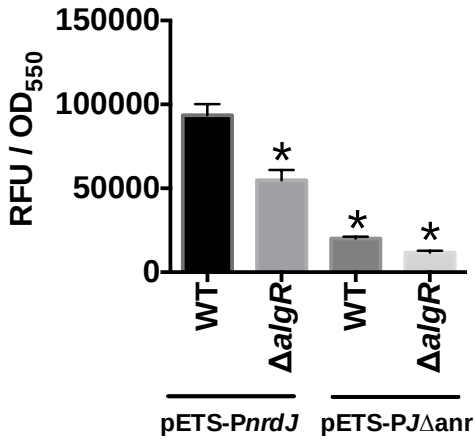


Table S1. Bacterial strains and plasmids used in this study. For each element, a general description is provided, together with an alternative self-explanatory name which will be commonly used in figures to make interpretation of the data easier for the reader. Throughout all the paper, a P before the name of a gene indicates the promoter controlling this gene (e.g., P*nrdA* for *nrdAB* operon promoter).

Name	Referred as…	Description	Source
Plasmids			
pGEM-T easy	pGEM-T easy	A/T cloning vector; Amp^R	Promega
pUCP20T	pUCP20T	Broad-host-range vector; Amp^R	(1)
pET28a	*pETS28a*	*Vector for His_6-tagged protein overexpression;* Km^R	Laboratory stock
pETS130-GFP	pETS130	Broad host range, promoterless GFP; Gm^R	(2)
pETS134	pETS-P*nrdA*	pETS130 derivative carrying *nrdA* promoter; Gm^R	(2)
pETS136	pETS-P*nrdD*	pETS130 derivative carrying *nrdD* promoter; Gm^R	(2)
pETS180	pETS-P*nrdJ*	pETS130 derivative carrying *nrdJ* promoter; Gm^R	(3)
pETS191	pETS-PJΔdnr	pETS130 derivative carrying Anr/Dnr box mutating in P*nrdJ*; Gm^R	(4)
pETS201	pETS201	pET28a derivative carrying *algR*, AlgR overproducer, Km^R	This work
pETS202	pETS202	pET28a derivative carrying algRD54N, AlgRD54N overproducer, Km^R	This work
pETS203	pUCP-AlgR	pUCP20T derivative carrying the *algR* gene; Cb^R	This work
pETS204	pUCP-D54N	pUCP20T derivative carrying the *algRD54N* gene; Cb^R	This work
pETS205	pETS-P*algD*	pETS130 derivative carrying *algD* promoter; Gm^R	This work
pETS206	pETS-P1157	pETS130 derivative carrying *PA1157* promoter; Gm^R	This work
pETS207	pETS-P*algR*	pETS130 derivative carrying *algR* promoter; Gm^R	This work
pETS208	pETS-PAΔbox1	pETS130 derivative carrying AlgR-box1 mutation in P*nrdA*, Gm^R	This work
pETS209	pETS-PJΔbox1	pETS130 derivative carrying AlgR-box1 mutation in P*nrdJ*, Gm^R	This work
pETS210	pETS-PJΔbox2	pETS130 derivative carrying AlgR-box2 mutation in P*nrdJ*, Gm^R	This work
pETS211	pETS-PJΔbox1+2	pETS130 derivative carrying AlgR-box1 and AlgR-box2 mutation in P*nrdJ*, Gm^R	This work
Strains			
E. coli			
DH5α	DH5α	*recA1 endA1 hsdR17 supE44 thi-1 relA1 Δ(lacZYA-argF)U169 deoR Φ80dlacZM15*	Laboratory stock
Rossetta(DE3)	Rosetta	*F^- ompT $hsdS_B$ (r_B^- m_B^-) gal dcm (DE3) pRARE (CamR)*	Merck Millipore
P. aeruginosa			
PAO1	PAO1 WT	Wild-type (ATCC 15692 / CECT 4122) - Spanish Type Culture Collection	Lab strain
PW9855	PAO1 Δ*algR*	*P. aeruginosa* PAO1 *algR*::ISphoA/hah; Tc^R	(5)
PAOMA	PAO1 Δ*mucA*	*P. aeruginosa mucA* strain	A. Oliver Lab

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Table S2. Primers and probes used in this study.

Name	Sequence (5'-3')	Application
M13-dir	GTTTTCCCAGTCACGAC	Check-Cloning
M13-rev	CAGGAAACAGCTATGACC	Check-Cloning
pUCP20T-up	CCTCTTCGCTATTACGCCAG	Cloning
pUCP20T-low	TCCGGCTCGTATGTTGTGTG	Cloning
pBBR1-up	CATCGCAGTCGGCCTATTGG	Cloning
pBBR1-low	CACTTTATGCTTCCGGCTCG	Cloning
AlgR-up	ACATATGAATGTCCTGATTGTCGATG	AlgR overproducer
AlgR-low	ATCAGAGCTGATGCATCAGAC	AlgR overproducer
AlgRD54N-up	GCGGATGTTCAGCAGGAC	AlgRD54N overproducer
AlgRD54N-low	GTCCTGCTGAACATCCGC	AlgRD54N overproducer
PfimSalgR-up	GGATCCTGTCTTCCTGGTTGTCCTTGTT	P*fimSalgR* cloning / AlgR complementation
PalgR-SmaI-GFP-low	TTCCCGGGCTTGAATCGGAT	P*fimSalgR* cloning
PA1157-up	AAGGATCCGGTATGCATGGGTGGGTATC	*PA1157* promoter cloning
PA1157-low	AACCCGGGTTCTTGCTCCACACAGCCTC	*PA1157* promoter cloning
PnrdA-BamHI-EcoRI-GFP-up	AGGATCCGAATTCTTGCTCCACACAGCCTC	P*nrdAB* cloning / EMSA P*nrdA* long / AFM
PnrdA-SmaI-GFP-low	ACCCGGGTTCTCGCGTGTGGTGTCG	P*nrdAB* cloning / AFM
PnrdA-EXT-low-M13	CTGGGCGTCGTTTTACGGCTCCTTGCGATGAG	EMSA P*nrdA* long
PnrdA-AlgR-EMSA-up	TACATATTGTGGGTAGGGTG	EMSA P*nrdA* short 1
PnrdA-AlgR-EMSA-low-M13	CTGGGCGTCGTTTTACGGATAAAGTGTGGGTCTTCT	EMSA P*nrdA* short 1
PnrdA-EMSA-up	TTTCCCCCAGACTGTCAC	EMSA P*nrdA* short 2
PnrdA-EMSA-low-M13	CTGGGCGTCGTTTTACTCAGAGTGGTCCGTGCG	EMSA P*nrdA* short 2
PnrdJ-AlgR-BamHI-EMSA-up	GGATCCTACGGGTTGCGCCATA	P*nrdJ* promoter cloning
PnrdJ-SmaI-GFP-low	AACCCGGGGACTGCGTTGCGTCTGTC	P*nrdJ* promoter cloning / AFM
PnrdJ-BamHI-GFP-up	GGATCCCGCGCCCAGCTGAAGGCC	EMSA P*nrdJ* long
PnrdJ-EXT-low-M13	CTGGGCGTCGTTTTACGGCCACCGTACGCAAC	EMSA P*nrdJ* long
PnrdJ-AlgR-EMSA-up	TACGGGTTGCGCCATA	EMSA P*nrdJ* short 1
PnrdJ-AlgR-EMSA-low-M13	CTGGGCGTCGTTTTACTTCGCTGAGGGTGTCG	EMSA P*nrdJ* short 1
PnrdJ-mid-up	CCGACACCCTCAGCGAAG	EMSA P*nrdJ* short 2
PnrdJ-mid-low-M13	CTGGGCGTCGTTTTACAGACAACCTTAGTCATCGGGA	EMSA P*nrdJ* short 2
PnrdJ-EMSA-up	TCCCGATGACTAAGGTTGTC	EMSA P*nrdJ* short 3
PnrdJ-EMSA-low-M13	CTGGGCGTCGTTTTACCTGATTAACCTCCCGATGG	EMSA P*nrdJ* short 3
PnrdJ-AFM-up	GCGCAAGTTCGTCAATTTCG	AFM
PnrdD-BamHI-GFP-up	AGGATCCCGCGACGCCCATTTC	EMSA P*nrdD* long
PnrdD-EMSA-low-M13	CTGGGCGTCGTTTTACCTTGAGCAGGGTGGCC	EMSA P*nrdD* long
PalgD-BamHI-GFP-up	GGATCCCTCCTCTTTCGGCAC	P*algD* cloning / EMSA positive control
PalgD-low-M13	CTGGGCGTCGTTTTACTTCCTTAATCTTCGACCCA	EMSA positive control / AFM
PalgD-SmaI-GFP-low	CCCGGGAGATGCTGATTCGCATC	P*algD* cloning
PalgD-BamHI-AFM-up	TGGATCCCCCTATCGACTGGAAATGG	AFM
Anr-EcoRI-up	GAATTCATGGCCGAAACCATCAAG	EMSA negative control
Anr-low-M13	CTGGGCGTCGTTTTACGCATCGGTGATGCTGAAG	EMSA negative control
DinB-AFM-up	CTGGTGATGCTGGTCGTG	AFM
DinB-low-M13	CTGGGCGTCGTTTTACCAGCTCCCGCAACCAC	AFM
PnrdA-mutAlgR1-up	GCTTCGCCTAACATTCTCCAGCGCTG	Mutagenesis P*nrdA* box1
PnrdA-mutAlgR1-low	TGTTAGGCGAAGCCCTCGGAAAGC	Mutagenesis P*nrdA* box1
PnrdJ-mutAlgR1-up	GGTTGCCGTAACGGTCTGCA	Mutagenesis P*nrdJ* box1
PnrdJ-mutAlgR1-low	CAGACCGTTACGGCAACCT	Mutagenesis P*nrdJ* box1
PnrdJ-mutAlgR2-up	GCTCTGAAAACTAGTTCCTGATATCCGC	Mutagenesis P*nrdJ* boxA1
PnrdJ-mutAlgR2-low	GCGCGGATATCAGGAACTAGTTT	Mutagenesis P*nrdJ* boxA1
PnrdJ-mutAlgR3-up	ATGGCCGCGAACGCTTGAGCG	Mutagenesis P*nrdJ* boxA2
PnrdJ-mutAlgR3-low	CGCTCAAGCGTTCGCGGCCAT	Mutagenesis P*nrdJ* boxA2
PnrdJ-mutAlgR4-up	CGAATTTGAAGGCTTAATGGAAAAGC	Mutagenesis P*nrdJ* box2
PnrdJ-mutAlgR4-low	TTCCATTAAGCCTTCAAATTCGC	Mutagenesis P*nrdJ* box2
WellRed-M13	[D3-PA]GTCACTGGGCGTCGTTTTAC	EMSA band infrared labelling

Table S3. PCR reactions and primer pairs used.

Primer	Forward primer	Reverse primer	Application
1	AlgR-D54N-up	AlgR-D54N-low	AlgR D54N directed mutagenesis
2	PfimSalgR-up	AlgR-low	AlgR complementation plasmids
3	PalgD-BamHI-GFP-up	PalgD-SmaI-GFP-low	P*algD*::*gfp* transcriptional fusion
4	PfimSalgR-up	PalgR-SmaI-GFP-low	P*algR*::*gfp* transcriptional fusion
5	PA1157-up	PA1157-low	P_{PA1157}::gfp transcriptional fusion
6	PnrdA-BamHI-EcoRI-GFP-up	PnrdA-SmaI-GFP-low	Outer primers in P*nrdA* promoter
7	PnrdJ-AlgR-BamHI-GFP-up	PnrdJ-SmaI-GFP-low	Outer primers in P*nrdJ* promoter
8	PnrdA-mutAlgR1-up	PnrdA-mutAlgR1-low	P*nrdA* AlgR box 1 mutagenesis
9	PnrdJ-mutAlgR1-up	PnrdJ-mutAlgR1-low	P*nrdJ* AlgR box 1 mutagenesis
10	PnrdJ-mutAlgR4-up	PnrdJ-mutAlgR4-low	P*nrdJ* AlgR box 2 mutagenesis
11	PnrdJ-mutAlgR2-up	PnrdJ-mutAlgR2-low	P*nrdJ* AlgR box A1 mutagenesis
12	PnrdJ-mutAlgR3-up	PnrdJ-mutAlgR3-low	P*nrdJ* AlgR box A2 mutagenesis
13	PnrdA-BamHI-EcoRI-GFP-up	PnrdA-EXT-low-M13	EMSA P*nrdA* long band
14	PnrdA-AlgR-EMSA-up	PnrdA-AlgR-EMSA-low-M13	EMSA P*nrdA* short 1 band
15	PnrdA-EMSA-up	PnrdA-EMSA-low-M13	EMSA P*nrdA* short 2 band
16	PnrdJ-BamHI-GFP-up	PnrdJ-EXT-low-M13	EMSA P*nrdJ* long band
17	PnrdJ-AlgR-EMSA-up	PnrdJ-AlgR-EMSA-low-M13	EMSA P*nrdJ* short 1 band
18	PnrdJ-mid-up	PnrdJ-mid-low-M13	EMSA P*nrdJ* short 2 band
19	PnrdJ-EMSA-up	PnrdJ-EMSA-low-M13	EMSA P*nrdJ* short 3 band
20	PalgD-BamHI-GFP-up	PalgD-low-M13	EMSA P*algD* positive control band
21	Anr-EcoRI-up	Anr-low-M13	EMSA *anr* negative control band
22	PnrdA-BamHI-EcoRI-GFP-up	PnrdA-SmaI-GFP-low	AFM P*nrdA* probe
23	PnrdJ-AFM-up	PnrdJ-SmaI-GFP-low	AFM P*nrdJ* probe
24	PalgD-BamHI-AFM-up	PalgD-low-M13	AFM P*algD* probe
25	PdinB-AFM-up	PdinB-low-M13	AFM P*dinB* probe

Article 3

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Chronic lung infections by the ubiquitous and extremely adaptable opportunistic pathogen *Pseudomonas aeruginosa* correlate with the formation of a biofilm, where bacteria grow in association with an extracellular matrix and display a wide range of changes in gene expression and metabolism. This leads to increased resistance to physical stress and antibiotic therapies, while enhancing cell-to-cell communication. Oxygen diffusion through the complex biofilm structure generates an oxygen concentration gradient, leading to the appearance of anaerobic microenvironments. Ribonucleotide reductases (RNRs) are a family of highly sophisticated enzymes responsible for the synthesis of the deoxyribonucleotides, and they constitute the only *de novo* pathway for the formation of the building blocks needed for DNA synthesis and repair. *P. aeruginosa* is one of the few bacteria encoding all three known RNR classes (Ia, II, and III). Class Ia RNRs are oxygen dependent, class II are oxygen independent, and class III are oxygen sensitive. A tight control of RNR activity is essential for anaerobic growth and therefore for biofilm development. In this work we explored the role of the different RNR classes in biofilm formation under aerobic and anaerobic initial conditions and using static and continuous-flow biofilm models. We demonstrated the importance of class II and III RNR for proper cell division in biofilm development and maturation. We also determined that these classes are transcriptionally induced during biofilm formation and under anaerobic conditions. The molecular mechanism of their anaerobic regulation was also studied, finding that the Anr/Dnr system is responsible for class II RNR induction. These data can be integrated with previous knowledge about biofilms in a model where these structures are understood as a set of layers determined by oxygen concentration and contain cells with different RNR expression profiles, bringing us a step closer to the understanding of this complex growth pattern, essential for *P. aeruginosa* chronic infections.

Keywords: ribonucleotide reductases, DNA synthesis, *Pseudomonas aeruginosa*, biofilm formation, anaerobic metabolism, oxygen diffusion, *nrd* genes, vitamin B_{12}

INTRODUCTION

Pseudomonas aeruginosa is a common Gram-negative bacterium that is recognized for its ubiquity and its advanced antibiotic resistance mechanisms. It is also relevant for its great adaptability, being able to inhabit many different environments; it can live free in soil and water and can grow in human and plant host-associated environments. This bacterium is related to clinically relevant human infections in immunocompromised patients and other risk groups. In particular, it causes severe chronic lung infections in patients suffering from cystic fibrosis (CF) or chronic obstructive pulmonary disease (COPD; Lyczak et al., 2002; Davies et al., 2007; Ito and Barnes, 2009).

The establishment of chronic *P. aeruginosa* infections correlates with the formation of biofilm, a structure with clusters of cells encapsulated in a complex extracellular polymeric matrix. Bacteria in biofilms display different patterns of gene expression and phenotypes, reducing their metabolic rate and increasing cell-to-cell communication (Costerton et al., 1999) while becoming less sensitive to chemical and physical stresses, and they show increased chances of developing new antibiotic resistances (Xu et al., 1998; Stewart and Franklin, 2008). Oxygen does not diffuse freely through the biofilm structure, leading to the formation of an oxygen concentration gradient, which generates anaerobic microenvironments (Xu et al., 1998; Werner et al., 2004; Stewart and Franklin, 2008). The oxygen (and other chemical compounds) gradients are major driving forces for regulating the morphogenesis of the biofilm (Dietrich et al., 2013; Kempes et al., 2014; Okegbe et al., 2014).

While usually listed as an obligate aerobe, *P. aeruginosa* is able to grow in the absence of oxygen via anaerobic respiration using nitrates or other oxidized forms of nitrogen (NO_2, NO) as electron acceptors in a chain of reductions ending in molecular nitrogen (N_2; Schobert and Jahn, 2010; Arat et al., 2015). The Anr, Dnr, and NarL transcriptional factors are essential for regulating the expression of genes that encode the enzymes needed for denitrification, as well as regulating other genes related to anaerobic metabolism (Schreiber et al., 2007; Arai, 2011). Anr acts as a global oxygen-sensing regulator, controlling essential enzymes such as arginine deiminase and nitrate reductase and controlling *dnr* and *narL* gene expression. Dnr is a NO sensor and is able to modulate the expression of several genes under anaerobic conditions, including the enzymes thought to be involved in dissimilatory nitrogen reduction. NarL is a member of the NarLX two-component system, also thought to be involved in the regulation of nitrate reduction (Benkert et al., 2008). Bioinformatic studies have failed to identify differences between the Anr and Dnr binding sites (Trunk et al., 2010).

Anaerobic growth in *P. aeruginosa* biofilms is thought to be essential for full biofilm establishment (Stewart and Franklin, 2008) and has proven to be clinically relevant. In chronic CF lung infections, it has been shown that *P. aeruginosa* grows in low-oxygen environments within mucus plugs or biofilms (Schobert and Jahn, 2010). Furthermore, it has been shown that microaerophilic and anaerobic conditions are predominant in the sputum of patients with CF (Yoon et al., 2002; Alvarez-Ortega and Harwood, 2007; Hassett et al., 2009).

As another manifestation of its metabolic versatility, *P. aeruginosa* is one of the few microorganisms that encodes the three different ribonucleotide reductase classes in its genome. ribonucleotide reductases (RNRs) are key enzymes that catalyze the reduction of all four ribonucleotides to their corresponding deoxyribonucleotides, providing the necessary precursor molecules for DNA synthesis and repair in all organisms (Cotruvo and Stubbe, 2011; Sjoberg and Torrents, 2011; Hofer et al., 2012; Torrents, 2014; Lundin et al., 2015). RNRs are divided into three classes (I, II, and III) based on their structural differences, metallocofactor requirements, and the mechanisms used for radical generation. Class I RNRs require oxygen to produce a tyrosyl radical using a diferric iron or a dimanganese iron center and, thereby, function only under aerobic conditions. Class II RNRs require adenosylcobalamin (AdoCob) for radical generation and do not depend on oxygen (Torrents et al., 2005; Sjoberg and Torrents, 2011). Class III RNR belongs to the family of glycyl radical enzymes. The radical is generated by an activating enzyme with a (4Fe-4S) cluster that catalyzes the reduction of *S*-adenosylmethionine (SAM). This class can only function under anaerobic conditions. Genes for active representatives of all three classes are present in *P. aeruginosa* metabolism: class I, subclass Ia (*nrdAB*), class II (*nrdJab*), and class III (*nrdDG*). Exceptionally the *P. aeruginosa* class II RNR is splitted and expressed in two different polypeptides (denoted as *nrdJa* and *nrdJb*; Torrents et al., 2005; Crona et al., 2015). The presence and coordinated activity of the three classes is essential to ensure a supply of precursor molecules for DNA synthesis under both aerobic and anaerobic conditions (Sjoberg and Torrents, 2011). However, specifically in *P. aeruginosa* the synthesis of vitamin B_{12} only occurs in aerobic conditions (Lee et al., 2012) and its availability determines the class II RNR activity. Unfortunately, the exact role of each class and how they are genetically regulated is not yet fully understood.

In this work we aimed to study the importance of the different *P. aeruginosa* RNR classes for biofilm formation. We assessed the effect of class II and class III RNR deletion on static and continuous-flow biofilm formation and examined the phenotypic effects of this inactivation to establish the essential roles of RNRs in proper biofilm development. We also studied the genetic regulation responsible for modulating class II and class III RNR gene expression in biofilms, and we incorporated our data into a model where the *P. aeruginosa* biofilm is considered a set of layers determined by oxygen concentration gradients, vitamin-B_{12} and cells with different RNR expression profiles.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

All bacterial strains and plasmids are listed in Supplementary Table S1. *Escherichia coli* and *P. aeruginosa* cells were routinely grown in Luria-Bertani broth (LB) at 37°C. Anaerobic growth occurred in LB medium containing KNO_3 (10 g/l; LBN medium) or 1 mM *S*-nitrosoglutathione (GSNO) in screw-cap tubes (Hungate Tubes) that were purged with N_2 (Garriga et al., 1996;

Arai, 2003). For the anaerobic culture of *P. aeruginosa anr, dnr,* and *narL* isogenic mutant strains, which are not able to grow anaerobically, cells were first grown under aerobic conditions in LB medium to a mid-exponential phase ($OD_{550} = 0.5$) and then the cultures were pelleted, resuspended in the same volume of LBN medium, and inoculated into screw-cap tubes containing anaerobic LBN medium. Finally, they were incubated for 3 h to induce anaerobic metabolism.

When necessary, antibiotics were added at the following concentrations: for *E. coli*, 10 μg/ml gentamicin and 50 μg/ml ampicillin and for *P. aeruginosa*, 150 μg/ml gentamicin, 300 μg/ml carbenicillin and 50 μg/ml tetracycline. Vitamin B_{12} was added when necessary at a concentration of 1 μg/mL.

DNA Manipulations and Construction of Plasmids and Strains

Recombinant DNA techniques were performed using standard procedures (Sambrook et al., 1989). DNA fragments were amplified via PCR using High-Fidelity PCR Enzyme Mix (Fermentas, Thermo Scientific). All primers used in this study are listed in Supplementary Table S2. DNA fragments were digested by the corresponding restriction enzymes (Fermentas, Thermo Scientific) and ligated with T4 DNA ligase (Fermentas, Thermo Scientific) according to the manufacturer's instructions. Plasmid DNA was isolated using the GeneJET Plasmid Miniprep Kit (Fermentas, Thermo Scientific). DNA was transferred into *P. aeruginosa* cells either via electroporation using a Gene Pulser Xcell™ electroporator (Bio-Rad) or via conjugation, as previously described (Crespo et al., 2015).

pETS191 and pETS192 plasmids were generated by applying PCR-based site-directed mutagenesis at the putative Anr/Dnr binding boxes of the P*nrdJ* and P*nrdD* promoter regions ($TTGA^{T}/_{C}NNNN^{A}/_{G}TCAA$, from the PRODORIC database[1]) and then cloning the resultant mutant promoters into pETS130-GFP plasmids. Anr/Dnr box mutagenesis was performed according to previously published procedures (Urban et al., 1997) using the following primers: for the P*nrdJ* promoter region, mutanrJ-up/mutanrJ-low as the inner primers and PnrdJ BamHI new-up/PnrdJ SmaI new-low as the outer primers; for the P*nrdD* promoter region, mutanrD-up/mutanrD-low as the inner primers and PnrdD-up/PnrdD new-low as the outer primers. The mutant fragments obtained from this process were cloned into pGEM-T Easy vectors, and the Anr/Dnr box mutation was verified via DNA sequencing. Finally, the fragments were digested with the corresponding restriction enzymes (BamHI/SmaI for P*nrdJ* and BamHI/ClaI for P*nrdD*) and cloned into pETS130-GFP plasmids.

For pETS193 generation, the *oprF* promoter region was amplified from *P. aeruginosa* PAO1 genomic DNA using the following primer pair: PoprFBHI-up/PoprFClaI-low. The amplicon (460 bp) was cloned into pGEM-T Easy vectors, verified via DNA sequencing, digested with BamHI/ClaI and cloned into pETS130-GFP plasmids.

For pETS195 generation, an amplicon containing the *dnr* promoter region and the full ORF (1128 bp) was amplified from *P. aeruginosa* PAO1 genomic DNA using the following primer pair: Pdnr-BHI up/Dnr-low. The amplicon was cloned into pGEM-T Easy vectors, verified via DNA sequencing, digested with BamHI/SalI and cloned into pUCP20T plasmids.

A *P. aeruginosa* Δ*nrdJ*Δ*nrdD* double mutant strain (ETS125) was constructed from the *P. aeruginosa* PAO1 *nrdD*::ΩTc; Tc^{R} (ETS103) strain (Sjoberg and Torrents, 2011) through the insertion of the gentamicin-resistance gene (*aacC1*) into the *nrdJ* gene using homologous recombination with the pETX100-Tlink vector, as previously described (Quenee et al., 2005). Briefly, two 400 bp areas surrounding the *nrdJ* gene were amplified via PCR with the following primer pairs: Jmut1HIIIup/Jmut2BIlw and Jmut3BIup/Jmut4SIlw. The two amplicons obtained were then cloned separately into pGEM-T Easy vectors. A plasmid containing both fragments was generated by BamHI/SacI digestion. The gentamicin resistance gene *aacC1* was obtained using *BamHI* digestion of pUCGmlox, and the corresponding cassette was ligated inside the two previous fragments. Next, the construct was cloned into the pEX100Tlink vector. The obtained plasmid pET100Tlink-*nrdJ*::ΩGm was transferred into the S17.1λ*pir* strain and conjugated to the *P. aeruginosa* ETS103 strain. Transformants were selected by plating them with tetracycline and gentamicin; 5% sucrose was added for *sacB*-mediated counterselection of the plasmids. The insertion of *aacC1* was screened via PCR with the primer pair Jmut1HIIIup/Jint-2-3lw and later confirmed via DNA sequencing.

Quantitative Reverse Transcription PCR (qRT-PCR)

RNA from *P. aeruginosa* PAO1 cells (either planktonic or from a biofilm) was isolated with RNAprotect Bacterial Reagent (Qiagen), according the manufacturer's instructions. RNA purification steps were carried out using the RNeasy Mini Kit (Qiagen), according the manufacturer's instructions. DNase I (Turbo DNA-free, Applied Biosystems) was used to remove the remaining DNA, and RNA samples were subjected to PCR to verify the absence of DNA. For cDNA synthesis, RNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific), and 0.5 μg of RNA was reverse transcribed with SuperScript III Reverse Transcriptase (Applied Biosystems) using the following primers for each gene: nrdATaqM2-low for *nrdA*, nrdJTaqM2-low for *nrdJa*, nrdDTaqM2-low for *nrdD* and gapTaqM-low for *gapA*. qRT-PCR quantification used nrdA-FAM, nrdJ-FAM, nrdD-FAM, and gap-FAM qRT-PCR probes (Crespo et al., 2015).

Western Immunoblot Analysis

Western blotting was carried out as previously described (Sjoberg and Torrents, 2011), using anti-NrdJ (Agrisera, Sweden; and Thermo Fisher, USA) at a 1:1000 dilution. The detection of primary antibodies was performed using donkey anti-rabbit (Bio-Rad) horseradish peroxidase-conjugated secondary antibodies at a 1/50,000 dilution. The antibody-antigen complex was detected using the Amersham™ ECL™ Prime western blotting reagent

[1]http://www.prodoric.de/vfp/

(GE Healthcare), according the manufacturer's protocol. Proteins were visualized and analyzed using an ImageQuant™ LAS4000 mini system (GE Healthcare).

Static and Continuous-Flow Biofilm Formation

To determine the biomass of static biofilms grown under aerobic conditions, cells were grown on 96-well plates (Nunclon Delta Surface, Thermo Scientific) in LB containing 0.2% glucose for 3 days at 37°C. Fully anaerobic static biofilms were grown in the same plates using LBN medium containing 0.2% glucose, and they were incubated inside GENbag ANAER (Biomerieux) devices. After the incubation period, the culture supernatant was removed, and both kinds of biofilm plates were washed three times with 1x phosphate buffered saline (PBS) to eliminate any remaining planktonic cells. Cells attached to the wells were then fixed with methanol and stained with 1% crystal violet (Cendra Mdel et al., 2012). After staining, excess crystal violet was eliminated with water, and 33% acetic acid was used to dissolve the remaining dye. Biofilm mass was finally determined as a function of the concentration of this dye based on the absorbance at 570 nm (A_{570}).

Continuous-flow biofilms were cultured as previously described (Christensen et al., 1999; Baelo et al., 2015) with the following modifications. Biofilms were grown into three-channel flow cells made of Perspex [poly(methyl methacrylate), channel size 40 mm × 4 mm × 1 mm; DTU Systems Biology, Technical University of Denmark) covered with a n°1 24 mm × 50 mm glass coverslip (Deltalab, ref. D102450) which served as the biofilm substratum. Flow cells were supplied with LB broth supplemented with 0.2% glucose, pumped by a high precision multichannel peristaltic pump (Ismatec ISM 943, Idex). Flow cells were inoculated using a 1-ml syringe with a 26 G needle and kept static for 1 h. After this point, flow was initiated at a rate of 3 ml/channel/hour. After 5–6 days of growth, biofilms were analyzed through staining the formed biofilm with the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Life Science), according to the manufacturer's instructions, and the biofilms were visualized with a Leica TCS SP5 confocal scanning laser microscope (CSLM; Leica Microsystems, Wetzlar, Germany). The excitation wavelength was 488 nm and the emission wavelength was 500 nm. Images were obtained using a 20×/0.70 air objective. Simulated fluorescence projections and sections were generated using ImageJ software, and COMSTAT 2 software was used to quantify the biomass and thickness of the biofilms (Weiss Nielsen et al., 2011).

Green Fluorescent Protein Gene Reporter Assay

Promoters of the different RNR genes fused to GFP in pETS130-GFP plasmids were used to determine RNR gene expression [pETS134 (P*nrdA*::GFP), pETS180 (P*nrdJa*::GFP) and pETS136 (P*nrdD*::GFP)]. pETS191 (P*nrdJ*ΔAnr/Dnr-box::GFP) and pETS192 (P*nrdD*ΔAnr/Dnr-box::GFP) plasmids were used to evaluate the effect of an Anr/Dnr box mutation on *nrdJ* and *nrdD* expression, respectively. pETS193 (P*oprF*::GFP) plasmids were used as a control.

For liquid culture experiments, GFP fluorescence was measured in 96-well plates (Costar® 96-Well Black Polystyrene Plate, Corning) on an Infinite 200 Pro Fluorescence Microplate Reader (Tecan), as previously described (Crespo et al., 2015). Briefly, three independent 1-ml samples of cells harboring the corresponding gene reporter assay plasmids grown to the mid-logarithmic phase (OD_{550}) were collected and pelleted. Cells were fixed with 1 ml of a freshly prepared 1x PBS solution containing 2% formaldehyde and stored in the dark at 4°C. Three measurements were performed for each independent sample.

To determine gene expression during biofilm formation, experiments were performed on static biofilms formed in 96-well plates (Nunclon Delta Surface, Thermo Scientific) after the incubation of a liquid culture of the corresponding strain in LB containing 0.2% glucose at 37°C. After incubating the plate for a specific time (from 3 to 72 h), the culture supernatant was removed, and each well was washed three times with PBS to eliminate and remaining planktonic cells. The biofilm cells attached to the wells were then fixed with PBS containing 2% formaldehyde. Finally, fluorescence measurements were performed on an Infinite 200 Pro Fluorescence Microplate Reader (Tecan).

RESULTS

Anaerobic RNR Classes Play an Important Role in *P. aeruginosa* Biofilm Formation

The *P. aeruginosa* genome encodes genes for three different ribonucleotide reductase (RNR) genes, two of them (class II and III) able to function enzymatically under anaerobic conditions, as previously described (Sjoberg and Torrents, 2011). The individual Δ*nrdJ* and Δ*nrdD* mutant strains showed a strong reduction in their anaerobic growth capacity (Supplementary Table S3). The Δ*nrdD* strain was able to grow under anaerobic conditions when supplemented with adenosylcobalamin or vitamin B_{12} to enhance class II RNR activity, agreeing with our previous report (Torrents et al., 2005; Sjoberg and Torrents, 2011). In this work, we generated a double class II (Δ*nrdJ*) and class III (Δ*nrdD*) RNR mutant (ETS125) that was unable to grow anaerobically (only growing to $OD_{550} = 0.05$ after a standard overnight anaerobic culture) and only was capable to grow under aerobic conditions ($OD_{550} = 3.8$). These growth patterns indicate the simultaneous need for class II and III RNRs in *P. aeruginosa* for anaerobic metabolism (Supplementary Table S3).

As anaerobic growth is needed to support full biofilm establishment (Stewart and Franklin, 2008), we explored the role of the different RNR classes in *P. aeruginosa* biofilm formation. The *P. aeruginosa* PAO1 wild-type strain, single Δ*nrdJ* (class II RNR) and Δ*nrdD* (class III RNR) isogenic mutant strains and double Δ*nrdJ*Δ*nrdD* mutant strain were assayed for their ability to form static biofilms under aerobic and anaerobic conditions (**Figure 1A**). The class I RNR mutation (Δ*nrdA*)

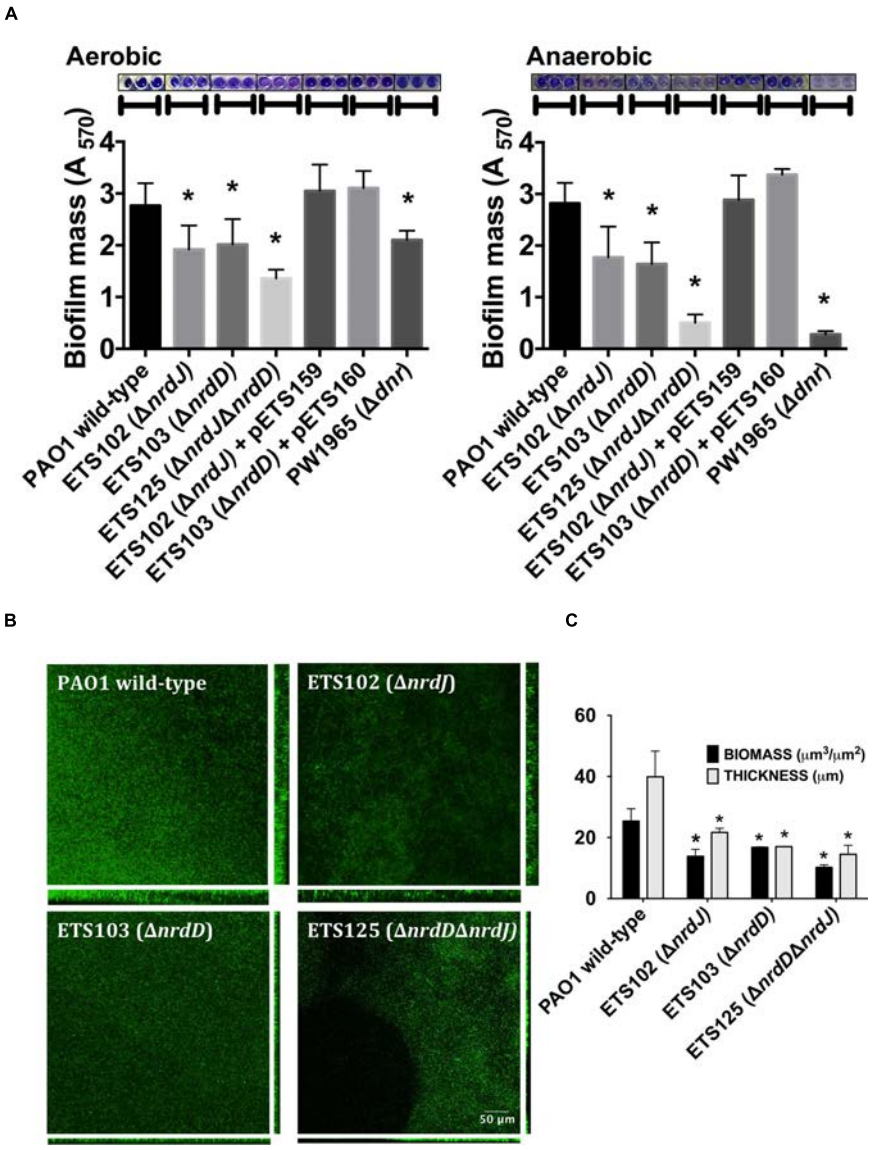

FIGURE 1 | Biofilm formation in *Pseudomonas aeruginosa* PAO1 wild-type and Ribonucleotide reductase (RNR) mutant strains. (A) Initially aerobic and fully anaerobic static biofilm biomass quantification after growing at 37°C for 4 days. Each value is accompanied by the corresponding crystal violet-stained biofilm image. More than 20 replicates were performed in three independent experiments. The *nrdJab* and *nrdDG* genes cloned into pETS159 and pETS160 plasmids were used to complement *nrdJ* and *nrdD* deficiencies in ETS102 and ETS103 strains. The Δ*dnr* strain was included to compare RNR mutant strains with a strain defective in anaerobic metabolism. **(B)** Confocal laser scanning microscopy (CLSM) images of continuous-flow biofilms (sum of stack images) and their corresponding orthogonal views for *P. aeruginosa* PAO1 wild-type, ETS102 Δ*nrdJ*, ETS103 Δ*nrdD* and ETS125 Δ*nrdD*Δ*nrdJ* strains. **(C)** Quantification of total biomass ($\mu m^3/\mu m^2$) and average thickness (μm) for the biofilms from the previous continuous-flow experiments. Data are the average of three independent experiments. *Significantly different from the wild-type strain in an unpaired *t*-test ($P < 0.05$).

strain is not viable and was not used in the current study (Sjoberg and Torrents, 2011). The results show that deficiencies in class II RNR activity (in ETS102 Δ*nrdJ* strain) and in class III RNR activity (in ETS103 Δ*nrdD* strain) resulted in decreased static biofilm formation under both aerobic and anaerobic initial conditions. Complementation of the mutation with a copy of the corresponding wild-type RNR gene (*nrdJ* or *nrdD* cloned into plasmids pEST159 and pETS160, respectively)

returned biofilm formation to a level similar to that of the wild-type strain. The double Δ*nrdJ*Δ*nrdD* RNR mutant (ETS125) showed almost no biofilm formation, and the decrease was even stronger in anaerobic biofilm formation experiments, demonstrating the key role of anaerobic RNR activity in *P. aeruginosa* biofilm formation. However, our results suggest that anaerobic RNR activity is needed for biofilm formation even when the experiment is performed under aerobic conditions. A *P. aeruginosa* Δ*dnr* mutant strain (PW1965), unable to grow anaerobically, was used to compare the results from the RNR mutant with those from a strain unable to perform general anaerobic metabolism. Dnr is a transcriptional factor that regulates the expression of essential genes during *P. aeruginosa* anaerobic growth (Trunk et al., 2010). As expected, the PW1965 strain showed strong differences in biofilm formation when compared with the wild-type strain, even when the initial culture conditions were aerobic, and its ability to form biofilms resembled the ability shown by the ETS125 double RNR mutant strain.

To further corroborate our previous static biofilm formation experiments, we explored the importance of the different RNR classes in continuous-flow biofilm formation performed in flow cells. This technique better mimics the biofilms found in nature and specifically in the mucus plaques within the lungs of CF patients (Weiss Nielsen et al., 2011; Lebeaux et al., 2013). Biofilm cultures of different strains were cultivated under a continuous flow of LB medium over 6 days to obtain a robust and mature biofilm. The formed biofilms were then stained and visualized using confocal laser scanning microscopy (CLSM), as described in the section "Materials and Methods."

Figure 1B shows the images obtained for the biofilms formed by the different strains that were evaluated and their corresponding orthogonal views. The thickness and total biomass values for each biofilm, estimated by COMSTAT software, are presented in **Figure 1C**. The biomass ($\mu m^3/\mu m^2$) and average thickness (μm) of the biofilms formed by all RNR mutant strains were decreased; biomass of the wild-type strain biofilm was 2.2, 1.8, and 2.7 times higher than the corresponding biomass of the anaerobic RNR mutant strains (ETS102 Δ*nrdJ*, ETS103 Δ*nrdD* and ETS125 Δ*nrdD*Δ*nrdJ*, respectively). The greatest thickness observed was in the *P. aeruginosa* wild-type strain biofilm (49.40 μm), while the different class II and III RNR mutant strains formed significantly thinner biofilms, with an average thickness of 24.84, 15.5, and 14.53 μm for ETS102 Δ*nrdJ*, ETS103 Δ*nrdD,* and ETS125 Δ*nrdD*Δ*nrdJ*, respectively. It is important to note that the *P. aeruginosa* double RNR class mutant (ETS125 Δ*nrdD*Δ*nrdJ*) grew in a discontinuous pattern and showed difficulties in attaching to the glass surface. These results confirm our previous observations in static biofilms, highlighting the importance of anaerobic RNRs in biofilm formation even when culture conditions are initially aerobic.

RNR Enzymes Contribute to Proper Cell Division in a Biofilm

Figure 2 shows the CLSM analysis of the longitudinal cell morphology in a structured biofilm formed by the different

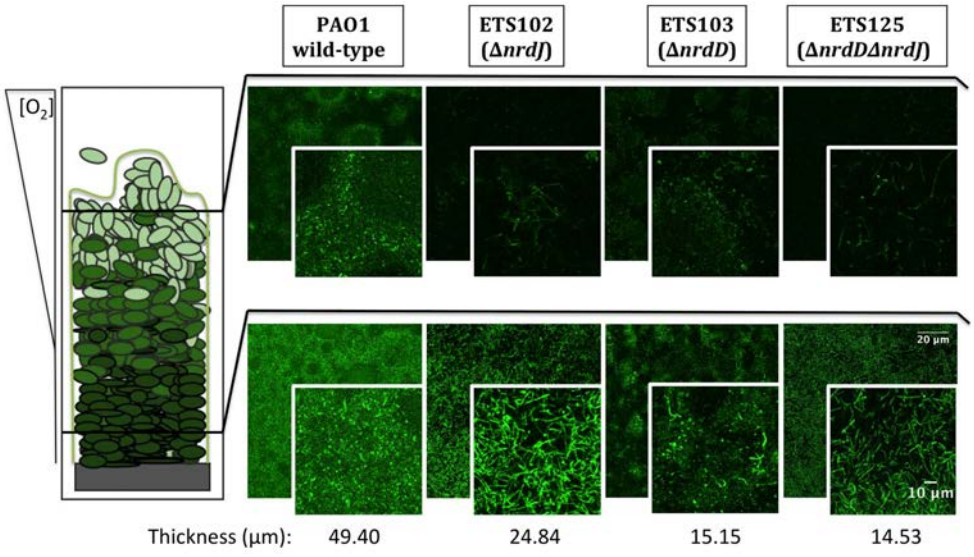

FIGURE 2 | Detailed microscopy observations of structured biofilms from *P. aeruginosa* PAO1 wild-type, ETS102, ETS103, and ETS125 strains. On the left side, a scheme of the longitudinal structure of *P. aeruginosa* biofilm is represented, labeled with indications of the oxygen concentration along the biofilm (Stewart and Franklin, 2008). On the right side, CLSM images are shown, which were taken from the aerobic region of the biofilm (top part, superficial biofilm) and from the anaerobic region (bottom part, deeper in the biofilm structure). An internal panel with a magnification of a local area is shown for each image. The corresponding average thickness of each strain is representative of three independent experiments.

P. aeruginosa strains (PAO1 wild-type, ETS102 Δ*nrdJ*, ETS103 Δ*nrdD* and ETS125 Δ*nrdD*Δ*nrdJ*). As described previously, the different RNR mutant strains showed elongated morphologies during anaerobiosis (Lee et al., 2012). The *P. aeruginosa* wild-type cells showed a normal rod-shape cell morphology throughout the biofilm in both the aerobic and anaerobic regions (top and bottom segments of the biofilm, respectively). However, the *P. aeruginosa* ETS102 Δ*nrdJ* mutant strain showed significant cell elongation in both the top and the bottom parts of the biofilm structure, indicating some disturbances in cell growth and division, as was clearly demonstrated in previous planktonic anaerobic cultures (Yoon et al., 2011; Lee et al., 2012). Some *P. aeruginosa* ETS103 Δ*nrdD* cells also showed cell elongation but only in the bottom layer of the biofilm (anaerobic region), while rod-shaped cells were found in the upper region that were similar to the shapes of the wild-type strain. Finally, the *P. aeruginosa* double mutant (ETS125 Δ*nrdJ*Δ*nrdD*) exhibited cell elongated along the entire span of the biofilm, similar to the results seen in the II RNR mutant (ETS102 Δ*nrdJ*).

During Biofilm Formation, Expression of the *nrdJ* and *nrdD* Genes Is Increased

Our previous results demonstrate the importance of class II and class III RNRs for anaerobic growth and biofilm formation in *P. aeruginosa* and show that these two processes are related as biofilm growth is characterized by a decrease oxygen tension that results in anaerobic conditions in the bottom regions of the structure (Werner et al., 2004; Stewart and Franklin, 2008). We hypothesized that the expression of class II and class III RNRs could be induced under these growing conditions. To explore this, we studied the induction of the different RNR genes using RT-PCR.

First, we explored the induction of RNR genes by comparing anaerobic growth with aerobic growth in liquid cultures at the stationary phase (**Table 1**). The results showed a strong increase in *nrdJa* and *nrdD* expression (85.2 and 110.6), while *nrdA* expression (2.1 times) was only slightly increased under anaerobic conditions.

TABLE 1 | Relative expression of ribonucleotide reductase (RNR) genes based on real-time PCR.

	Differential expression (fold-change)		
	nrdA	***nrdJa***	***nrdD***
Planktonic anaerobic vs. Planktonic aerobic	2.1 ± 0.4	85.2 ± 5.0	110.6 ± 19.2
Biofilm aerobic vs. Planktonic aerobic	13.1 ± 6.2	1500 ± 150	128.2 ± 5.1
Biofilm aerobic vs. Planktonic anaerobic	2.4 ± 1.0	51.6 ± 7.3	−12.3 ± 1.6

Fold change in Pseudomonas aeruginosa PAO1 nrdA, nrdJa, and nrdD transcription determined using real time PCR from 16-h-old planktonic cells grown aerobically or anaerobically and 4-day-old cells growing in biofilms. "Biofilm aerobic" refers to biofilms grown under initially aerobic conditions. The gap gene was used as an internal standard. The results shown represent the average of three independent experiments ± standard deviation.

We also explored the effect of biofilm growth itself on RNR expression. To do this, we analyzed the RNA expression of each RNR class in aerobic planktonic cells (at the stationary phase) relative to the RNR expression in cells growing in aerobically made biofilms (a 4-day-old biofilm; **Table 1**) using RT-PCR. The results obtained in the *P. aeruginosa* wild-type strain showed significant differences in RNR expression between the two conditions: expression levels of *nrdA* showed a slight increase, but the expression of *nrdJa* and *nrdD* were both highly induced in the cells forming a robust biofilm relative to expression in the planktonic culture.

The induction of *nrdJa* and *nrdD* gene expression shown in biofilm formation and under anaerobic conditions could be due to control by factors related to anaerobic metabolism (i.e., factors acting in anaerobic cultures and in the anaerobic areas of biofilms) or/and due to specific biofilm-related factors. As a first approach to exploring this control, we examined the patterns of our previous RT-PCR results (**Table 1**). When comparing the results in the initially aerobic biofilm conditions with those of the anaerobic planktonic conditions, it is clear that *nrdJa* expression was highly increased during biofilm formation (1500 fold-change vs. 85), while *nrdD* expression was increased to a higher rate by factors related to anaerobic metabolism (almost same fold-change levels 110 vs. 128).

Class II RNRs Are Transcriptionally Activated by a *dnr* Transcription Factor

To this point, we have demonstrated that class II and class III RNRs are of great importance for biofilm formation and that biofilm growth and an anaerobic environment strongly induce their expression. Therefore, the key transcriptional factors involved in *P. aeruginosa* anaerobic metabolism (Anr, Dnr, and NarL) were studied as putative transcriptional regulators for inducing RNR anaerobic expression (Arai, 2011; Arat et al., 2015).

The different transcription factors (Anr, Dnr, and NarL) are responsible for the regulation of different parts of the reduction chain in anaerobic respiration in *P. aeruginosa* [from NO_3 to N_2, through NO_2 and NO (Trunk et al., 2010)]. Therefore, in the anaerobic transcriptional regulation study, *P. aeruginosa* was grown using KNO_3 and GSNO (a NO donor) as final electron acceptors to obtain more information about which transcriptional regulator might be involved in the RNR transcriptional regulation under anaerobic conditions.

Transcriptional fusions of the *nrdJ* and *nrdD* promoter regions to GFP (present in the pETS180 and pETS136 plasmids, respectively) were transformed in different *P. aeruginosa* strains (PAO1, PW3784 Δ*anr*, PW1965 Δ*dnr* and PW7549 Δ*narL*) and used for gene reporter assays (see Materials and Methods). As seen in **Figure 3A**, the results show an increased expression of both *nrdJ* and *nrdD* under anaerobic growth in the presence of both NO_3^- (321 and 188, respectively) and GSNO (351 and 163, respectively) compared to the expression during aerobic growth (146 and 101, respectively).

Comparing *nrdJ* expression between the *P. aeruginosa anr*, *dnr*, and *narL* knockout mutant strains and the wild-type strain

A

	Strains	Relative fluorescence units (kRfu)		
		O_2	NO_3	GSNO
pETS180 (P*nrdJ*)	PAO1 wild-type	146 ± 8	321 ± 34	351 ± 13
	PW3784 (Δ*anr*)	132 ± 18	181 ± 18*	248 ± 16*
	PW1965 (Δ*dnr*)	136 ± 10	204 ± 34*	242 ± 25*
	PW7549 (Δ*narL*)	123 ± 24	315 ± 27	344 ± 21
pETS136 (P*nrdD*)	PAO1 wild-type	101 ± 3	188 ± 22	163 ± 6
	PW3784 (Δ*anr*)	106 ± 18	217 ± 26	101 ± 9
	PW1965 (Δ*dnr*)	96 ± 18	221 ± 17	102 ± 11
	PW7549 (Δ*narL*)	100 ± 7	188 ± 12	244 ± 12

B

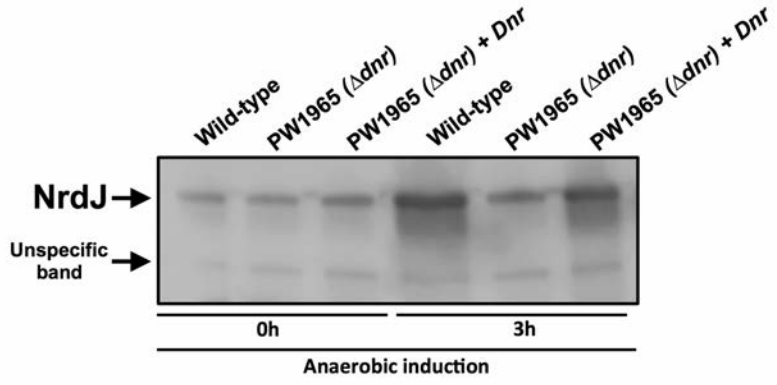

FIGURE 3 | Transcriptional regulation of *nrdJ* and *nrdD* during anaerobic growth. (A) Transcriptional expression of *nrdJ* (pETS180) and *nrdD* (pETS136) promoters in *P. aeruginosa* wild-type, PW3785 Δanr, PW1965 Δdnr and PW7549 ΔnarL strains. Cells were grown aerobically to a mid-logarithmic phase (A550 0.5) and then were grown anaerobically (with NO3 or GSNO as electron acceptors) for 3 h to induce anaerobic metabolism. Values are the means SD from more than three independent experiments. *Significantly different from the *P. aeruginosa* wild-type strain in an unpaired *t*-test (*p*-value 0.05). kRfu 1000 relative fluorescence units. **(B)** NrdJ protein expression analysis in PAO1 wild-type, PW1965 Δ*dnr* and PW1965 Δ*dnr* + pETS195 (*dnr* complementation plasmid) via western blot analysis performed at a mid-logarithmic phase after 0 h or 3 h of anaerobic induction. A representative blot of three independent western blot analyses is shown. An unknown, unspecific band that is present at an almost constant intensity in all samples is shown in the blot and served as a loading control.

(**Figure 3A**), we identified a reduced anaerobic induction of *nrdJ* expression in the Δ*anr* (PW3784) and Δ*dnr* (PW1965) mutant strains compared with the values of the wild-type strain. No effect was observed on *nrdJ* transcription when the *narL* gene was mutated. Our results show the dependence of the anaerobic induction of *nrdJ* gene expression on Anr and Dnr transcriptional regulators. As the effect is shown when any of these two genes are mutated and Dnr is controlled by Anr (which acts early in the regulatory chain of anaerobic metabolism), Dnr was considered the most likely candidate for being responsible for regulating *nrdJ*.

This control of *nrdJ* expression by Dnr was later verified at the protein level using a western blotting assay (**Figure 3B**). Although no differences were found in the amount of NrdJ protein between *P. aeruginosa* wild-type and PW1965 Δ*dnr* mutant strains when measured during aerobic growth, 3 h of anaerobic metabolism induced a strong reduction in NrdJ levels in the Δ*dnr* strain relative to expression in the wild-type cells. This effect was reverted back to near wild-type levels by Dnr complementation using the pETS195 complementation plasmid.

We failed to identify any regulation on *nrdD* expression by anaerobiosis-related Anr, Dnr, or NarL factors, as demonstrated in our results (**Figure 3A**).

To determine if the anaerobiosis-related transcriptional factors bind specifically on the RNR promoters, a bioinformatic search of putative Anr-Dnr binding sites was performed on the P*nrdJ* and P*nrdD* promoter regions. One Anr/Dnr-box was identified on both P*nrdJ* and P*nrdD* promoters (see Materials and Methods) according to the $TTGA^{T}/_{C}NNNN^{A}/_{G}TCAA$ consensus present in PRODORIC database. The putative Anr/Dnr boxes identified are shown in **Figure 4**.

To confirm the binding of Anr/Dnr to the promoters, we specifically mutated the essential nucleotides of the putative Anr/Dnr-boxes identified, fused the mutant promoters to GFP

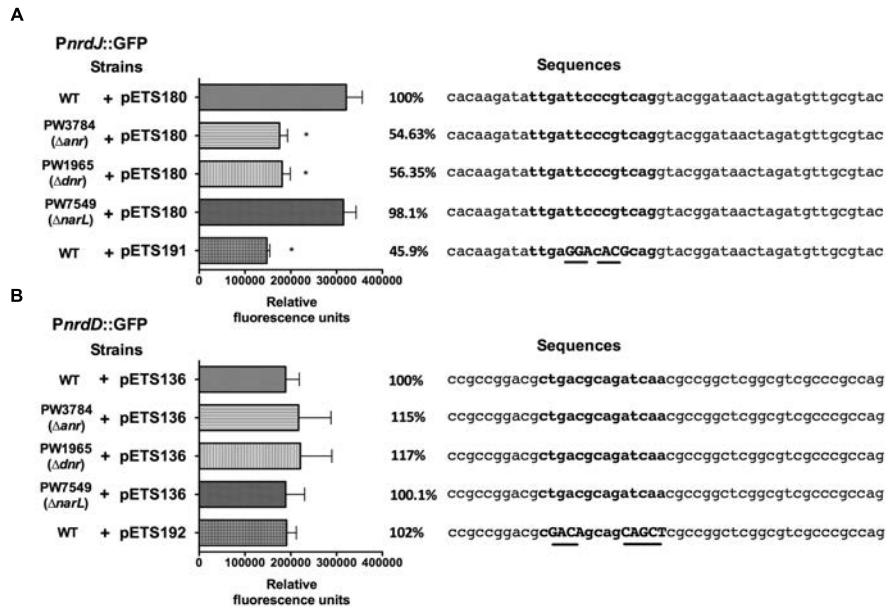

FIGURE 4 | *nrdJ* and *nrdD* expression in *P. aeruginosa* wild-type, PW3784 Δ*anr*, PW1965 Δ*dnr* and PW7549 Δ*narL* strains. GFP fluorescence of **(A)** P*nrdJ* and P*nrdJ* ΔAnr/Dnr-box and **(B)** of P*nrdD* and P*nrdD* ΔAnr/Dnr-box measured in the wild-type, PW3784 Δ*anr*, PW1965 Δ*dnr* and PW7549 Δ*narL* strains at a mid-logarithmic phase after 3 h of anaerobic induction. A fragment of the sequence of the corresponding promoter regions surrounding the putative Anr/Dnr-box is added at the right, and the sequence of the box is indicated in bold letters. In the mutant Anr/Dnr-boxes, mutated nucleotides are indicated in capital letters and underlined. The Anr/Dnr box is centered at −84 and −98 bp from the translation start site of the *nrdJ* and *nrdD* genes, respectively. *Significantly different from the wild-type strain in an unpaired *t*-test ($P < 0.05$). Error bars represent the standard deviation for three independent experiments.

and constructed plasmids for gene reporter assays (pETS191 for the *nrdJ* promoter and pETS192 for the *nrdD* promoter). In the corresponding assay, P*nrdJ* expression decreased when the Anr/Dnr-box was mutated. Moreover, the ΔAnr/Dnr-box P*nrdJ* (pETS191) expression was similar to that found in Δ*anr* and Δ*dnr* mutant strains (**Figure 4A**). However, no significant results were obtained when mutating the P*nrdD* Anr/Dnr-box (**Figure 4B**).

The Presence of Anaerobic Environments in the Biofilm Increases *nrdJ* Expression Through *dnr* Activation

Our previous results demonstrate that class II and class III RNRs are of great importance for biofilm formation and anaerobic growth in *P. aeruginosa* and that their expression is specifically induced under these conditions. As the induction of anaerobic metabolism increases as biofilm growth advances, we expected to detect a progressive induction of the expression of both RNRs during biofilm establishment and maturation.

To determine this, a GFP-based gene reporter assay was performed on a static biofilm culture over time. The expression of wild-type P*nrdA*, *PnrdJ,* and P*nrdD* was determined together with that of the mutant versions of P*nrdJ* and P*nrdD* (carrying mutant Anr/Dnr-boxes); a promoterless GFP plasmid (pETS130) was used as a negative control, and the *oprF* promoter (P*oprF*) was used as a positive control for anaerobic induction. OprF is a membrane protein that has its highest expression under anaerobic conditions, and it can be used as a marker of infection in a CF patient's lung or sputum (Yoon et al., 2002; Eichner et al., 2014).

As expected (**Figure 5A**), P*oprF* expression increased greatly during mature biofilm development, demonstrating the progressive establishment of anaerobic conditions in the deep layers of the biofilm structure. Simultaneously, the P*nrdJ* and P*nrdD* promoter expression increased, although P*nrdD* expression increased only in the later stages when a mature and robust biofilm was formed. Mutating the Anr/Dnr-box severely reduced P*nrdJ* expression, while the anaerobic induction of P*nrdD* remained unaffected.

As a *P. aeruginosa* Dnr deficient strain is still able to grow as a biofilm, we further explored how Dnr controls RNR expression during biofilm formation by comparing *nrdA*, *nrdJ* and *nrdD* transcription in planktonic cells with that in biofilm-forming cells using the PW1965 Δ*dnr* mutant strain. Comparing the results obtained (**Figure 5B**) with those from a *P. aeruginosa* wild-type biofilm vs. planktonic comparison (**Table 1**), we can see that when mutating the Dnr gene, the induction of *nrdJ* expression in biofilms becomes severely reduced but not completely abolished. Surprisingly, we also noticed that the

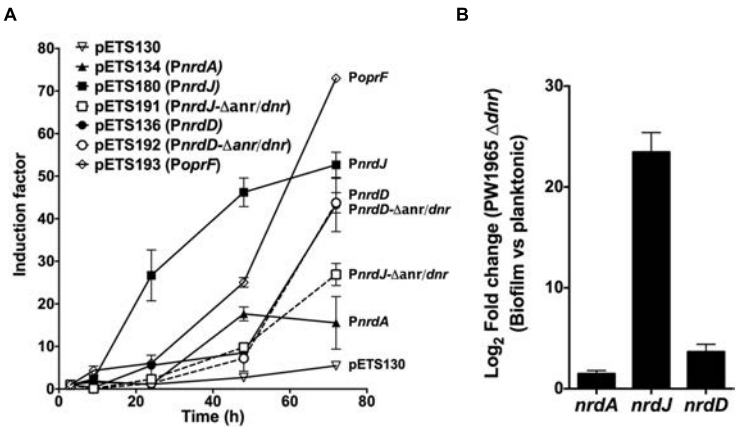

FIGURE 5 | Regulation of RNR expression during biofilm formation. (A) GFP-based gene reporter assay of *P. aeruginosa* cells growing as a static biofilm. The induction factor is expressed as the quotient of the fluorescence units measured from one strain at one point in time relative to the corresponding value measured at the first time point (3 h of culture). Each strain was monitored between 3 and 72 h of biofilm growth. Wild-type RNR promoters (P*nrdA*, P*nrdJ*, and P*nrdD*) are represented in continuous lines, and mutant RNR promoters (P*nrdJ* and P*nrdD* carrying a mutant version of the putative Anr/Dnr-box identified) are plotted in dotted lines. A promoterless GFP in pETS130 plasmids was used as a negative control, and the P*oprF* promoter in pETS193 plasmids was used as a positive control for anaerobic induction of gene expression. **(B)** Fold change in *P. aeruginosa* PW1965 Δ*dnr* P*nrdA*, P*nrdJ,* and P*nrdD* promoter transcription was determined through real time PCR in 4-day-old cells grown as a static biofilm and compared with transcription in 16-h-old planktonic cells, both of which were cultured under aerobic conditions. The *gap* gene was used as an internal standard. The results shown represent the mean of three independent experiments ± standard deviation.

induction of *nrdD* expression was also strongly reduced in the PW1965 Δ*dnr* strain, which is in contrast with what was observed in the gene reporter assays (**Figures 3A** and **4B**).

DISCUSSION

Pseudomonas aeruginosa is well known for its genetic diversity. It has a relatively large genome (6.3 Mb) for a bacterium, and contains a large number of genes involved in different metabolic activities, which might contribute to the environmental adaptability of this bacterium. Its ability to grow in the absence of oxygen using nitrates or other forms of oxidized nitrogen as electron acceptors is an important example of *P. aeruginosa's* anaerobic growth capacity (Trunk et al., 2010; Arat et al., 2015), which opens up a wide range of environments in which *P. aeruginosa* can grow.

Such anaerobic environments are present in a mature biofilm, in which different nutrient gradients and differential physical properties appear. Previous reports have highlighted the oxygen concentration heterogeneity in biofilms using microelectrodes, and have described the oxygen diffusion profiles in continuous biofilms (Werner et al., 2004). The oxygen concentration throughout the biofilm is thus a crucial parameter for bacterial growth in a mature biofilm (Stewart and Franklin, 2008) and strongly defines its morphogenesis and final structure (Dietrich et al., 2013; Kempes et al., 2014; Okegbe et al., 2014). Metabolites and oxygen easily diffuse in the outer layers of the biofilm; however, the free oxygen concentration becomes reduced in lower layers, resulting in strict anaerobic conditions in the depths of the mature biofilm. The three ribonucleotide reductase classes encoded by *P. aeruginosa* (class Ia, encoded in *nrdA* and *nrdB*; class II, encoded in *nrdJa* and *nrdJb*; and class III, encoded in *nrdD* and *nrdG*) are likely to increase the capacity of this bacterium to grow in the different environments generated throughout biofilms (Torrents et al., 2005; Sjoberg and Torrents, 2011).

Class Ia activity is strictly oxygen dependent, while class III is oxygen sensitive and can only function under strict anaerobic conditions. Class II is oxygen independent but needs vitamin B_{12} (*S*-adenosylcobalamin) for the completion of its catalytic cycle (Torrents et al., 2005). In accordance of these different levels of oxygen dependence, we hypothesized that all three RNR classes would have a predominant role in the progressively deeper layers of the biofilm structure, with class II and class III RNRs essential for anaerobic growth and therefore for the establishment of fully mature biofilms.

The most basic study was performed to analyze the differential ability of Δ*nrdJ* and Δ*nrdD* mutant strains and a Δ*nrdJ*Δ*nrdD* double mutant strain to grow in aerobic and anaerobic liquid cultures. The large reduction in anaerobic growth found after altering class II or class III RNRs highlights the importance of both RNR classes for anaerobic growth (Supplementary Table S3). In addition, the ability of class II RNRs alone to sustain anaerobic growth when the culture was supplemented with exogenous *S*-adenosylcobalamin suggests that class II RNRs can theoretically synthesize enough dNTPs to maintain normal growth rates, with *S*-adenosylcobalamin levels under anaerobic conditions being the limiting step.

The next step was to study how these same effects act on the natural formation of the anaerobic environments that appear during biofilm formation. Static biofilm formation was severely diminished when class II or class III RNRs were mutated (**Figure 1A**). This effect was higher when biofilms were built directly under anaerobic conditions but was also present under aerobic conditions. We associated this effect to the formation of anaerobic microenvironments in the biofilm depths that will undoubtedly occur if biofilms grow thick enough, and this was demonstrated by the analogous effect observed when mutating the *dnr* gene, which is one of the main transcriptional regulators of anaerobic metabolism (Schreiber et al., 2007). In this case, the impaired anaerobic metabolism implies that biofilm biomass will be reduced even when conditions are initially aerobic.

As static biofilm formation in microplates can be considered an artificial lab condition, we also studied the effect of class II and class III RNR alterations on continuous-flow biofilm formation, a technique that is thought to better the mimic biofilms present in nature and in clinically relevant cases, such as lung infections in CF patients (Weiss Nielsen et al., 2011; Lebeaux et al., 2013). Agreeing with our previous results, both biofilm biomass and thickness were considerably reduced when mutating the class II and/or class III RNRs (**Figures 1B,C**). The structure of the so-formed biofilm also changed compared with that of the wild-type biofilm. It is particularly important that in the Δ*nrdJ* Δ*nrdD* double mutant strain, a growth pattern of discontinuous patches appeared, showing the dependence on aeration of this strain.

All these data can be incorporated into a model in which the biofilm is considered a set of layers where the free oxygen concentration is progressively reduced with depth (**Figure 6**). Interestingly, vitamin B_{12} can only be synthesized under aerobic conditions (Lee et al., 2012); to our knowledge the diffusion properties of vitamin B_{12} in the biofilm have never been formally determined, but we can expect it to be gradually diffused throughout the biofilm layers and actively consumed when crossing them, therefore the deeper layers would not only be anaerobic but also limited in adenosylcobalamin. Therefore, at the top of the biofilm, class I RNRs would be the main enzyme responsible for dNTP synthesis, while class II RNRs would gain more importance in the middle layers (characterized by reduced oxygen levels but within the range of vitamin B_{12} diffusion) and class III RNRs would support growth in the lower layers as it does not depend on oxygen or metabolite diffusion from the outer regions.

Additionally, we studied the cell morphology in the different layers of the biofilm. A cell elongation phenotype is associated with impaired cell division, which can be triggered by depletion of the dNTP pool when RNR metabolism is affected. According to our model, the Δ*nrdJ* mutant strain and the Δ*nrdJ*Δ*nrdD* double mutant strain showed elongated cells throughout almost the entire biofilm depth, while the Δ*nrdD* strain only presented elongation in the lower layers (**Figure 2**). These results must be interpreted by also taking into account the fact that reductions in biofilm biomass and thickness were also happening, so the thin layer in which class the III RNR mutation seems to affect cell morphology means only that strictly anaerobic areas were unable to form.

Given the importance of class II and class III RNRs for anaerobic growth and biofilm formation, we expected an up-regulation of these enzymes under these conditions. It

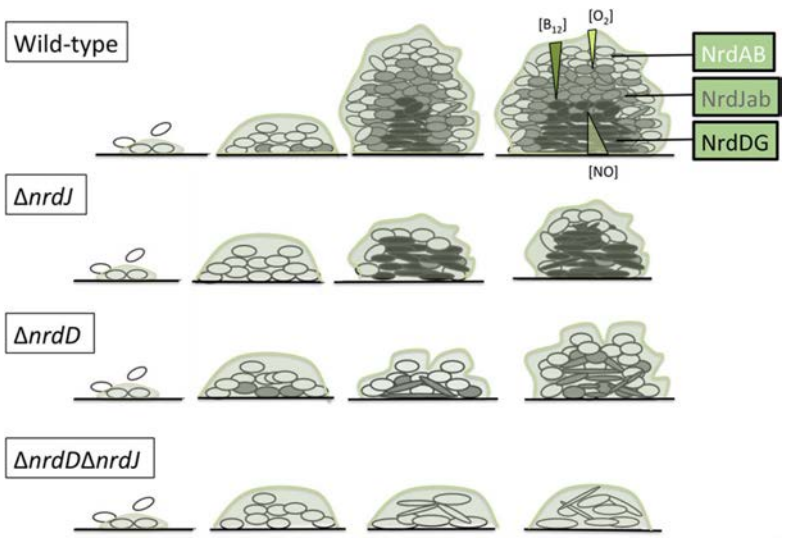

FIGURE 6 | Model of ribonucleotide reductases expression during *P. aeruginosa* biofilm formation. Oxygen concentration is progressively reduced throughout the biofilm structure in a well-established gradient (Xu et al., 1998; Stewart and Franklin, 2008). Vitamin B_{12} and NO gradient concentrations also presented here are hypothetical (see the Discussion section).

is known that as much as half of the *P. aeruginosa* genome is differentially expressed during biofilm development, including many genes involved in anaerobic metabolism, which are up-regulated in mature biofilms (Waite et al., 2006). Some studies have highlighted that the differential gene expression of class II RNRs depends on levels of oxygenation and have shown a 3.2-fold up-regulation under anaerobic conditions compared with expression during aerobiosis (Filiatrault et al., 2005). *nrdJ* up-regulation has also been noticed in anaerobic sputum (Palmer et al., 2007), and NrdJ and NrdD proteins were also identified to have an increased concentration under anaerobic conditions (Wu et al., 2005).

In agreement with these observations, we observed a large increase in *nrdJ* and *nrdD* mRNA levels under anaerobic conditions (compared with aerobiosis) and in biofilm-forming cells (compared with planktonic cells; **Table 1**). These results imply the existence of a direct or indirect mechanism to activate *nrdJ* and *nrdD* transcription as a result of anaerobic metabolism and/or due to specific biofilm-related factors. The comparison between the expression in initially aerobic biofilm cells and in anaerobic planktonic cells shows that *nrdD* transcription was mainly activated by anaerobiosis, while *nrdJ* expression levels appeared to also be regulated by specific biofilm factors, as *nrdJ* induction in the biofilm (where only some anaerobic and microaerophilic areas are present) is higher than in fully anaerobic planktonic cultures.

To sustain anaerobic metabolism, *P. aeruginosa* uses NO_3 or other more oxidized forms of nitrogen (NO_2, NO) as final electron acceptors for anaerobic respiration: the final product of the full chain of reductions is molecular nitrogen (N_2; Schreiber et al., 2007). Anr acts as a general regulator of all anaerobic metabolism, activating the transcription of all metabolic enzymes thought to be involved in the pathway and that of the more specific regulators *dnr* and *narL*. NarL and Dnr transcription factors are in turn responsible for the control of the enzymes acting in the first reduction (from NO_3 to NO_2) and in the whole pathway, respectively. When analyzing the effects of mutations of these transcription factors on the RNR expression levels measured in a gene reporter assay, we observed a strong reduction in the anaerobic induction of *nrdJ* expression in the Δ*dnr* and Δ*anr* mutant strains, while no effect was observed when mutating the *narL* gene, and *nrdD* expression was not altered (**Figure 3**).

Therefore, we suggest that regulation by Anr/Dnr is partially responsible for class II RNR anaerobic induction. If Anr is active in the upper part of the regulation cascade, a simple transcriptional activation by Dnr would be the easiest explanation for the results obtained. Furthermore, as *nrdJ* expression was increased when GSNO, as an NO donor, was used as an electron acceptor, and NO levels affect the denitrification process by modulating Dnr regulation (Van Alst et al., 2007; Castiglione et al., 2009), these findings support the hypothesis of transcriptional control of *nrdJ* expression by Dnr. According to the biofilm reaction-diffusion theory (Stewart, 2003) we hypothesize that NO, described to be the main metabolite accumulated as a consequence of anaerobic metabolisms (Ye et al., 1994), should see its concentration increased in the lower layers, enhancing the effect of Dnr regulation (see **Figure 6**).

The genes belonging to the Anr/Dnr regulons are associated with Anr and Dnr binding boxes (Trunk et al., 2010), although the binding sites are still not well determined and more studies are needed to distinguish between them. Surprisingly, we identified a putative Anr/Dnr binding box in both class II and class III RNR promoters (P*nrdJ* and P*nrdD*; **Figure 4**). In our gene reporter assays, we determined that the mutation of the putative Anr/Dnr box in P*nrdJ* dramatically reduced the anaerobic induction of class II RNR expression (resembling the effect of *dnr* or *anr* gene mutation), while mutating the putative Anr/Dnr box in P*nrdD* had no significant effect.

According to these results, we can assume that under anaerobic conditions or in the anaerobic and microaerophilic environments generated during biofilm formation, NrdJ activity is essential for proper growth and that it is activated under these conditions by Dnr or Anr/Dnr via direct binding with its promoter. However, further studies are needed to determine if there are other specific biofilm-related factors activating NrdJ transcription and to define the mechanism for class III RNR anaerobic induction. This could be due to other factors that have not yet been studied, or it could even be related to Anr/Dnr pathways (as suggested the putative box found in the promoter) that may only be detectable under specific conditions that have not yet been tested.

Integrating our experiments on the effects of RNR mutation on biofilm formation and on RNR regulation in biofilm growth and under anaerobic conditions, we performed a gene reporter assay during biofilm formation, which supported our model: as the *P. aeruginosa* PAO1 wild-type biofilm structure matured, anaerobic areas were generated (as defined by the induction of the control promoter P*oprF*) and P*nrdJ* and P*nrdD* were consequently induced (**Figure 5A**). Again, mutating the putative Anr/Dnr boxes reduced class II RNR induction and had no effect on class III RNRs. However, analyzing the difference in expression in a PAO1 Δ*dnr* mutant strain between biofilm forming cells and planktonic cells, we not only observed a reduced anaerobic induction of class II RNRs but also, surprisingly, a considerably reduced induction of class III RNRs (**Figure 5B**), reinforcing the hypothesis that there could be an as-yet-undefined direct or indirect mechanisms by which Anr/Dnr controls *PnrdD* expression.

The model of a *P. aeruginosa* biofilm as a set of layers with different RNR expression profiles that are determined by oxygen concentration and B_{12} diffusion gradients and by cells with specific genetic regulation to support the differential RNR activities is of great importance for our understanding of this particular growth pattern. These results could play an important role in understanding the virulence of bacterial biofilms as it has been shown that the growth conditions in the lungs of CF patients include oxygen-limited growth and anaerobic environments (Schobert and Jahn, 2010) and that susceptibility to antibiotics in biofilms is modulated by limited oxygen availability (Borriello et al., 2006).

AUTHOR CONTRIBUTIONS

AC, LP, and ET designed the study. AC, LP, and JA performed the experiments. All authors analyzed the data, wrote the paper, read and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00688

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Material

Pseudomonas aeruginosa exhibits deficient biofilm formation in the absence of specifically activated class II and III ribonucleotide reductases

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Supplementary Tables

- **Supplementary Table S1. Bacterial strains and plasmids used in this study.**
- **Supplementary Table S2. Primers and probes used in this study.**
- **Supplementary Table S3. Growth of *P. aeruginosa* wild-type and mutant strains under aerobic and anaerobic conditions.**

Supplementary Table S1. Bacterial strains and plasmids used in this study.

Strain or plasmid	***Description***	***Source***
Plasmids		
pGEM-T easy	A/T cloning vecto;, AmpR	Promega
pUCP20T	Broad-host-range vector; AmpR	(West et al., 1994)
pUCGmlox	pUC18-based vector containing the lox flanked aacC1 gene; AmpR, GmR	(Quenee et al., 2005)
pEX100Tlink	*Pseudomonas* suicide vector pEX100T with a MCS, sacB, oriT; AmpR	(Quenee et al., 2005)
pETS130-GFP	Broad host range, promoterless GFP; GmR	(Sjoberg and Torrents, 2011)
pETS134	pETS130 derivative carrying *nrdA* promoter; GmR	(Sjoberg and Torrents, 2011)
pETS136	pETS130 derivative carrying *nrdD* promoter; GmR	(Sjoberg and Torrents, 2011)
pETS159	pBBR1 derivative carrying *nrdJab* operon; GmR	(Sjoberg and Torrents, 2011)
pETS160	pBBR1 derivative carrying *nrdDG* operon; GmR	(Sjoberg and Torrents, 2011)
pETS180	pETS130 derivative carrying *nrdJ* promoter; GmR	(Crespo et al., 2015)
pETS191	pETS130 derivative carrying mutant Anr/Dnr box in *nrdJ* promoter; GmR	This work
pETS192	pETS130 derivative carrying mutant Anr/Dnr box in *nrdD* promoter; GmR	This work
pETS193	pETS130 derivative carrying *oprF* promoter; GmR	This work
pETS195	pUCP20T derivative carrying *dnr* gene; AmpR	This work
pETS196	pET100Tlink-nrdJ::ΩGm	This work
Strains		
E. coli		
DH5	*recA1 endA1 hsdR17 supE44 thi-1 relA1 (lacZYA-argF)U169 deoR 80dlacZM15*	Laboratory stock
S17.1λpir	*recA thi pro hsdR- M+RP4::2-Tc::Mu::Km Tn7 Tpr Smr Xpir*	(de Lorenzo et al., 1993)
P. aeruginosa		
PAO1	Wild-type (ATCC 15692 / CECT 4122)- Spanish Type Culture Collection	Lab strain
PW3784	*P. aeruginosa* PAO1 *anr:*:ISlacZ/hah; TcR	(Jacobs et al., 2003)
PW1965	*P. aeruginosa* PAO1 *dnr*::ISlacZ/hah; TcR	(Jacobs et al., 2003)
PW7549	*P. aeruginosa* PAO1 *narL* ::ISlacZ/hah; TcR	(Jacobs et al., 2003)
ETS102	*P. aeruginosa* PAO1 *nrdJ* :: ΩTc; TcR	(Sjoberg and Torrents, 2011)
ETS103	*P. aeruginosa* PAO1 *nrdD* ::ΩTc; TcR	(Sjoberg and Torrents, 2011)
ETS125	*P. aeruginosa* PAO1 *nrdD* ::ΩTc; TcR, *nrdJ* ::ΩGm; GmR	This work

Supplementary Table S2. Primers and probes used in this study.

Name	Sequence (5'→3')	Application
M13-dir	GTTTTCCCAGTCACGAC	Check-Cloning
M13-rev	CAGGAAACAGCTATGACC	Check-Cloning
pUCP20T-up	CCTCTTCGCTATTACGCCAG	Cloning
pUCP20T-low	TCCGGCTCGTATGTTGTGTG	Cloning
pBBR1-up	CATCGCAGTCGGCCTATTGG	Cloning
pBBR1-low	CACTTTATGCTTCCGGCTCG	Cloning
PnrdA-up	AGGATCCGAATTCTTGCTCCACACAGCCTC	Cloning
PnrdA-low	ACCCGGGTTCTCGCGTGTGGTGTCG	Cloning
PnrdJ BamHI new-up	GGATCCCGCGCCCAGCTGAAGGCC	*PnrdJ* promoter cloning
PnrdJ SmaI new-low	AACCCGGGGACTGCGTTGCGTCTGTC	*PnrdJ* promoter cloning
PnrdD-up	AGGATCCGAATTCGCCCGCCTCGCCCAGG	*PnrdD* promoter cloning
PnrdD new-low	AATCGATCAGGGTGGCCGGCCAGGTAG	*PnrdD* promoter cloning
nrdATaqM2-low	TGTTCATGTCGTGGGTACG	qRT-PCR
nrdJTaqM2-low	GTAAACACCCGCACCACTTC	qRT-PCR
nrdDTaqM2-low	CCGAGTTGAGGAAGTTCTGG	qRT-PCR
gapTaqM-low	GAGGTTCTGGTCGTTGGT	qRT-PCR
nrdA-FAM	CTGGCACCTGGACATC	qRT-PCR probe
nrdJ-FAM	TCGGCTCGGTCAACCT	qRT-PCR probe
nrdD-FAM	CCCGACCTACAACATC	qRT-PCR probe
gap-FAM	CCTGCACCACCAACTG	qRT-PCR probe
mutanrJ-up	TATTGAGGACACGCAGGTACGGA	Mutation of Anr box in P*nrdJ*
mutanrJ-low	TCCGTACCTGCGTGTCCTCAATA	Mutation of Anr box in P*nrdJ*
mutanrD-up	GACGCGACAGCAGCAGCTCGCCGGC	Mutation of Anr box in P*nrdD*
mutanrD-low	GCCGGCGAGCTGCTGCTGTCGCGTC	Mutation of Anr box in P*nrdD*
Jmut1HIIIup	AAAGCTTCCCGTCAGGTACGGATAAC	*nrdJ* gene mutation
Jmut2BIlw	AAAAGGATCCATGGAGTCCTGGATGGTCC	*nrdJ* gene mutation
Jmut3BIup	AAAAGGATCCTATTACGGCAAGTACTGAGG	*nrdJ* gene mutation
Jmut4SIlw	AGAGCTCGACAAGGAAGGTGCAGTC	*nrdJ* gene mutation
Jint-2-3lw	TAGATGTCCATGAACGACAGC	checking *nrdJ* gene mutation
PoprFBHI-up	GGATCCCAACGAGTGCATCACG	P*oprF* promoter cloning
PoprFClaI-low	ATCGATGGTGTTCTTCAGTTTCAT	*PoprF* promoter cloning
Pdnr-BHI-up	GGATCCACGGCAGATGCACT	dnr cloning for complementation
Dnr-low	ATCACTCGAAGCACTCCAGGC	dnr cloning for complementation

Supplementary Table S3. Growth of *P. aeruginosa* wild-type and mutant strains under aerobic and anaerobic conditions. Δ*nrdJ*, Δ*nrdD*, Δ*nrdD*Δ*nrdJ* and wild-type PAO1 strains were grown for 16 h under aerobic and anaerobic conditions in LB and LBN, respectively. Bacterial growth was measured by reading the optical density at 550 (OD_{550}). Vitamin B_{12} was added when necessary at a concentration of 1 µg/mL. Final OD_{550} values are listed in the table.

		OD_{550}			
	B_{12}	**PAO1 wild-type**	**ETS102 Δ*nrdJ***	**ETS103 Δ*nrdD***	**ETS125 Δ*nrdD*Δ*nrdJ***
Aerobic	-	4.00	3.70	3.90	3.80
	+	3.90	3.80	3.87	3.76
Anaerobic	-	1.58	0.13	0.17	0.05
	+	1.91	0.13	2.00	0.07

Article 4

Deficient anaerobic growth and infection in Pseudomonas aeruginosa PAO1 strain due to a single point mutation in the class III ribonucleotide reductase promoter.

Deficient anaerobic growth and infection in *Pseudomonas aeruginosa* PAO1 strain due to a single point mutation in the class III ribonucleotide reductase promoter.

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Running title: Class III ribonucleotide reductase from *Pseudomonas aeruginosa*.

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Keywords: ribonucleotide reductases; *Pseudomonas aeruginosa*; NrdD; clinical isolates; anaerobic metabolism

Abstract

200 words

Pseudomonas aeruginosa strain PAO1 has become the reference strain in many laboratories. One enzyme that is essential for its cell division is the ribonucleotide reductase (RNR) enzyme that supplies the deoxynucleotides required for DNA synthesis and repair. *P. aeruginosa* is one of the few microorganisms that encodes three different RNR classes (Ia, II and III) in its genome, enabling it to grow and adapt to diverse environmental conditions, including during infection.

In this work, we demonstrate that a lack of RNR activity induces cell elongation in *P. aeruginosa* PAO1. Moreover, RNR gene expression differs among *P. aeruginosa* strains, with class III highly expressed in *P. aeruginosa* clinical isolates relative to the laboratory *P. aeruginosa* PAO1 strain. A single point mutation was identified in the class III RNR promoter region that disrupts its anaerobic transcription by the Dnr regulator. The induction of class III RNR expression allows *P. aeruginosa* PAO1 anaerobic growth and increases its virulence to resemble that of clinical strains. Therefore, our results demonstrate that *P. aeruginosa* PAO1 is adapted to laboratory conditions and is not the best reference strain for anaerobic or infection studies.

Introduction

Pseudomonas aeruginosa is a gram-negative opportunistic pathogen that is responsible for several acute and chronic infections. It presents a significant problem for patients with chronic wounds, cystic fibrosis (CF) and other immunocompromised diseases. The ability of *P. aeruginosa* to adapt to diverse environmental conditions and to cause infections relies on its ability to control gene expression in response to environmental stimuli. Additionally, the bacterial mutation rate within the host is a key factor in determining the potential for the bacterial pathogens to genetically adapt to the host immune system and evade drug therapies [1].

The genome of the wild-type *P. aeruginosa* PAO1 strain is relatively large (6.3 Mbp) and contains paralogues of various genes that perform different metabolic activities, thereby allowing for adaption to and exploration of different ecological niches. *P. aeruginosa* is one of the few microorganisms that simultaneously encodes three different ribonucleotide reductase (RNR) classes in its genome (class Ia, II and III), allowing it to grow under different specific environmental conditions. RNR enzymes reduce the four different ribonucleotides (NTPs) into their corresponding deoxynucleotides (dNTPs), which are the principal monomers for DNA synthesis and repair; RNR is thus essential for cell division. The genes encoding the three different RNR classes (class I, II and III) share only 10% of their nucleotide composition but perform the same enzymatic activities [2].

Class I RNRs require oxygen to generate a tyrosyl radical by interacting with a di-ferric iron or a di-manganese iron center and therefore function only under aerobic conditions. Based on sequence identity, the metal cofactor center and allosteric properties, class I RNRs are subdivided into classes Ia, Ib and Ic, which are encoded by the *nrdAB*, *nrdHIEF* and *nrdAB* genes, respectively. Class II RNR enzymes, encoded by the *nrdJab* genes, require S-adenosylcobalamin (AdoCob) or a vitamin B_{12} cofactor for radical generation and do not depend on oxygen for enzymatic activity. Members of the class III RNR, encoded by the *nrdDG* genes, carry a stable but oxygen-sensitive glycyl residue plus an iron-sulfur center that catalyzes the reduction of S-adenosylmethionine to generate its radical. This class can only be active under anaerobic conditions [3].

During the infection process, *P. aeruginosa* acts under anaerobic conditions [4,5,6]. However, the *P. aeruginosa* strain PAO1 cannot properly grow anaerobically[7]. This common reference laboratory strain is a spontaneous chloramphenicol-resistant mutant strain that was isolated in 1954 from a patient wound in Melbourne, Australia (American Type Culture Collection ATCC 15692) [8] and was found to be equipped with endogenous virulence-suppression mechanisms and to be highly adapted to growth under laboratory conditions. Nevertheless, it is the reference strain in many laboratories for *Pseudomonas* genetics, physiology, identification of virulence factors, metabolic studies and for the identification of specific *Pseudomonas* inhibitors. A debate currently exists concerning whether this laboratory strain is similar to or behaves in the same manner as naturally occurring *P. aeruginosa* strains from clinical or natural sources. Other *P. aeruginosa* strains that have been more recently isolated, such as *P. aeruginosa* PA14, are more adapted to infection conditions and show greater virulence than the PAO1 strain [9,10]. Specifically, *P. aeruginosa* PA14 preserves two *P. aeruginosa* pathogenicity islands (PAPI-1 and PAPI-2) in its genome [11]. These pathogenic islands occur in several cystic fibrosis (CF) *P. aeruginosa* isolates but are absent from *P. aeruginosa* PAO1.

We have previously published work on the involvement of the different RNR enzymes during laboratory growth, during biofilm formation and in the virulence of *P. aeruginosa* [12,13,14]. The presence of a class Ia RNR is necessary during aerobic *P. aeruginosa* PAO1 growth under laboratory conditions. However, class II and class III RNRs are involved in anaerobic growth, biofilm formation and virulence, although it is difficult to discern the specific importance or role of each RNR class under these specific conditions. Therefore, in this work, we were particularly interested in the specific role of the class III RNR in *P. aeruginosa* growth and infection and sought to understand the relative importance of this enzyme in clinical isolates compared to the laboratory strain PAO1. A goal of this study is to clarify the differences in pathogenesis and virulence

associated with each *P. aeruginosa* strain.

Results

Different cell morphologies of *P. aeruginosa* strains under anaerobic conditions.

P. aeruginosa PAO1 cells showed a clear filamentous morphology phenotype (> 19 μm) (Fig. 1a) when grown under anaerobic conditions, indicating impaired DNA replication, as we and other authors have previously suggested [7,12]. Surprisingly, different *P. aeruginosa* clinical isolate strains (from cystic fibrosis patients, PAET1 and PAET2; from *P. aeruginosa* acute infections, PA54 and PA166; and the laboratory reference strain PA14) showed rod-shaped morphologies (approximately 2 μm) that were markedly different from that of *P. aeruginosa* PAO1 (Fig. 1a). In this case, DNA replication in clinical isolates and PA14 appears unimpaired under anaerobic conditions, in contrast to PAO1.

Low activity of class III RNR enzymes causes cell elongation in *P. aeruginosa* PAO1.

Next, we asked why only PAO1 cells were elongated under anaerobic conditions. As expected, PAO1 cells were elongated under anaerobic conditions (average length of 19.2 μm) but were restored to their normal rod shape when the class II RNR cofactor vitamin B_{12} was added to the culture medium (average length of 2.5 μm) (Fig. 1b), indicating that the enzymatic activation of this RNR class restores proper DNA synthesis and replication. No morphological changes were observed in the clinical isolates growing in the absence or presence of vitamin B_{12} (data not shown). When the class II and III RNR genes were mutated (strains ETS102, ET103 and ET125), no anaerobic growth was observed (with an OD of approximately 0.1), and the few visualized cells showed extremely elongated morphologies (from 8.9 to 92 μm), demonstrating the requirement of anaerobic RNR activity to restore DNA synthesis impairment under anaerobic growth conditions. Indeed, class II RNR activity in the complemented strain (ETS102 + NrdJ) was only restored when the vitamin B_{12} cofactor was present in the culture medium (average length of 1.5 μm). Furthermore, class III RNR complementation (in PAO1 $NrdDG^+$, ETS102 $NrdDG^+$, ETS103 $NrdDG^+$ and ETS125 $NrdDG^+$) was sufficient to promote *P. aeruginosa* PAO1 growth with rod-shaped cell morphology (average length of 1.5 μm) (Fig. 1b) without requiring vitamin B_{12}. This experiment revealed the relatively low class III RNR expression and activity in *P. aeruginosa* PAO1 compared with that in clinical isolates. Our results clearly demonstrated deficient *P. aeruginosa* PAO1 DNA replication under anaerobic conditions that was reversed by increasing class III RNR expression levels or gene copy number.

nrdD expression is impaired in _P. aeruginosa_ PAO1 compared to the clinical isolates.

To understand the variation in cell morphology among strains, we analyzed the expression of the genes encoding different RNR classes in *P. aeruginosa* laboratory strains (PAO1 and PA14) and in clinical isolates (PAET1, PAET2, PA166 and PA54) under aerobic or anaerobic growth conditions by qRT-PCR (Fig. 2a).

As previously described, class I RNR (*nrdA*) showed the highest expression level when PAO1 was grown under aerobic conditions, while under anaerobic conditions, class II (*nrdJ*) and III (*nrdD*) RNRs are highly expressed [15]. However, under aerobic conditions, most clinical isolates showed decreased expression of the three *nrd* genes (*nrdA, nrdJ* and *nrdD*) relative to PAO1 (Fig. 2a, negative values). The same results were observed under anaerobic conditions, with *nrdA* and *nrdJ* showing reduced expression tendency in the clinical isolates compared with that in the laboratory strain PAO1 (Fig. 2a). However, *nrdD* gene expression was anaerobically upregulated in all the different clinical isolates (9.69 times for PAET1, 9.25 times for PAET2, 3.04 times for PA166, 2.12 times for PA54 and 1.51 times for PA14) compared with that in PAO1, which showed remarkably low expression levels (Fig. 2a).

When we specifically compared the difference in *nrd* expression during anaerobic versus aerobic growth, *nrdD* expression was significantly higher (from 40 to 100 times) in all of the analyzed strains than in strain PAO1 (Fig. 2b). This result reveals a marked increase in class III RNR expression in clinical isolates compared with that in PAO1 that is responsible for proper anaerobic growth with optimum DNA replication, thereby permitting the rod-shaped morphology observed in Fig. 1.

A single point mutation renders _P. aeruginosa_ PAO1 deficient for anaerobic growth.

Low levels of class III RNR (NrdD) activity were clearly responsible for the deficient growth of *P. aeruginosa* PAO1 under anaerobic conditions. To explain the different *nrdD* transcription levels of the clinical isolates relative to the laboratory PAO1 strain, we analyzed their promoter regions in detail. We sequenced the *nrdD* promoter region from all of the different clinical isolates we tested (PAET1, PAET2, PAET4, PAET6, PA1016, PA166 and PA54) and all available laboratory PAO1 strains (PAO1-CECT, PAO1-UW, PAO1-JPN), and the resulting DNA sequences were compared to other known, sequenced *P. aeruginosa* strains (PAO1-PAdb, PA14, PA7 and LESB58) and to other related *Pseudomonas* species (*P. fluorescens, P. chloraphis* and *P. alicagenes*) (Fig. 3a). The alignment showed nearly 100% identity among the different *P. aeruginosa* strains, while some differences were observed when comparing sequences from other

non-related *Pseudomonas* species. By using the Virtual Footprint tool from the PRODORIC database [16], we identified in all *P. aeruginosa* strains a putative Anr/Dnr binding box in this promoter region, located at -98 bp from the translation start site in strain PAO1. Surprisingly, this position showed the only base-pair mismatch we identified between PAO1 (**C**TGACGCAGATCAA) and the clinical isolates and other laboratory strains such as PA14 (**T**TGACGCAGATCAA) (Fig. 3a).

To verify that this nucleotide mismatch influences promoter activity, we analyzed PAO1 strains carrying *nrdD* transcriptional fusions to GFP with different Dnr transcription factor binding box signatures. We transformed *P. aeruginosa* PAO1 cells with plasmids bearing the *nrdD* promoter with single point mutations in the Dnr signature (pETS136-**C**TGACGCAGATCAA or with the clinical-isolate *nrdD* promoter containing the T substitution P*nrdD* (*C*>T)) (pETS196-**T**TGACGCAGATCAA) (see Material and Methods). As shown in Fig. 3b, in all analyzed strains, increased P*nrdD* activity was observed for the pETS196-T vector compared with that observed for the vector (pETS136-C) carrying the native PAO1 promoter. Moreover, when we measured the P*nrdD* expression of pETS136-C (PAO1 *PnrdD*) in the clinical isolates, we observed a decrease in its expression to the same level observed for the PAO1 strain. Therefore, maximal *nrdD* expression is associated with a promoter bearing the Dnr(T)-box signature (**T**TGACGCAGATCAA), which is typically observed in the naturally occurring *P. aeruginosa* strains.

The Anr and Dnr transcription factors share a consensus binding box but activate different specific promoters [17]. Anr activates the transcription of the *dnr* gene, following a regulatory cascade[18]. Thus, to determine which transcription factor is responsible for regulating *nrdD*, we analyzed *nrdD* expression (pETS196-T) in isogenic PAO1 strains carrying Δ*anr* and Δ*dnr* mutations. Complementation of the *dnr* mutation with a plasmid-borne *dnr* gene (pETS195) showed that Dnr specifically induced *nrdD* expression (in pETS196-T with P*nrdD* (C>T)), as we previously observed for the *nrdJ* gene[14]. Notably, *nrdD* was expressed at the same level from the pETS196-T vector in the Δ*anr* and Δ*dnr* mutant strains as from the pETS136-C vector with the PAO1 promoter.

nrdD expression and its role during infection

We analyzed *nrd* expression *in vivo* by measuring the relative fluorescence during *P. aeruginosa* PAO1 infection in the zebrafish (*Danio rerio*) model. The GFP intensity results showed that the *nrdJ* and *nrdD* genes were highly expressed during the course of infection (from 6 until 25 hours post-infection; hpi), with *nrdD* showing the highest expression (Fig. 4a) and following the same expression pattern as observed previously in fly-infection experiments[12]. As shown in Fig. 4b,

the expression of *nrdD* was significantly higher in all strains analyzed (PAO1, PA14 and the clinical isolate PAET1) when the promoter contained the C>T modification (pETS196-**T**; P*nrdD* (C>T)) than when it had the native PAO1 sequence (pETS136-**C** (P*nrdD*)).

Finally, we were interested in evaluating whether this single mutation in the *nrdD* promoter, found specifically in *P. aeruginosa* PAO1, affects its virulence relative to other strains. We infected zebrafish with wild-type PAO1 strains engineered with the different constructs that showed different *nrdD* expression. We used two *nrdD* merodiploid strains (i.e., with two chromosomal copies), one with the PAO1 wild-type promoter (ETS129; PAO1 NrdDG^{+}) and another with the modified P*nrdD* promoter carrying the point mutation of the clinical isolates (ETS130; PAO1 NrdDG (C>T)$^{+}$). We also used strains complemented for the Δ*nrdD* mutation (ETS127, Δ*nrdD* PAO1 NrdDG^{+}, and ETS128, Δ*nrdD* PAO1 NrdDG (C>T)$^{+}$). The *nrdD* expression of each strain was validated and measured using qRT-PCR, as shown in Fig S1a, demonstrating that the increased *nrdD* transcription of these strains relative to that from the single copy present in wild-type PAO1 was associated with the rod-shaped morphology found in PA14 and the clinical isolates (Fig S1b).

As expected, the mortality of *D. rerio* decreased when we used *P. aeruginosa* with an inactivated *nrdD* gene (ETS103; Δ*nrdD*) (18.2 hpi for 50% survival) relative to the mortality after infection with the wild-type strains PAO1 or PA14 (14.03 or 12.7 hpi for 50% survival, respectively) (Fig. 4c). Infection with strains chromosomally complemented for the *nrdD* mutation (strains ETS127, Δ*nrdD* PAO1 NrdDG^{+} and ETS128, Δ*nrdD* PAO1 NrdDG (C>T)$^{+}$) resulted in survival resembling that of PAO1-infected fish. Furthermore, ETS128 (Δ*nrdD* PAO1 NrdDG (C>T)$^{+}$) yielded a similar survival percentage as PA14, but it was significantly increased (P<0.05) over that of PAO1. As previously observed, PA14 showed higher virulence than PAO1 [11]. Interestingly, the virulence of the ETS130 (PAO1 NrdDG (C>T)$^{+}$) merodiploid strain was significantly increased (P<0.0001) compared with that of the PAO1 and PA14 wild-type strains due to the elevated *nrdD* expression that increases its anaerobic RNR activity.

Discussion

P. aeruginosa is an aerobic bacterium with a versatile metabolism, but its host-infection process is considered to occur under anoxic conditions[4,6,19]. Most *P. aeruginosa* patient isolates exhibit strain-dependent differences in response to specific niches and have diverse metabolic activities due to growth in different environments with low nutrients and/or low oxygen gradients[20]. Its large genome and genetic flexibility allow *P. aeruginosa* to respond to selective pressure in the host environment by generating mutations in specific genes that allow survival in and adaptation to

a variety of infection environments (low oxygen gradients in planktonic and biofilm growth, etc.)[1,21,22].

Ribonucleotide reductases (specifically, class II and class III RNRs) are described as key enzymes in *P. aeruginosa* anaerobic growth and virulence[12,14,23,24,25]. Previous studies have shown impaired cell division accompanied by an elongated-cell (filamentous) phenotype during *P. aeruginosa* PAO1 growth in anaerobic conditions due to the induction of cellular stress by NO levels[24,26]. During denitrification metabolism, nitric oxide (NO) is produced as an intermediate molecule that inhibits the vitamin B_{12} biosynthesis pathway and disrupts class II RNR activity[7,24]. Therefore, addition of vitamin B_{12} (the cofactor of NrdJ) into an anaerobic PAO1 culture prevents cell elongation by increasing NrdJ activity and thus increasing the dNTP synthesis that allows appropriate cell division.

In this study, we showed that *P. aeruginosa* clinical isolates have rod-shaped cells under anaerobic conditions, in contrast to filamentous PAO1 cells. We also demonstrated that PAO1 cells had a filamentous phenotype due to a general lack of RNR activity that blocks DNA synthesis in anaerobic conditions (Fig. 1). Under such conditions, the only way for PAO1 cells to grow properly is by the addition of exogenous vitamin B_{12} into the medium to allow fully functional and active class II RNR activity. However, overexpression of the class III RNR system also overcomes the dNTP deficiency and permits the DNA replication required for rod-shaped cell morphology (Fig. 1). Clinical isolates did not show RNR activity deficiency during anaerobic growth, as they show the clear rod-shaped phenotype that is typical of cells with proper cell division.

The reason why clinical isolates show a very different cell morphotype than PAO1 remains unclear. Strikingly, evaluating the expression of different RNR genes under anaerobic conditions showed that *nrdD* expression was elevated, with a concomitant reduction in *nrdJ* (class II RNR) and *nrdA* (class Ia RNR) expression, in the clinical isolates relative to PAO1. By analyzing this extreme difference, we observed that it was due to a single-base mutation in the promoter region of the *nrdDG* operon that specifically affects the binding of the Dnr transcriptional factor, which is an important regulator of gene induction during anaerobic growth. The PAO1 promoter contains a cytosine (**C**) at the first position of the consensus Dnr box (**C**TGACGCAGATCAA) rather than the thymine (**T**) that is found in the clinical-isolate *nrdD* promoters (Fig. 3a). A substitution of the PAO1 *nrdD* promoter with this specific T nucleotide (pETS196-T, Fig. 3b) returns *nrdD* expression levels to that of the clinical isolates, probably due to more-optimal Dnr transcriptional factor positioning at its binding region. Notably, the affinity of Anr-Dnr for the *arcDABC* promoter and the regulation of the *arcDABC* genes via these transcriptional factors are decreased if the cytosine

(C) at the first position of the Anr-Dnr binding box is mutated, implying the same situation as at the *P. aeruginosa* PAO1 *nrdD* promoter[27].

Anr and Dnr are anaerobic transcriptional factors that control most of the genes that are important for anaerobic growth. The global oxygen-sensing regulator is Anr (anaerobic regulator of arginine deiminase and nitrate reductase), which controls *dnr* gene expression. The Dnr regulator is an NO sensor and induces the expression of several genes under anaerobic growth conditions, including during infection[4,28,29]. We have previously demonstrated that Dnr is involved in class II RNR anaerobic expression[14], and *Escherichia coli* Fnr (an Anr homologue*)* controls class III RNR expression[30,31].

We also studied the importance of Dnr in the transcriptional regulation of a class III RNR (*nrdD*) during biofilm formation, as we have previously shown its importance for regulating a class II RNR[14]. We clearly observed its importance under this condition (Fig. S2a); however, specifically during biofilm formation, class II RNR is the most highly expressed RNR (Fig. S2b) [14].

Some studies have found that the class III RNR (NrdDG) is an important protein for bacterial virulence in *E. coli* LF82[32], *Porphyromonas gingivalis*[33], *Staphylococcus aureus*[34,35], *Streptococcus pneumoniae*[36] and *Streptococcus sanguinis*[37]. For this reason, we attempted to learn whether the single-nucleotide substitution in the *nrdD* promoter region that is specifically found in PAO1 affects its virulence relative to other laboratory strains, such as PA14. PA14 is known to display greater virulence than PAO1[11,38]. Therefore, we analyzed virulence in a *D. rerio* animal model of infection using *P. aeruginosa* strains with different *nrdD* expression levels (Fig. 4c). As previously observed[12], mutation of the *nrdD* gene reduces *P. aeruginosa* virulence (Fig. 4c). However, the strains that displayed higher *nrdD* expression also showed virulence that was significantly increased relative to wild-type PAO1 and was even higher than that of PA14.

Clearly, in this work, we have seen that *P. aeruginosa* uses the class III RNR (NrdDG) for dNTP synthesis, which is important for DNA replication during anaerobic growth and during infection. We specifically identified a single point mutation in the *nrdDG* promoter region that causes PAO1 to grow inefficiently during anaerobic growth and during infection, in contrast to other laboratory *P. aeruginosa* strains (e.g., PA14).

Our results indicate that *P. aeruginosa* PAO1 is neither appropriate for virulence studies and experiments that require anaerobic metabolism nor in searches for new antimicrobial compounds that involve anaerobic conditions.

Methods

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table S1. *E. coli* and *P. aeruginosa* cells were grown in Luria-Bertani (LB) medium at 37°C. Anaerobic growth was performed in screw-cap tubes (Hungate Tubes) in LBN medium (LB medium + 10 g/l KNO_3) [15]. When necessary, antibiotics were added, including ampicillin (Ap) 50 μg/ml, tetracycline (Tc) 12.5 μg/ml and gentamicin (Gm) 10 μg/ml for *E. coli* and carbenicillin (Cb) 300 μg/ml, Tc 100 μg/ml and Gm 50 μg/ml for *P. aeruginosa*.

DNA manipulation

Recombinant DNA techniques were performed using standard procedures[15,19]. Plasmids were isolated using the GeneJET Plasmid Miniprep Kit, and DNA fragments were purified using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Inc) according to the manufacturer's instructions. The *P. aeruginosa* PAO1 strain that was genetically modified in this work was the PAO1-CECT strain (Table S1). DNA was transformed into *P. aeruginosa* cells by conjugation or electroporation as previously described[15].

Using site-directed mutagenesis as previously described[15], the identified Anr/Dnr-box at the *nrdD* promoter (see Results section) was mutated at a single nucleotide using the PD-Dnr-T up/PD-Dnr-T low primer pair. The mutated amplicon was cloned into the pETS130 plasmid to generate pETS196-T (P*nrdD* (C>T).

To complement the *nrdDG* deficiency, the complete *nrdDG* genes with their native promoter regions were amplified by PCR using PfuIIDGSacI-up/PfuIIDG-low-BamHI, cloned into pJET1.2, and then further cloned into pUCP20T to generate pUCP20T-DG (pETS197). In addition, the *nrdDG* fragment was also used to construct a merodiploid *P. aeruginosa* strain by cloning this DNA fragment into the transposon-containing pBAM-Gm plasmid to generate pBAM-Gm-DG (pETS199). The *nrdDG* (C>T) fragment was generated using PfuIIDGSacI-up/PD-Dnr-T low and PD-Dnr-T up/PfuIIDG-low-BamHI to construct a merodiploid *P. aeruginosa* strain with a mutated Anr/Dnr box by cloning this DNA fragment into the transposon-containing pBAM-Gm plasmid to generate pBAM-Gm-DG (C>T) (pETS200). The resulting merodiploid strains were ETS129 (PAO1 NrdDG$^+$) and ETS130 (NrdDG (C>T)$^+$). Both pBAM-Gm-DG (pETS199) and pBAM-Gm-DG (C>T) (pETS200) were also used for complementation of the Δ*nrdD* strain, thus producing ETS127 (Δ*nrdD::Tc* PAO1 NrdDG$^+$) and ETS128 (Δ*nrdD::Tc* PAO1 NrdDG (C>T)$^+$). Constructs were validated by PCR and DNA sequencing.

RNA extraction, reverse transcription and real-time PCR

Strains of interest were grown to mid-logarithmic phase (OD_{550}=0.5), and total RNA was extracted using the RNAprotect Bacteria Reagent (Qiagen) and RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. To further remove DNA contamination, the eluted RNA was treated with RNAse-free Turbo DNase (Thermo Scientific). The amount of RNA was determined from its 260-nm absorption (NanoDrop spectrophotometer ND-1000, NanoDrop). Reverse transcription-PCR (RT-PCR) was performed using the SuperScript III First-Strand Synthesis System (Thermo Scientific). Quantitative real-time PCR measurements were conducted using SYBR-green primers (Table S2), and detection was performed using an ABI Step One Plus detection system (Applied Biosystems). The *gapA* sequence was used as an internal standard.

Gene reporter assay for cells grown under anaerobic and biofilm conditions

Strains containing derivatives of the pETS130-GFP plasmid were grown under aerobic conditions to mid-log phase (OD_{550}=0.5) before different samples were pelleted and inoculated with a needle. The samples were added into Hungate screw-cap tubes containing anaerobic LBN medium for 3 hours to induce anaerobic metabolism. Three independent samples from three independent cultures were collected and fixed with 1X PBS containing 2% formaldehyde. Fluorescence was then measured in 96-well plates using an Infinite 200 Pro fluorescence microplate reader (Tecan). Three measurements were performed for each independent sample.

Gene expression during biofilm formation was determined by growing biofilms as previously described[14]. After 4 days of incubation, the 96-well plate (Nunclon Delta Surface, Thermo Scientific) containing the biofilm was washed to eliminate remaining planktonic cells, and the attached biofilm cells in the wells were fixed with PBS containing 2% formaldehyde. The fluorescence was measured on an Infinite 200 Pro Fluorescence microplate reader.

Fluorescence microscopic imaging and analysis

Anaerobic overnight cultures of *P. aeruginosa* strains were stained using the LIVE/DEAD BacLight viability kit (Thermo Scientific) for 15 minutes at room temperature in the dark. Fluorescent bacteria were visualized with a Nikon E600 microscope (Nikon) coupled with an Olympus DP72 camera. Live cells were visualized in green (SYTO 9 dye), and dead cells were visualized in red (propidium iodide dye). ImageJ software was used for image analysis.

nrdD promoter region sequencing and multiple sequence alignment

The *nrdD* DNA promoter region from different *P. aeruginosa* clinical isolates, PA14 and different PAO1 strains from different laboratories (PAO1-CECT from the Spanish Type Culture Collection, PAO1-UW from the Monoil laboratory, and PAO1-JPN from the Japan collection) were amplified by PCR (using the primer pair PnrdD3up/PnrdD-new-low) and then further sequenced using the primer PnrdD3up by the scientific services at the University of Barcelona. We also obtained the *P. aeruginosa* PAO1-PAdb, PA14, LESB58, *Pseudomonas fluorescens, Pseudomonas chlororaphis* and *Pseudomonas alcaligenes* sequences from the *Pseudomonas* Genome Database[40]. Sequence alignments were performed with CLUSTALW Omega using the default parameters in the CLC Main Workbench software (ver. 6.9.1).

P. aeruginosa infection of *Danio rerio*

The zebrafish (*D. rerio*) is a well-established host for studying bacterial virulence mechanisms[41]. Zebrafish embryos from the AB line were a kind gift from Prof. Angel Raya (Center of Regenerative Medicine in Barcelona, Spain). Embryos were kept at 29°C and staged at 48 hours post-fertilization, and were then dechorionated and anesthetized with 66 µg/mL of ethyl 3-aminobenzoate methanesulfonate (Sigma-Aldrich) prior to infection. Bacterial cells (1,000 cfu) were microinjected into the yolk circulation valley using borosilicate glass capillaries (World Precision Instruments, FL) with a microinjector (TriTech Research, CA). The exact inoculum size in 2-5 nl was determined by viable cell counts from the transfer of cells in the needle to PBS.

For the experiments to determine the expression of the *nrd* genes during fish infection with *P. aeruginosa* cells, we used *P. aeruginosa* PAO1, PA14 and PAET1 containing the different promoter probe vectors (pETS130, pETS134, pETS136-C, pETS180 and pETS196-T). Fluorescence was measured until 24 hours post-infection (hpi) using a stereo fluorescence microscope (Leica MZ16F) and was analyzed using Nikon Nis-Elements software.

Three independent experiments were performed for the zebrafish survival curve using 100 zebrafish per infecting strain, including PAO1, PA14, ETS103 (Δ*nrdD),* ETS127 (Δ*nrdD* PAO1 NrdDG^{+}), ETS128 (Δ*nrdD* PAO1 NrdDG (C>T)$^{+}$), ETS129 (PAO1 NrdDG^{+}) and ETS130 (PAO1 NrdDG (C>T)$^{+}$), over 42 hours post-infection (hpi). The results were plotted in GraphPad Prism 6.0 software using Kaplan-Meier analysis and the log-rank (Mantel-Cox) test to evaluate statistical differences.

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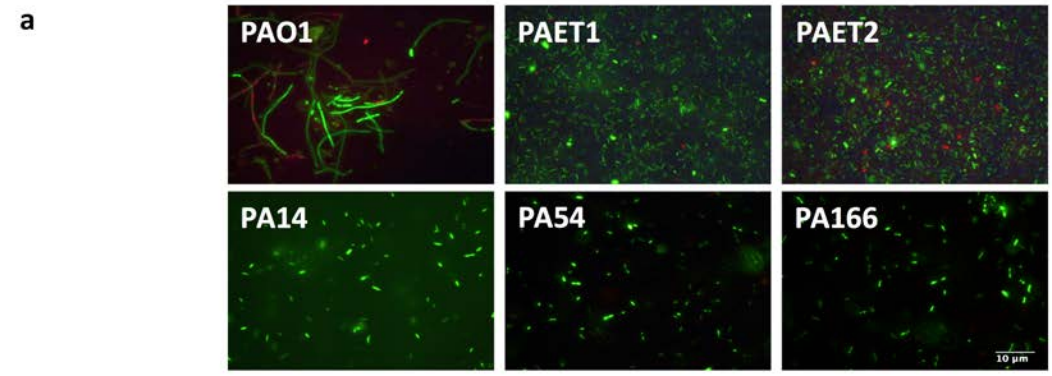
Figure 1: Elongated cell morphology of *P. aeruginosa* PAO1 under anaerobic conditions. (**a**) Fluorescence micrographs of *P. aeruginosa* PAO1, PAET1, PAET2, PA14, PA54, and PA166 cells grown anaerobically (to an OD_{550} of approximately 0.5) and stained with the LIVE/DEAD assay. (**b**) RNR mutant strains (Δ) of *P. aeruginosa* PAO1 (ETS102 (Δ*nrdJ*), ETS103 (Δ*nrdD*), and ETS125 (Δ*nrdJ*Δ*nrdD*) that contain complementation vectors (pETS159, pBBR1-NrdJab; pETS160, pBBR1-NrdDG; or pETS197, pUCP20T-DG)). Cells were stationary-cultured in the presence or absence of 1 µM vitamin B_{12}. Live cells stained with the LIVE/DEAD assay were visualized under a fluorescence microscope (1,000X magnification). The images are representative of three independent experiments with three replicates each. Cell length (mean ± standard deviation) was determined with ImageJ software. Scale bars, 10 µm.



b

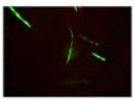
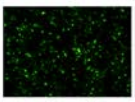
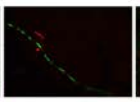
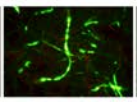
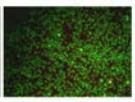
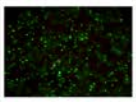
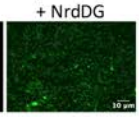
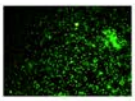
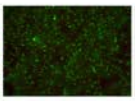
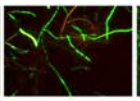
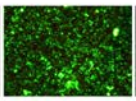
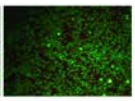
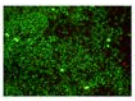
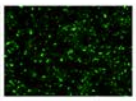
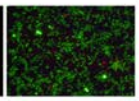
	PAO1 wild-type	PAO1 wt + NrdDG	PAO1 Δ*nrdJ* (ETS102)	PAO1 Δ*nrdJ* (ETS102) + NrdJab	PAO1 Δ*nrdJ* (ETS102) + NrdDG	PAO1 Δ*nrdD* (ETS103)	PAO1 Δ*nrdD* (ETS103) + NrdDG	PAO1 Δ*nrdD*Δ*nrdJ* (ETS125)	PAO1 Δ*nrdD*Δ*nrdJ* (ETS125) + NrdDG
-									
OD_{550}	1.6 ± 0.1	1.9 ± 0.07	0.1 ± 0.02	1.6 ± 0.12	1.8 ± 0.10	0.2 ± 0.10	2.0 ± 0.20	0.1 ± 0.03	1.7 ± 0.30
length (µm)	19.2 ± 8	1.5 ± 0.02	92 ± 15	58.7 ± 7.1	1.7 ± 0.2	8.9 ± 4.1	1.9 ± 0.3	13.5 ± 5	1.5 ± 0.1
+ vit B_{12}									
OD_{550}	1.9 ± 0.10	1.8 ± 0.07	0.1 ± 0.02	2.0 ± 0.10	1.8 ± 0.20	2.0 ± 0.02	1.9 ± 0.05	0.05 ± 0.01	1.6 ± 0.08
length (µm)	2.5 ± 1.3	1.6 ± 0.2	22.0 ± 4.9	1.3 ± 0.5	1.7 ± 0.2	1.2 ± 0.2	1.3 ± 0.2	11.1 ± 2.5	1.1 ± 0.03

Figure 2: Relative *nrd* gene expression in clinical isolates and laboratory *P. aeruginosa* strains. (**a**) Differences in the expression levels of *nrd* genes in PAO1 and in clinical isolate cells grown anaerobically or aerobically at mid-exponential phase (OD_{550}=0.5). The values in bold represent up-regulated *nrdD* gene. The log fold-change is shown as the mean ± standard deviation of three independent experiments. (**b**) Different RNR (*nrd*) gene expression levels in cells grown anaerobically *versus* cells grown aerobically. The induction expression factor of the *nrdA* (in white), *nrdJ* (in gray) and the *nrdD* (in black) in clinical isolates compared to PAO1 strain. The error bars represent the standard error of the mean. Significantly different from *P. aeruginosa* PAO1 in an unpaired *t*-test (*, P<0.05 and **, P<0.0001).

a

	fold change		
	class Ia *nrdA*	class II *nrdJa*	class III *nrdD*
Anaerobic			
PAET1 vs PAO1	2.11 ± 0.5	-1.54 ± 0.1	**9.69 ± 0.9**
PAET2 vs PAO1	1.47 ± 0.2	-1.49 ± 0.3	**9.25 ± 1.1**
PA166 vs PAO1	3.25 ± 0.8	1.50 ± 0.2	**3.04 ± 0.7**
PA54 vs PAO1	-2.51 ± 0.3	1.39 ± 0.3	**2.12 ± 0.5**
PA14 vs PAO1	-3.68 ± 0.2	-3.26 ± 0.6	**1.51 ± 0.4**
Aerobic			
PAET1 vs PAO1	-2.25 ± 0.2	-1.89 ± 0.4	-7.62 ± 0.7
PAET2 vs PAO1	-1.98 ± 0.5	-3.37 ± 0.3	-8.37 ± 0.5
PA166 vs PAO1	3.18 ± 0.7	4.04 ± 1.1	-34.05 ± 0.3
PA54 vs PAO1	-2.08 ± 0.3	1.91 ± 0.4	-21.40 ± 0.3
PA14 vs PAO1	-1.82 ± 0.1	-2.24 ± 0.5	-24.80 ± 0.4

b

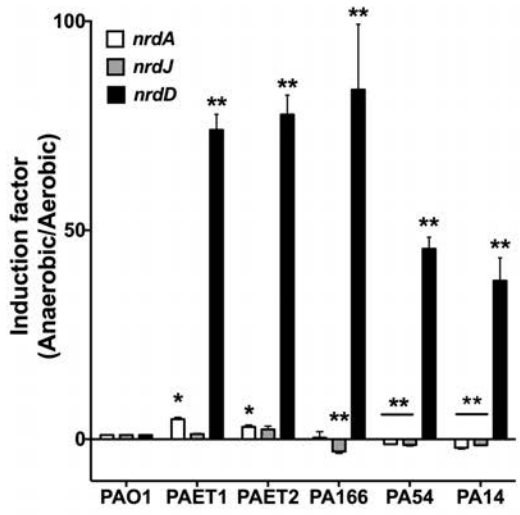


Figure 3: *nrdD* promoter variations in different *P. aeruginosa* strains. (**a**) Multiple alignment of the *nrdD* promoter region sequences from different *P. aeruginosa* PAO1 strains (PAO1-CECT, PAO1-UW, PAO1-JPN) and from strains isolated from patients with CF (PAET) and with acute infections (extensively drug resistant; XDR). *P. aeruginosa* PAO1-PAdb, PA7, PA14 and PA-LESB58 sequences, along with *P. fluorescens* and *P. alcaligenes* sequences, were obtained from the *Pseudomonas* database. The gray background indicates a mismatch in the sequence. Twenty nucleotides of P*nrdD*, corresponding to the Anr-Dnr binding box that predicted the different *Pseudomonas* strains, are magnified. The percentage conservation is indicated in the bars. (**b**) Relative fluorescence units of *nrdD* promoter activity (pETS136-C (P*nrdD* of PAO1) or pETS196-T (P*nrdD* C>T)) in *P. aeruginosa* PAO1, PAET1, PAET2, PA166, PA54 and PA14 strains. *P. aeruginosa* PAO1 isogenic Δ*anr* and Δ*dnr* mutants were used as controls for Anr/Dnr binding. A plasmid carrying an extra copy of the *dnr* gene (pETS195) was used to complement the Δ*dnr* mutation. Three independent experiments were performed, and the mean ± standard deviation is shown. *, values for pETS196-T (P*nrdD* C>T) significantly differ from those for pETS136 (P*nrdD* of PAO1) in an unpaired t-test ($P<0.05$).

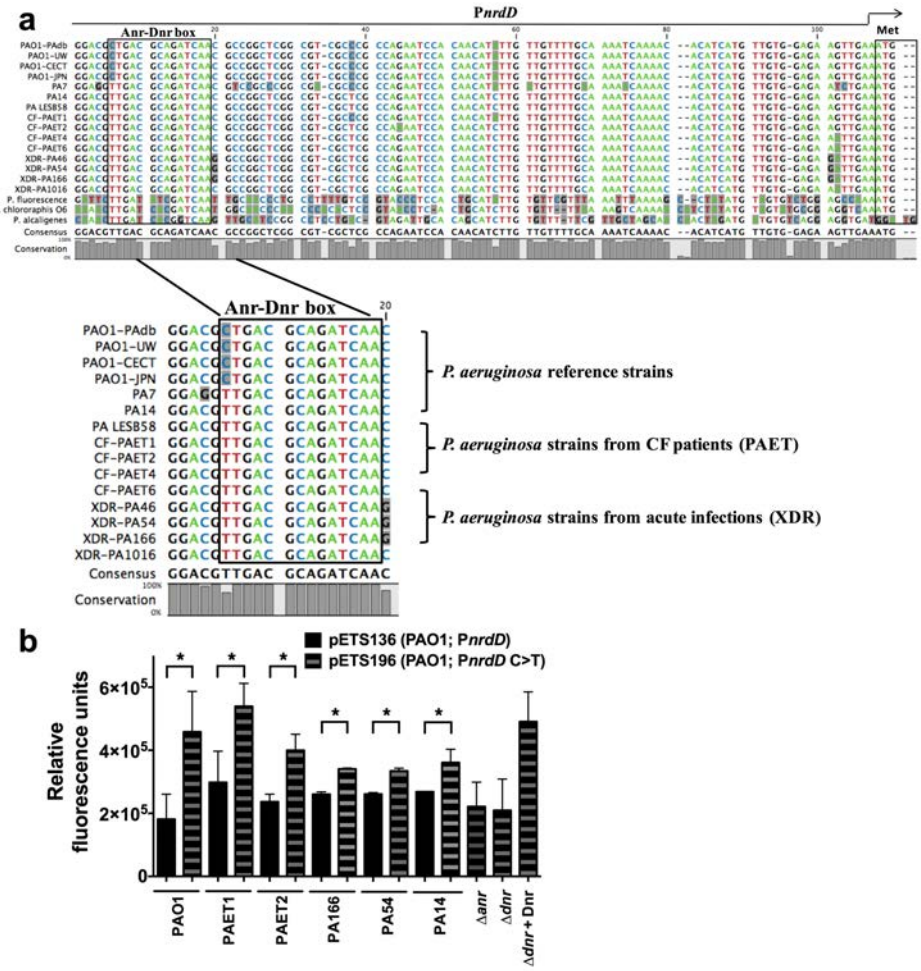


Figure 4: Role of NrdDG in infection. Mean fluorescence intensity values (sum of intensity/area of measurement) in individual embryos infected with (**a**) *P. aeruginosa* PAO1 containing the pETS130, pETS134 (*PnrdA*), pETS180 (*PnrdJ*), or pETS136 (*PnrdD*) vectors or (**b**) with different *P. aeruginosa* strains (PAO1, PA14 and PAET1) containing the pETS136-C and pETS196-T vectors over 24 h post-infection (hpi). The data represent three independent experiments, with 100 fish analyzed per strain. Statistics were performed to compare strains carrying pETS196-T with strains carrying pETS136-C in an unpaired *t*-test (*, $P<0.05$ and **, $P<0.001$). (**c**) Fluorescent and overlaid images of *D. rerio* embryos infected with PAO1, PA14 and PAET1 containing the pETS136-C or pETS196-T fluorescent reporter vectors at 16 hpi. Fluorescence was visualized with a fluorescence microscope (Leica MZ16F), quantified with Nikon Nis-element software and processed with ImageJ software. Bars represent 500 µm. (**d**) Kaplan-Meier plots of a survival experiment in *D. rerio* infected with different *P. aeruginosa* strains (*P. aeruginosa* PAO1, PA14, ETS103 (Δ*nrdD*), ETS127 (Δ*nrdD* PAO1 NrdDG^{+}), ETS128 (Δ*nrdD* PAO1 NrdDG (C>T)$^{+}$), ETS129 (PAO1 NrdDG^{+}) and ETS130 (PAO1 NrdDG (C>T)$^{+}$)). The graph corresponds to a single representative experiment from a total of three independent experiments performed (each using 100 fish per condition). The number of hours post-infection (hpi) at which 50% of zebrafish survived are listed with standard deviation. Statistics were performed to compare different strains to *P. aeruginosa* PAO1 in a Mantel-Cox test. *$P<0.05$, **$P>0.001$ and ****$P<0.0001$; N.S., no significant difference).

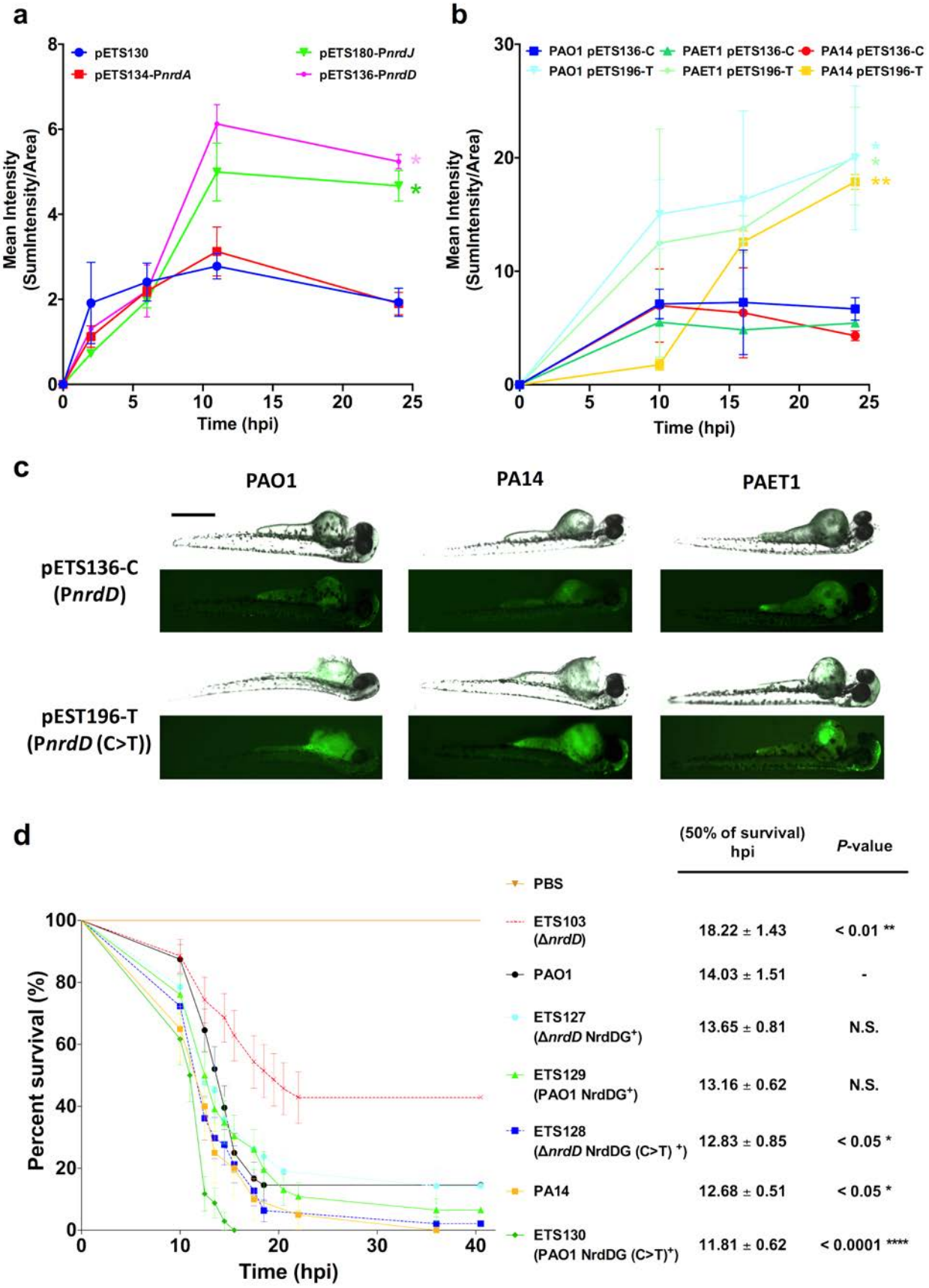

a
pETS130
pETS134-PnrdA
pETS180-PnrdJ
pETS136-PnrdD
Mean Intensity (SumIntensity/Area)
Time (hpi)
b
PAO1 pETS136-C
PAET1 pETS136-C
PA14 pETS136-C
PAO1 pETS196-T
PAET1 pETS196-T
PA14 pETS196-T
Mean Intensity (SumIntensity/Area)
Time (hpi)
c
PAO1
PA14
PAET1
pETS136-C (PnrdD)
pEST196-T (PnrdD (C>T))
d
Percent survival (%)
Time (hpi)
PBS
ETS103 (ΔnrdD)
PAO1
ETS127 (ΔnrdD NrdDG+)
ETS129 (PAO1 NrdDG+)
ETS128 (ΔnrdD NrdDG (C>T) +)
PA14
ETS130 (PAO1 NrdDG (C>T)+)
(50% of survival) hpi
P-value
18.22 ± 1.43
< 0.01 **
14.03 ± 1.51
-
13.65 ± 0.81
N.S.
13.16 ± 0.62
N.S.
12.83 ± 0.85
< 0.05 *
12.68 ± 0.51
< 0.05 *
11.81 ± 0.62
< 0.0001 ****

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Figure S1: NrdD expression analysis in different *P. aeruginosa* PAO1 strains; microscopic examination of reporter expression and cell morphology. A) Relative *nrd* expression in PAO1, ETS129 (PAO1 NrdDG^{+}), ETS130 (NrdDG (C>T)$^{+}$), ETS127 (Δ*nrdD* PAO1 NrdDG^{+}), ETS128 (Δ*nrdD* PAO1 NrdDG (C>T)$^{+}$) and ETS203 (Δ*nrdD* PAO1) strains as assessed by qRT-PCR. Three independent experiments were performed, and the mean ± standard deviation is shown. **B)** Fluorescence micrographs of an NrdD reporter in *P. aeruginosa* PAO1 strains under anaerobic conditions (at 16 h) using a fluorescence microscope (Leica MZ16F). Cell lengths of wild-type PAO1 (23 µm), ETS127 (Δ*nrdD* PAO1 NrdDG^{+}; 1.6 µm), ETS128 (Δ*nrdD* PAO1 NrdDG (C>T)$^{+}$; 1.4 µm), ETS129 (PAO1 NrdDG^{+}; 1.2 µm), ETS130 (NrdDG (C>T)$^{+}$; 1.3 µm) and ETS203 (Δ*nrdD*; 11 µm)) were measured with ImageJ software. Bars represent 20 µm. The images are representative of three independent experiments.

a)

	Log-fold change		
	nrdA	***nrdJ***	***nrdD***
ETS103 vs PAO1	3.47 ± 0.58	2.64 ± 0.60	-83.96 ± 22.20
ETS127 vs PAO1	-2.23 ± 0.65	-32.18 ± 4.70	1.03 ± 0.20
ETS128 vs PAO1	-1.09 ± 0.25	-10.93 ± 3.40	1.41 ± 0.11
ETS129 vs PAO1	-2.18 ± 0.11	-20.81 ± 3.65	2.01 ± 0.29
ETS130 vs PAO1	-1.33 ± 0.32	-19.01 ± 5.51	2.22 ± 0.16

b)

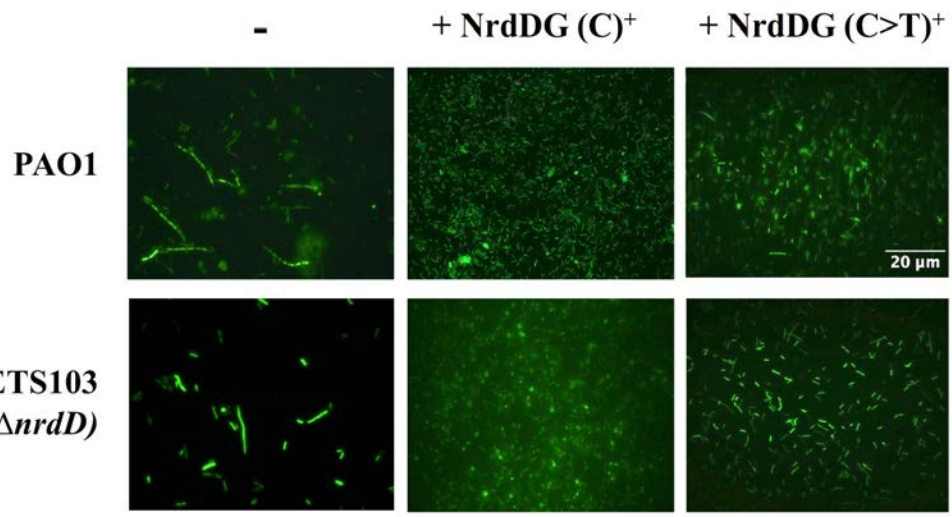

Figure S2: NrdDG during biofilm formation. (**a**) Relative fluorescence of *nrdD* expression from pETS136-C (P*nrdD* of PAO1) and pETS196-T (P*nrdD* (C>T)) after 4 days of PAO1 and PAET1 biofilm growth under aerobic or anaerobic conditions. *, significantly different values for pETS196-T (P*nrdD* C>T) and pETS136 (P*nrdD* of PAO1) in an unpaired t-test ($p<0.05$). (**b**) Relative expression of the *nrdJ* and *nrdD* genes at 4 days of *P. aeruginosa* PAO1, PAET1, PAET2, PA14 or PA1016 biofilm formation compared with planktonic expression by qRT-PCR. The *gapA* gene was used as an internal standard. Three independent experiments were performed, and data with standard deviations were plotted. *, significantly different values for *nrdJ* and *nrdD* expression in an unpaired t-test ($p<0.05$).

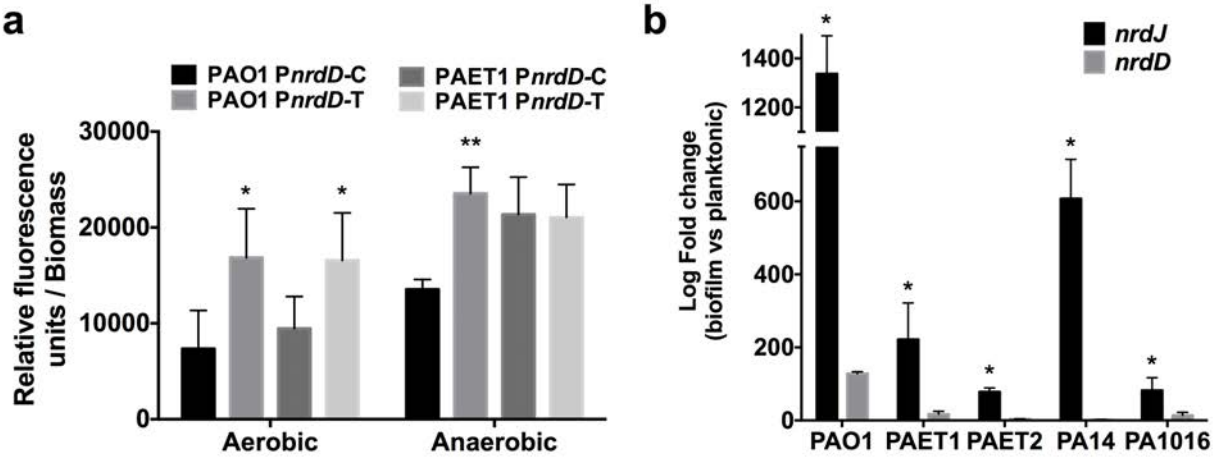

Table S1: Plasmids and strains used in this study.

Strain or plasmid	Description	Source
Plasmids		
pBAM-Gm	pBAM derivative carrying Gm^R	This work
pETS130-GFP	Broad host range, promoterless GFP, Gm^R	12
pETS134	pETS130 derivative carrying *nrdA* promoter, Gm^R	12
pETS136	pETS130 derivative carrying *nrdD* promoter, Gm^R	12
pETS159	pBBR1 derivative carrying *nrdJab* operon, Gm^R	12
pETS160	pBBR1 derivative carrying *nrdDG* operon, Gm^R	12
pETS180	pETS130 derivative carrying *nrdJ* promoter, Gm^R	15
pETS195	pUCP20T derivative carrying *dnr* gene, Ap^R	14
pETS196	pETS130 derivative carrying *nrdD* promoter (C>T) , Gm^R	This work
pETS197	pUCP20T derivative carrying *nrdDG* gene, Ap^R	This work
pETS199	pBAM-Gm derivative carrying promoter (C) and *nrdDG* genes, Gm^R	This work
pETS200	pBAM-Gm derivative carrying promoter (C>T) and *nrdDG* genes, Gm^R	This work
pJET1.2/blunt	*Blunt-end vector,* Ap^R	*Thermo Scientific*
pUCP20T	Broad-host-range vector, Ap^R	42
Strains		
E. coli		
DH5α	*recA1 endA1 hsdR17 supE44 thi-1 relA1 Δ(lacZYA-argF)U169 deoR Φ80dlacZM15*	Laboratory stock
S17.1 λ*pir*	*recA thi pro hsdR- M+RP4::2-Tc::Mu::Km Tn7 Tpr Smr Xpir*	43
P. aeruginosa		
PAO1-CECT	Wild-type (CECT 4122 / ATCC 15692)-Spanish Type Culture Collection	Laboratory stock
PAO1-JPN	Wild-type (ATCC 15692)- Nobuhiki Nomura laboratory	44
PAO1-UW	Wild-type (ATCC 15692)- Colin Manoil laboratory	45
PA14	Wild-type *P. aeruginosa* PA14	Laboratory stock
PAET1	CF strain isolated from recurrent infection in chronic patient	Laboratory stock
PAET2	CF strain isolated from recurrent infection in chronic patient	Laboratory stock
PAET4	CF strain isolated from recurrent infection in chronic patient	Laboratory stock
PAET6	CF strain isolated from recurrent infection in chronic patient	Laboratory stock
PA1016 (ST175)	Extensively drug resistant strain (XDR)- J. Gavaldà Lab (VHIR)	46
PA166 (ST111)	Extensively drug resistant strain (XDR)- J. Gavaldà Lab (VHIR)	46
PA54 (ST111)	Extensively drug resistant strain (XDR)- J. Gavaldà Lab (VHIR)	46
PW3784 (Δ*anr*)	*P. aeruginosa* PAO1 *anr*::*ISlacZ*/hah, Tc^R	45
PW1965 (Δ*dnr*)	*P. aeruginosa* PAO1 *dnr*::*ISlacZ*/hah, Tc^R	45
ETS103 (Δ*nrdD*)	*P. aeruginosa* PAO1 *nrdD*::ΩTc; Tc^R	12
ETS102 (Δ*nrdJ*)	*P. aeruginosa* PAO1 *nrdJ*::ΩTc; Tc^R	12
ETS125 (Δ*nrdJ*Δ*nrdD*)	*P. aeruginosa* PAO1 *nrdD*::ΩTc; Tc^R *nrdJ*::ΩGm; Gm^R	14
ETS127 (Δ*nrdD* PAO1+NrdDG$^+$)	*P. aeruginosa* PAO1 *nrdD*::ΩTc,*nrdDG*$^+$ merodiploid,Gm^R Tc^R	This work
ETS128 (Δ*nrdD* PAO1+NrdDG (C>T)$^+$)	*P. aeruginosa* PAO1 *nrdD*::ΩTc; Tc^R,*nrdDG (C>T)*$^+$ merodiploid, Gm^R Tc^R	This work
ETS129 (PAO1+NrdDG$^+$)	*P. aeruginosa* PAO1 *nrdDG*$^+$ merodiploid, Gm^R	This work
ETS130 (PAO1+NrdDG (C>T)$^+$)	*P. aeruginosa* PAO1 *nrdDG (C>T)*$^+$ merodiploid, Gm^R	This work

Table S2. **Primers and probes used in this study.**

Name	**Sequence (5'→3')**	**Application**
greenQRT_PAO-gapA_rv	GGTCATCAGGCCGTGCTC	qRT-PCR
greenQRT_PAO-gapA_fw	CCTCCCATCGGATCGTCTC	qRT-PCR
gapTaqM-low	GAGGTTCTGGTCGTTGGT	cDNA
greenQRT_PAO-nrdA_rv	TGTGGATGAAGTAGCGGTCG	qRT-PCR
greenQRT_PAO-nrdA_fw	ACCTGGAGAAACTGGGCAAG	qRT-PCR
nrdATaqM2-low	TGTTCATGTCGTGGGTACG	cDNA
greenQRT_PAO-nrdD_rv	GGGTGATGTTGTAGGTCGGG	qRT-PCR
greenQRT_PAO-nrdD_fw	AGATGGACCTGATCAACCGC	qRT-PCR
nrdDTaqM2-low	CCGAGTTGAGGAAGTTCTGG	cDNA
greenQRT_PAO-nrdJ_rv	TCCACCGCCTGCATGAAC	qRT-PCR
greenQRT_PAO-nrdJ_fw	CGAATTCATCCGCGCCAAG	qRT-PCR
nrdJTaqM2-low	GTAAACACCCGCACCACTTC	cDNA
pBAM-low	GGAACACTTAACGGCTGACAT	Cloning
pBAM-up	ACGAACCGAACAGGCTTATG	Cloning
PD-Dnr-T low	GATCTGCGTCAACGTCCGGC	Cloning
PD-Dnr-T up	GCCGGACGTTGACGCAGATC	Cloning
PfuIIDG-low-BamHI	AAGGATCCTGAGTCTTGTGAAGGACAGGCC	Cloning
PfuIIDGSacI-up	AAGAGCTCTGGACAACTACGTCGTCTTCGC	Cloning
pJET-rev	AAGAACATCGATTTTCCATGGCAG	Check-Cloning
pJET-up	CGACTCACTATAGGGAGAGCGGC	Check-Cloning
PnrdD new-low	AATCGATCAGGGTGGCCGGCCAGGTAG	Cloning
PnrdD-up	AGGATCCGAATTCGCCCGCCTCGCCCAGG	Cloning
PnrdD3-up	TGCTCGAACGCTTCCCGGCGGC	Sequencing
pUCP20T-low	TCCGGCTCGTATGTTGTGTG	Cloning
pUCP20T-up	CCTCTTCGCTATTACGCCAG	Cloning

Article 5

Aerobic vitamin B_{12} biosynthesis is essential for Pseudomonas aeruginosa class II ribonucleotide reductase activity during planktonic and biofilm growth

Aerobic vitamin B_{12} biosynthesis is essential for *Pseudomonas aeruginosa* class II ribonucleotide reductase activity during planktonic and biofilm growth

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Running title: Class II RNR activity depends on vitamin B_{12} availability.

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Keywords: Vitamin B_{12}, Ribonucleotide Reductases; *Pseudomonas aeruginosa*; NrdJ; bacterial growth; biofilm.

ABSTRACT

P. aeruginosa is one of the major pathogen bacteria involved in chronic infections, and it is a model organism used to study biofilms. *P. aeruginosa* is considereted an aerobic bacterium, but in the presence of nitrate, it is also able to grow in anaerobic conditions. The oxygen diffusion through the biofilm generates metabolic and genetic diversity in *P. aeruginosa* growth, such as, in the ribonucleotide reductases activity. These essential enzymes are necessary for DNA synthesis and repair. Oxygen availability determines the activity of the three ribonucleotide reductases classes. Class II and III RNRs are active in the absence of oxygen. However, class II RNR, which is important in *P. aeruginosa* biofilm growth, require the vitamin B_{12} cofactor for their enzymatic activity.

Therefore, in this work, we elucidated the conditions in which class II RNR is active due to the synthesis of vitamin B_{12}. We have demonstrated an increase of vitamin B_{12} levels during biofilm growth that activates class II RNR activity. Our results unravel the *P. aeruginosa* mechanisms for dNTPs synthesis during biofilm growth.

INTRODUCTION

P. aeruginosa is an opportunistic pathogen causing severe chronic infections in immunocompromised patients and other risk groups, like cystic fibrosis (CF) or chronic obstructive pulmonary disease (COPD) patients. The key to the survival of *P. aeruginosa* in environments ranging from soil to various living host organisms is its metabolic versatility. It can subsist on a variety of different carbon sources for energy, can utilize nitrogen as a terminal electron acceptor to respire under anaerobic conditions, has minimal nutrient requirements, and grows at temperatures of up to 42°C. *P. aeruginosa* uses the anaerobic metabolism to reduce nitrogen (N_2) via the denitrification process [1, 2], as an essential metabolic condition during chronic infection and biofilm growth [3-5].

During *P. aeruginosa* infections, bacteria need to multiply inside the body, requiring active DNA synthesis for bacterial cell division. Ribonucleotide reductase (RNR) enzymes provide all living organisms with the deoxyribonucleotides triphosphates (dNTP) supplying the monomers for DNA synthesis. There are three different RNR classes (class I, subdivided in Ia, Ib and Ic; class II and class III) that differ in their overall protein structure and cofactor requirement but have in common an allosteric regulation and the use of an organic radical to initiate catalysis through free radical chemistry [6, 7]. *P. aeruginosa* is one of the few organisms that encode for the three different RNR classes; the oxygen dependent class Ia (encoded by the *nrdAB* gene), the oxygen independent class II (encoded by the *nrdJab* genes) and the oxygen sensitive class III (encoded by

the *nrdDG* genes). Specifically, class II RNR activity depends on an external cofactor, the adenosylcobalamin (AdoCob) or vitamin B_{12}, for the generation of its radical independently of oxygen to reduce the different ribonucleotides to their corresponding deoxyribonucleotides.

Vitamin B_{12} is one of the most structurally complex cofactors synthesised by bacteria [8]. However, not all microorganisms encode for the around 25 genes needed for its complete biosynthesis pathway. In nature, there exists two vitamin B_{12} biosynthesis pathways: the aerobic or the late cobalt insertion pathway and the anaerobic or early cobalt insertion pathway [8]. One of the genes involved in the aerobic pathway which participates in the cobalt insertion is the *cobN* gene described extensively in *Pseudomonas denitrificans* [8]. On the other hand, the most studied anaerobic biosynthetic pathway involved in the early cobalt insertion was described in *Salmonella typhymurium* [9].

P. aeruginosa PAO1 grows in a filament cell morphology under anaerobic conditions due to low expression levels of class III RNR [10,11] and the high nitric oxide levels, one of the intermediates in the denitrification process that interacts with a cobalamin precursor [12-14]. Therefore, cell filamentation is the result of DNA replication impairment that affects *P. aeruginosa* cell division thus affecting infection [11,15] and biofilm growth [5,15]. Class II and III RNRs enzymes perform their activity under anaerobic conditions and also during biofilm growth conditions [5]. In *P. aeruginosa* class II RNR (NrdJab) activity is oxygen independent, but it is strictly dependent on vitamin B_{12} availability. But nowadays, the link is unknown between internal vitamin B_{12} biosynthesis or availability from the environment and the real class II RNR activity important for many essential physiological growth conditions for *P. aeruginosa* such as during anaerobic growth or during biofilm formation [6,16].

In this work, we analysed *P. aeruginosa* vitamin B_{12} biosynthesis during aerobic and anaerobic growth as well as during biofilm formation. We also determined the relation between the vitamin B_{12} biosynthesis and the activity of class II RNR activity under different growing conditions.

MATERIAL AND METHODS

Bacterial strains and growth conditions

P. aeruginosa and *Escherichia coli* strains, listed in Table S1, were grown in Luria-Bertani broth (LB) or Minimum Medium (MM) [17] at 37ºC. MM containing 1% KNO_3 (MMN) was used for anaerobic liquid growth in screw-cap tubes (Hungate tubes) [5, 18] or in anaerobic plates using the GENbag system (bioMérieux) according to the manufacturer's instructions.

The medium was supplemented, when necessary, with the following antibiotics: 100 μg/ml or 50 μg/mL gentamycin, 300 μg/ml carbenicillin and 40 μg/ml tetracycline for *P. aeruginosa;* 10 μg/ml gentamycin and 50 μg/ml ampicillin for *E. coli.*

Construction of cobN deletion mutant strain

The *P. aeruginosa* PAO1 with a mutation in the *cobN* gene (ETS126; Δ*cobN*) was constructed by an insertion of the gentamicin-resistance gene (*aacC1*) into the *cobN* gene by homologous recombination using the pEX18Tc vector, as previously was described [19]. Briefly, two 400 bp areas surrounding the *P. aeruginosa* PAO1 *cobN* gene were amplified by PCR (High-Fidelity PCR Enzyme Mix (Thermo Scientific)) with the following primer pairs: CobN1HIII-up/CobN2BI-low and CobNBI-up/CobN4SI-low, listed in Table S2. The two amplicons obtained were cloned separately into the pJET1.2 vector (Thermo Scientific). A plasmid containing both fragments was generated by *BamHI/SacI* digestion. The gentamicin resistance gene *aacC1* was obtained using *Bam*HI digestion of pUCGmlox, and the corresponding cassette was ligated to the two fragments. The construct was cloned into the *sac*B gene-based counter-selection pEX18Tc vector and transferred into the S17.1λ*pir* strain for *P. aeruginosa* PAO1 conjugation as previously described [5]. Transconjugants were selected by plating them with tetracycline, gentamycin and sucrose (5%), used for *sacB*-mediated plasmid counter selection. The insertion of *aacC1* was screened and verified by PCR with the primer pair CobN1HIII-up/CobN-5-low and later confirmed by DNA sequencing.

Quantitative Real-Time PCR (qRT-PCR)

Transcripts of RNR genes (*nrdA, nrdJ* and *nrdD)* were quantified using quantitative Real-Time PCR (qRT-PCR). *P. aeruginosa* was grown in planktonic conditions at the mid-exponential phase of growth in which samples were treated with RNAprotect Bacterial Reagent (Qiagen). RNeasy Mini Kit (Qiagen) was used for RNA total isolation and purification with extra DNA removal using DNase I (Turbo DNA-free, Applied Biosystems) according to the manufacturer's instructions. DNA contaminations were verified by PCR. cDNA was synthetized using 0.5 μg RNA with SuperScript III Reverse Transcriptase (Thermo Scientific). The primers used are listed in Table S2 [5]. RNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific). *gapA* gene was used to normalise the transcript levels of genes.

Green fluorescent protein gene reporter assay

GFP fluorescence expressed in plasmids pETS134 (P*nrdA*), pETS180 (P*nrdJ*) and pETS136 (P*nrdD*) was measured for determining the promoter activity of each RNR gene. *P. aeruginosa* containing the *nrd* promoter fusion was grown until exponential phase, and three independent 1 ml samples were analysed. Cells were fixed with 1 ml of freshly prepared PBS 1x solution containing 2% formaldehyde (Sigma) and stored in the dark at 4ºC. GFP fluorescence was measured in a 96-well plate (Costar® 96-Well Black Polystyrene Plate, Corning) on an Infinite 200 Pro Fluorescence Microplate Reader (Tecan), as previously described [20]. Three measurements were performed for each independent sample.

Continuous-flow biofilm formation

Continuous-flow cell biofilms were grown in MM + 0.2% glucose and performed as previously described [5, 21]. These *in vitro* formed biofilms are more natural-like mature biofilm with clear oxygen concentration stratification [22]. Briefly, biofilms were grown in a three-channel flow cell with a constant flow rate of 42 µl per minute for each channel using an Ismatec ISM 943 pump (Ismatec). After five days of growth, biofilms were stained with LIVE/DEAD BacLight Bacterial Viability Kit (Thermofisher) and visualised by a Confocal Scanning Laser Microscope (CSLM) (Leica TCS SP5, Leica Microsystems, Wetzlar Germany). Images were generated using ImageJ Fiji software, and the COMSTAT 2 software was used for biomass and thickness biofilm quantification.

Vitamin B_{12} quantification by HPLC-MS

P. aeruginosa PAO1, PAET1 and PA14 strains were grown in MM or MMN medium for 20 h, in aerobic or anaerobic conditions, or for five days in a continuous-flow cell biofilm growth system. Cells were lysed using lysozyme (50 mg/ml) (Sigma) and sonicated in ice five times using 6 mm sonication probe at 32% power for 20 sec. After centrifugation (4000 x g at 4ºC), the supernatants were filtrated with a 10 kDa Centricon column (Millipore). Samples were manipulated in the dark to avoid vitamin B_{12} degradation. Finally, 1 % ammonium formate was added to each sample before HPLC-MS quantification. Samples of 10 µl were injected into the Luna 5 µm C18 100 Å (150x2 mm) column for HPLC-MS (4000 QTRAP (AB SCIEX) in an Aligent 1200 Series) at the Separation Techniques platform of Scientific Center Services of the Scientific Park of Barcelona (PCB). A calibration curve was done for vitamin B_{12} measured in the range of 0.1-100 ng/ml. Values were normalised with the concentration of protein measured with a Bradford assay (Bio-Rad).

RESULTS and DISCUSSION

Active vitamin B_{12} biosynthesis is essential for class II RNR activity

Vitamin B_{12} or adenosylcobalamin (AdoCob) acts as a radical generator for class II RNR activity, but the link between vitamin B_{12} biosynthesis and class II RNR enzyme (NrdJ) activity is not understood, and further investigations are required.

We analysed, first, the essentiality and role of the class II RNR (NrdJ) enzyme under aerobic and anaerobic conditions depending on vitamin B_{12} availability. We used diverse *P. aeruginosa* PAO1 strains deficient for different RNR classes (ETS102, Δ*nrdJ*; ETS103, Δ*nrdD* and ETS125, Δ*nrdJ*Δ*nrdD*). Also, we used a mutant strain for the vitamin B_{12} biosynthesis pathway involved in the cobalt insertion under aerobic conditions (ETS126, Δ*cobN*). As the *nrdA* mutation is not viable, we added 30 mM hydroxyurea (HU) to inhibit its activity and mimic a *nrdA* mutant strain. Aerobically, class Ia RNR inhibition by HU decreased *P. aeruginosa* PAO1 wild-type growth in minimal medium, also previously described [16, 23], but after 48 h aerobic incubation, there was some growth (see the undiluted sample 0 with HU) (Fig. 1A). However, *P. aeruginosa* Δ*nrdJ*, Δ*nrdJ*Δ*nrdD* and Δ*cobN* strains treated with HU did not grow, even after more than 48 h of incubation. Nevertheless, the addition of vitamin B_{12} into the minimal medium containing 30 mM HU, re-establishes the optimal aerobic growth in the strains encoding an active class II RNR (NrdJ) enzyme (*P. aeruginosa* PAO1 wild-type; Δ*cobN*, ETS126 and Δ*nrdD*, ETS103 strains). Therefore, vitamin B_{12} availability supports class II RNR activity when we inhibit class Ia RNR.

Under anaerobic conditions, class II and III RNR inactivation (Δ*nrdJ*, Δ*nrdD,* and Δ*nrdJ*Δ*nrdD*) displayed slightly lower growth compared to the *P. aeruginosa* PAO1 wild-type and Δ*cobN* deficient strains (Fig. 1A). The growth of the Δ*cobN* mutant strain was not affected anaerobically (undiluted sample, 0). This result suggested that *cobN* gene was not involved in *P. aeruginosa* vitamin B_{12} biosynthesis under anaerobic conditions. Thus, *P. aeruginosa* PAO1 strain cannot sustain an adequate growth in anaerobic conditions unless increasing RNR activity by adding vitamin B_{12} to the medium (1 μg/ml) or increasing class III RNR gene copy number (+NrdDG) as it was previously seen [11].

Therefore, we demonstrated that NrdJ is active in both aerobic and anaerobic conditions if vitamin B_{12} is present in the medium. However, class Ia and III RNR supplies the dNTPs required for aerobic [15] or anaerobic [11] bacterial DNA replication. Lack of class Ia and III RNR activity, due to class Ia RNR activity inhibition by HU or by the low *nrdD* expression levels, causes cell filamentation growth in *P. aeruginosa* PAO1 strain [11, 15] increasing its *nrd* expression (Fig. S1). But, the addition of vitamin B_{12} decreases the expression of the three *nrd* genes (Fig. S1), independently of B_{12}-riboswitch regulation [24], and returned its cellular morphology to rod-

shaped, by restoring their DNA replication impairment [10]. Other vitamin B_{12} dependent enzymes (methionine, cobalamin biosynthesis and some ribonucleotide reductase from other microorganisms) are usually regulated via a B_{12}-riboswitch on their promoter regions [24, 25].

P. aeruginosa PAO1 cells treated with HU for two hours in minimal medium cause filamentous morphology under aerobic conditions, however, at 24 h of post-HU treatment (at late stationary phase), *P. aeruginosa* PAO1 cells return to their rod-shaped morphology without the addition of external vitamin B_{12} (Fig 1B). The *cobN* gene mutation in the *P. aeruginosa* PAO1 causes filamentous cells even at 24h of HU treatment due to the vitamin B_{12} biosynthesis disruption. These results highlight active vitamin B_{12} biosynthesis in *P. aeruginosa* PAO1 which specifically requires the *cobN* gene under aerobic conditions. However, vitamin B_{12} levels are not enough during the initial hours of *P. aeruginosa* PAO1 growth and probably reach optimal physiological levels after 24 h of growth.

On the other hand, under anaerobic conditions, *P. aeruginosa* PAO1 cells have filamentous morphology due to the low activity of class III RNR [11]. *P. aeruginosa* PAO1 Δ*cobN* mutant cell morphology and growth was similar to the *P. aeruginosa* PAO1 strain, suggesting no implication of this gene during anaerobiosis Fig. S2. Hence, in anaerobic conditions, *P. aeruginosa* PAO1 growth needs an external vitamin B_{12} supplement for optimal NrdJ activity because we did not observe any anaerobic vitamin B_{12} biosynthesis.

Biofilm formation depends on vitamin B_{12} synthesis.

Class II and III RNR enzymes are necessary for biofilm formation being class II RNR particularly highly expressed [5]. Today it is not known whether vitamin B_{12} is synthetized and affects class II RNR activity under biofilm conditions. Thus, we analysed different *P. aeruginosa* strains (wild-type and isogenic mutant strains for *nrdJ, nrdD* and *cobN* genes) grown in a continuous-flow cell biofilm. Fig. 2 shows that biofilm formation in minimal media, measured as total biomass and average thickness, decreases when class II and III RNR were mutated. A similar result we previously reported in biofilm cells growing in LB rich media [5]. We also observed a decrease in the *P. aeruginosa* PAO1 Δ*cobN* strain biofilm formation, at similar levels produced in any *P. aeruginosa* deficient for class II RNR (Δ*nrdJ* and Δ*nrdJ*Δ*nrdD*), compared to the *P. aeruginosa* PAO1 wild-type strain (Fig 2).

Furthermore, the biomass and thickness levels in the *P. aeruginosa* PAO1 Δ*nrdJ* and Δ*nrdJ*Δ*nrdD* mutants did not arrive at the wild-type strain levels even with the addition of vitamin $B_{12.}$ However, the thickness in the *P. aeruginosa* PAO1 Δ*nrdD* and Δ*cobN* mutant strains biofilms increase considerably. These results suggest that a supply of vitamin B_{12} enable an optimum *P.*

aeruginosa PAO1 biofilm, activating class II RNR in those biofilm layers where there is not active vitamin B_{12} biosynthesis due to oxygen concentrations strengths. As expected, cell filamentation morphology was restored with the addition of vitamin B_{12} into the continuous-flow biofilm (Fig. 2B).

Vitamin B_{12} availability during P. aeruginosa aerobic and biofilm growth

We have described here that *P. aeruginosa* needs to synthetize vitamin B_{12} during aerobic and biofilm growth, to allow class II RNR enzymatic activity. For this reason, we aim to elucidate the amount of vitamin B_{12} available for *P. aeruginosa* growth under different growing conditions, including biofilm formation.

We only detected vitamin B_{12} levels in cells grown aerobically at stationary phase (Table 1). At exponential growth, vitamin B_{12} detected was below the technique detection limit (data not shown). Surprisingly, under five-days-old continuous-flow biofilm *P. aeruginosa* PAO1 growth, cells produced a ten-fold increased in vitamin B_{12} levels compared to aerobic growth indicating activation of this biosynthetic pathway. However, we suggested that vitamin B_{12} biosynthesis in biofilm is only produced in the upper biofilm layer because we did not detect any vitamin B_{12} in cells grown anaerobically. Some studies suggested that genes of vitamin B_{12} (*cob*) aerobic synthesis are expressed during biofilm growth [26], in mucoid phenotype [27] and in stationary phase [28], with down-regulation in anoxic conditions [29].

Also, different vitamin B_{12} levels were detected in other *P. aeruginosa* strains distinct from the PAO1 laboratory strain. We identified in *P. aeruginosa* PA14 and PAET1 strains increased vitamin B_{12} levels under aerobic conditions (1.6 and 2.1 times, respectively) (Table 1) and less vitamin B_{12} levels under biofilm growth conditions compared to the *P. aeruginosa* PAO1 strain. These different vitamin B_{12} levels could affect RNR activity, but further experiments need to be done to validate this hypothesis. Previously, these strains showed different RNR activity than the *P. aeruginosa* PAO1 strain in aerobic and anaerobic conditions [11].

P. aeruginosa clinical isolates are resistant to hydroxyurea inhibition.

The increased vitamin B_{12} availability in the aerobic *P. aeruginosa* PA14 and PAET1 cells suggest higher class II RNR activity. We evaluated the growth of these strains in cells with class Ia RNR inhibited by the addition of 30 mM HU thus only growing with an active class II RNR. Hydroxyurea apparently interferes with the *P. aeruginosa* PAO1 growth arresting DNA replication [10, 15], as we can see also in the growth curve, but can be restored with the vitamin B_{12} addition (Fig. 3A). Surprisingly, the growth of *P. aeruginosa* PA14 and PAET1 strains were not disturbed

by HU treatment. Additionally, at four hours of HU treatment, *P. aeruginosa* PAO1 cells showed filamentous growth (around 10 μm). However, the HU treatment of *P. aeruginosa* PA14 and PAET1 displayed rod-shaped cell morphology (1.4 and 1.7 μm) suggesting that their DNA replication it is not impaired, although there is increased *nrd* expression (Fig. S1). This result was corroborated by cell viability under HU and HU with vitamin B_{12} treatment. *P. aeruginosa* PA14 and PAET1 grew as early as in 20 hours in the presence of HU (Fig 3B) compared with the 48 hours needed for *P. aeruginosa* PAO1 strain.

On top of that, we proposed that this resistance is due to high levels of vitamin B_{12} in aerobic conditions that activate the class II RNR enzyme. However, more experiments are needed to understand the mechanism of HU resistance in clinical isolates strains. Given this, there is ground for the belief that an administration of vitamin B_{12} could increase the *P. aeruginosa* cell division in patients with biofilm infection.

CONCLUDING REMARKS

Integrating our experiments, we have demonstrated that the synthesis of vitamin B_{12} is under *P. aeruginosa* aerobic planktonic growth conditions with an active class Ia RNR that supplies dNTPs required for DNA replication (Fig. 4A). Vitamin B_{12} cannot be synthesised under anaerobic conditions being *P. aeruginosa* cells growing with class III RNR (Fig. 4B). Class II RNR is enzymatically active when vitamin B_{12} is available through their internal biosynthesis or from an external source.

The situation of *P. aeruginosa* growing in biofilm is different and requires a deeper analysis. Oxygen diffusion through the complex biofilm structure generates an oxygen concentration gradient with an apparent cell distribution with different RNR class activity (Fig. 4C). We suggest, in the superficial biofilm layers, the cells express class Ia RNR whereas the internal layers are anaerobic thus expressing class III RNR. But, class II RNR is oxygen independent and vitamin B_{12} dependent, highly expressed during biofilm formation and anaerobic environments, so under which conditions is this RNR class enzymatically active?

We believe that the external biofilm cells, which are in contact with aerobic environments, can synthesise vitamin B_{12} and this can diffuse through the biofilm structure creating a vitamin B_{12} concentration gradient along the biofilm structure. In this sense class II RNR can be active in parts with microaerophilic conditions in which class I or class III RNR are not active (Fig. 4C).

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Figure 1: Effect of hydroxyurea and vitamin B_{12} on *P. aeruginosa* PAO1 wild-type, Δ*nrdJ*, Δ*nrdD*, Δ*nrdJ*Δ*nrdD* and Δ*cobN* strains growth. A) 5 µl drops were plated from 0, 10^{-4} and 10^{-8} dilution into a solid medium containing 30 mM hydroxyurea (HU) and 1 µg/ml vitamin B_{12} (vit B_{12}) for 48 h. Pictures are representative of three independent experiments. **B)** Fluorescence images from aerobic *P. aeruginosa* PAO1 wild-type and Δ*cobN* growth were visualised with Nikon E600 microscope (Nikon) coupled with a DP72 Olympus camera after 2 h and 24 h of HU treatment. Cells were stained with LIVE/DEAD BacLight Bacterial Viability Kit, and the ImageJ software was used to image analysing. The images were representative of at least three different experiments. Scale bars, 20 µm.

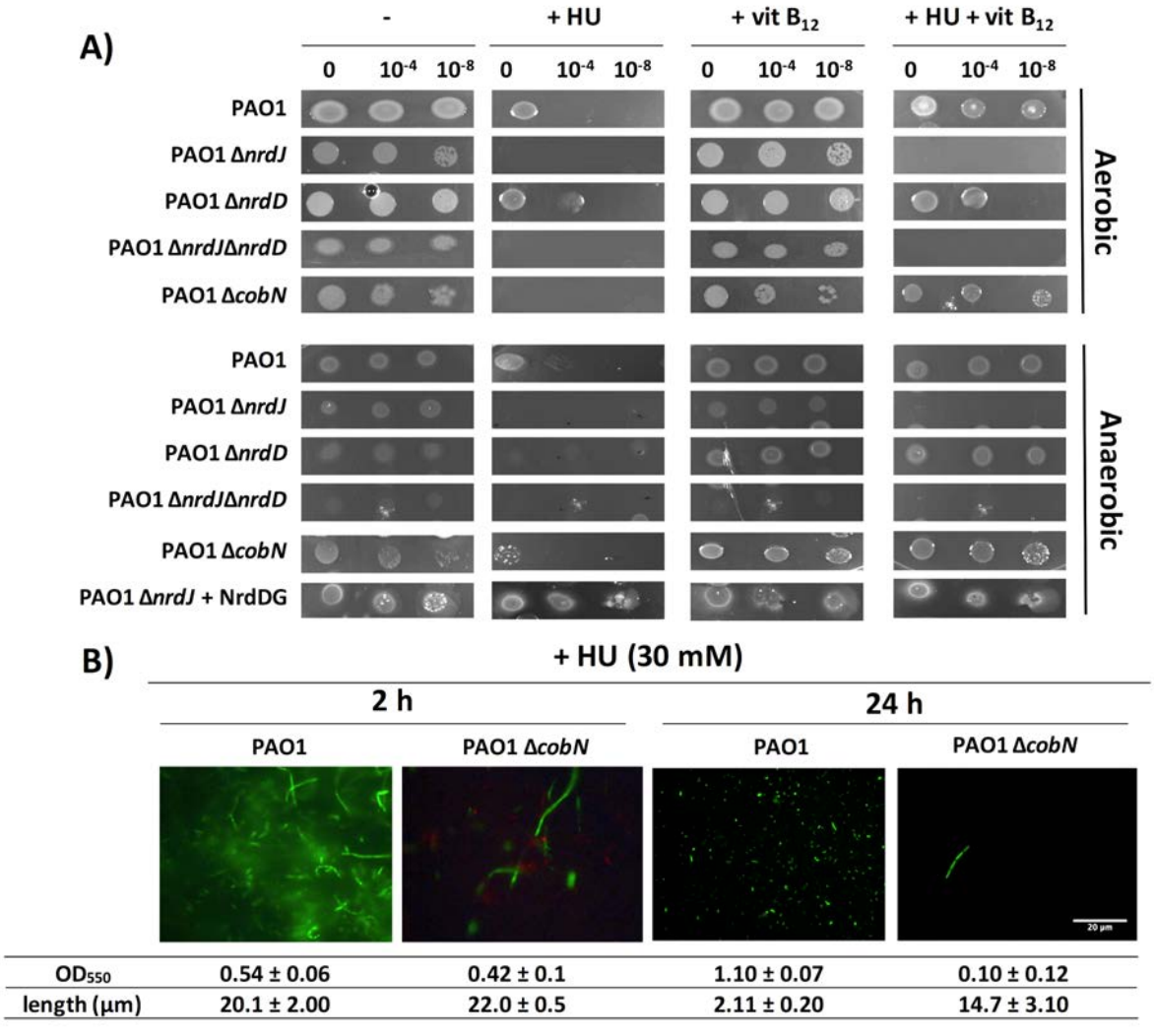


Figure 2: Vitamin B_{12} enables *P. aeruginosa* biofilm formation thought class II RNR activity. **A)** The continuous-flow cell biofilm of *P. aeruginosa* PAO1 wild-type, Δ*nrdJ,* Δ*nrdD,* Δ*nrdJ*Δ*nrdD* and Δ*cobN* strains was grown during 5 days in MM ± vitamin B_{12} (1 µg/ml). Biomass and thickness were calculated using Image J software. Data are the average of three independent experiments. Asterisks over bars (*) indicates statistically different (p<0.005 in pairwise *t*-test calculated with GraphPad 6.0). **B)** Pictures of the sum and the orthogonal views of biofilm stained with LIVE/DEAD BacLight Bacterial Viability Kit before visualisation using CLSM. Images are representative of three independent experiments. Bar represent 200 µm.

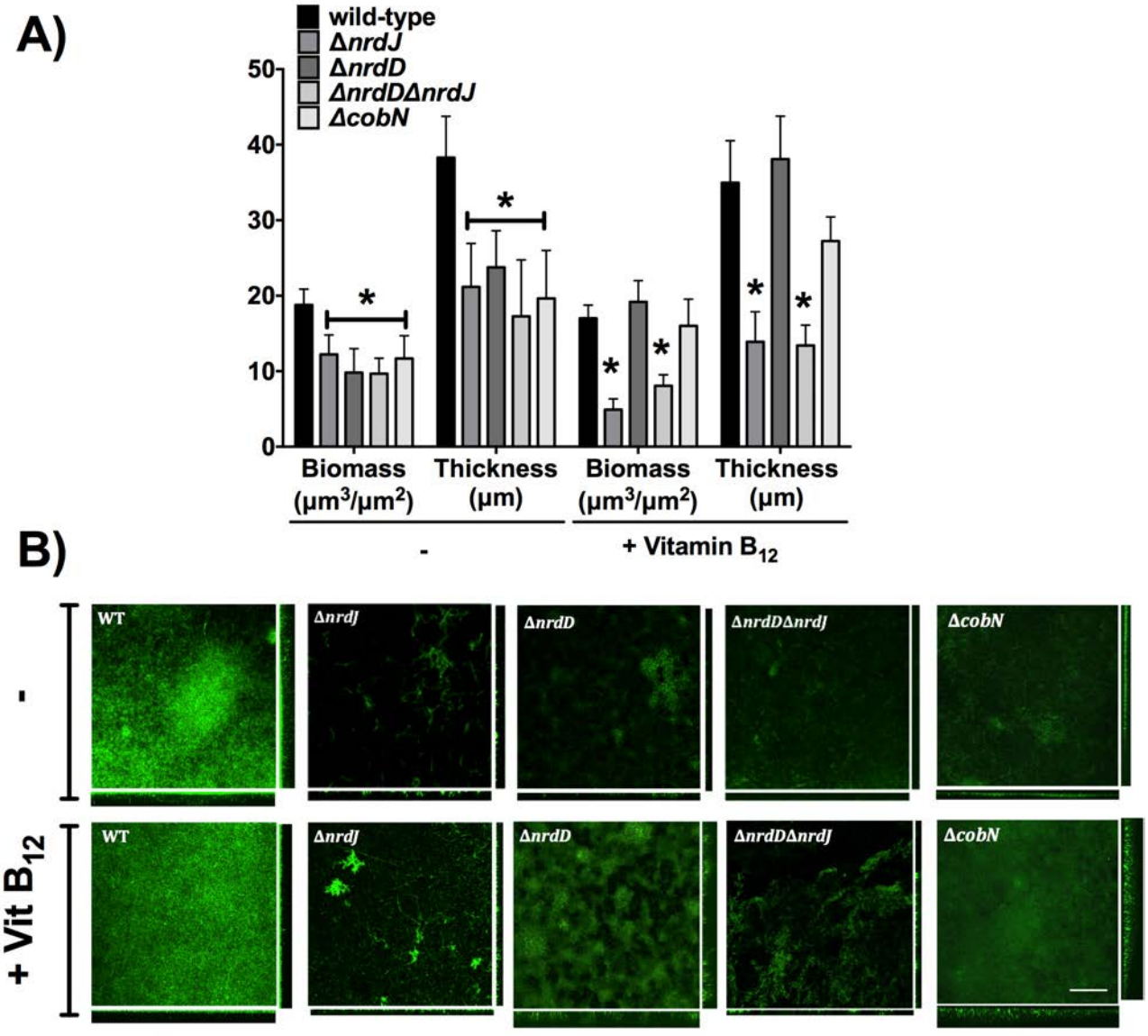


Table 1. Quantification of vitamin B_{12} levels by HPLC-MS. The growth of *P. aeruginosa* PAO1, PAET1 and PA14 strains in aerobic and anaerobic conditions during 16 hours and in biofilm formation conditions. Values were normalised by protein concentration. Two independent experiments were performed, and the mean ± standard deviation is shown.

	ng Vitamin B_{12} / mg protein		
	Aerobic	**Anaerobic**	**Biofilm**
PAO1	0.32 ± 0.05	0[1]	3.72 ± 0.01
PAET1	0.67 ± 0.06	0[1]	1.72 ± 0.05
PA14	0.51 ± 0.02	0[1]	0.84± 0.01

[1]Not-detected, below the technique detection limit.

Figure 3: *P. aeruginosa* PAET1 and PA14 strains are resistant to HU. A) Growth curve of *P. aeruginosa* PAO1, PAET1 and PA14 strains in MM containing 30 mM hydroxyurea (HU) and 1 µg/ml vitamin B_{12}. Cells were visualised at 4 hours after HU treatment. Images and values represent a representative experiment of three independent experiments ± standard deviation. Bar represent 20 µm. **B)** Aerobic *P. aeruginosa* PAO1, PAET1 and PA14 strains cell viability with HU and vitamin B_{12} at 20 or 48 hours of incubation. Pictures are representative of three independent experiments. Cultures were diluted to 0, -4 and -8 for the growth.

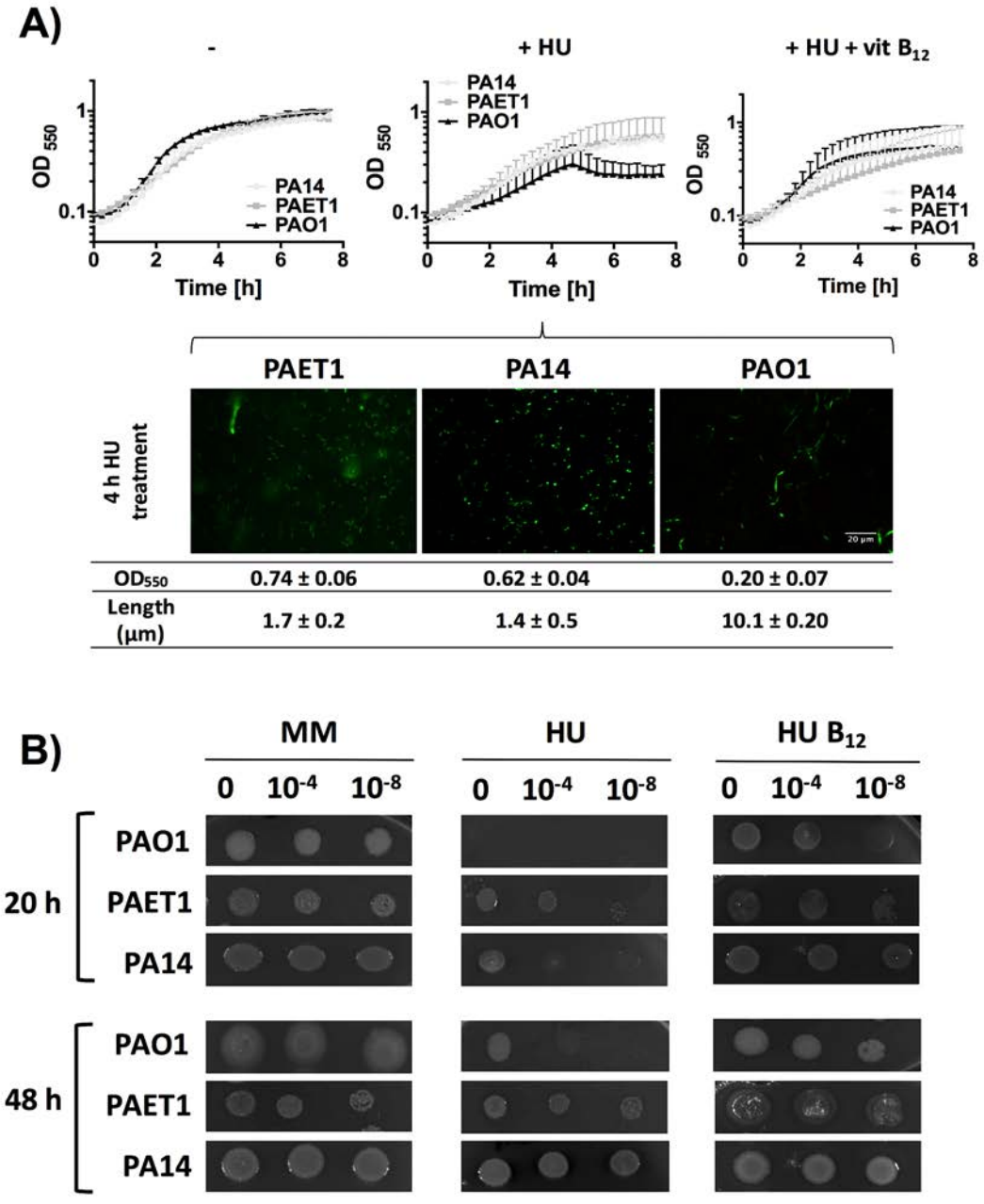
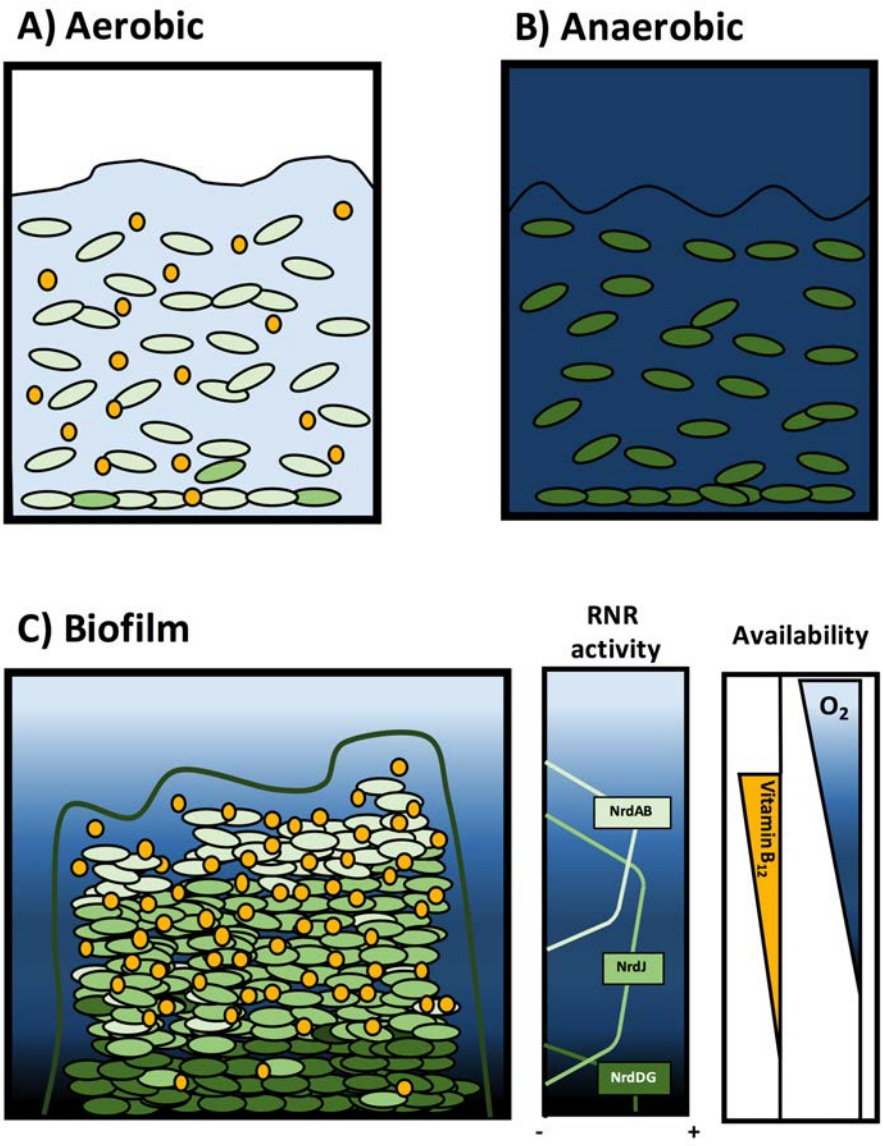


Figure 4: Model of ribonucleotide reductase activity and vitamin B_{12} availability during *P. aeruginosa* planktonic and biofilm growth. Scheme of *P. aeruginosa* growing in **A**) aerobic planktonic culture, **B**) anaerobic planktonic culture and **C**) biofilm. Orange circles represents vitamin B_{12}, blue gradient colour indicates oxygen concentration gradients and in green the different RNR activity.



SUPPLEMENTARY MATERIAL

Figure S1. Expression analysis of the different RNR classes under HU and vitamin B_{12} treatment in aerobic and anaerobic conditions. Cultures were treated in the presence of HU (30 mM) and vitamin B_{12} (1 μg/ml) during 20 min before to measure the relative fluorescence units of **(A)** *PnrdA* (pETS134), **(B)** *PnrdJ* (pETS180*) and* **(C)** *PnrdD* (pETS136). Results are the mean of three independent experiments ± standard deviation. Asterisks over bars (*) indicates statistically different (p<0.05 in pairwise *t*-test calculated with GraphPad 6.0).

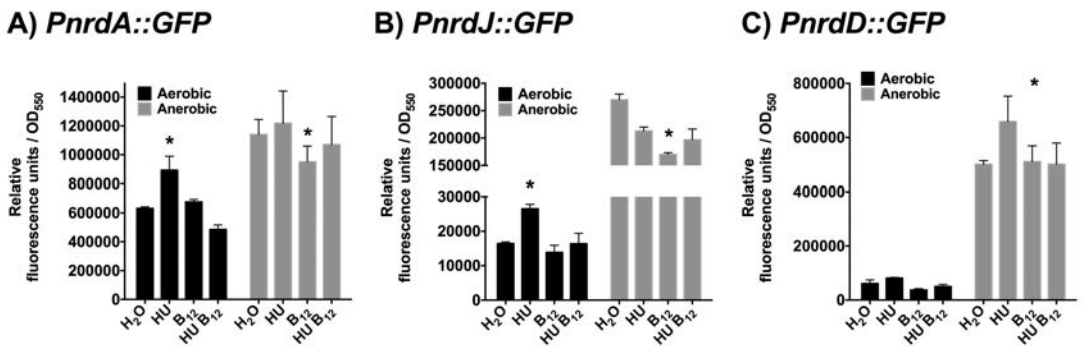
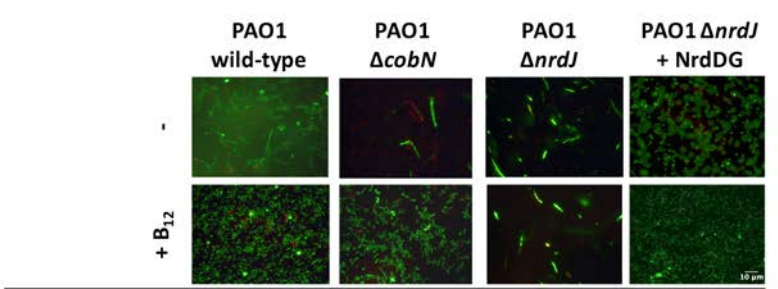


Figure S2: CobN is not involved in *P. aeruginosa* anaerobic growth. Fluorescence microscopy pictures of *P. aeruginosa* PAO1 wild-type, Δ*cobN*, Δ*nrdJ and* Δ*nrdJ* + NrdDG cells stained with Syto 9 dye at 16 h of anaerobic growth in MM ± vitamin B_{12}. The length was measured using Image J software. Scale bars, 10 μm.



		PAO1 wild-type	PAO1 Δ*cobN*	PAO1 Δ*nrdJ*	PAO1 Δ*nrdJ* + NrdDG
-	OD_{550}	1.65 ± 0.30	1.57 ± 0.1	0.20 ± 0.20	1.87 ± 0.30
	length (μm)	23.1 ± 0.18	18.9 ± 4.0	39.0 ± 0.70	2.17 ± 0.30
B_{12}	OD_{550}	1.95 ± 0.30	2.17 ± 0.2	0.30 ± 0.05	1.07 ± 0.02
	length (μm)	1.10 ± 0.18	1.90 ± 0.3	50.0 ± 3.00	1.70 ± 0.50

Fig. S3: Expression of *nrdA*, *nrdJ* and *nrdD* genes of *P. aeruginosa* PAO1 and clinical isolates (PAET1 and PA14) strains with HU. Fold-change of *nrd* genes using qRT-PCR at 20 min of treatment with 30 mM HU compared with H_2O. The *gap* gene was used as an internal standard. Two independent experiments were analysed, and the standard deviation was annotated.

	log-fold change (HU vs H_2O)		
	class Ia *nrdA*	**class Ia *nrdJ***	**class III *nrdD***
PAO1	9.19 ± 2.1	31.65 ± 3	50.46 ± 7.2
PAET1	27.74 ± 2.1	7.63 ± 1.1	16.38 ± 0.5
PA14	4.91 ± 0.8	1.18 ± 0.2	32.7 ± 0.9

Table S1: Plasmids and strains

Strain or plasmid	Description	Source
Plasmids		
pJET1.2/blunt	*Blunt-end vector, AmpR*	*Thermo*
pETS130-GFP	Broad host range, promoterless GFP, Gm^R	[15]
pUCGmlox	Ap^R, Gm^R; source of Gm^R cassette	[30]
pEX18Tc	*sac*B based counter-selection vector, Tc^R	[30]
pETS160	pBBR1 derivative carrying *nrdDG* operon, Gm^R	[15]
pETS134	pETS130 derivative carring *nrdA* promoter, Gm^R	[15]
pETS136	pETS130 derivative carring *nrdD* promoter, Gm^R	[15]
pETS180	pETS130 derivative carrying *nrdJ* promoter, Gm^R	[20]
Strains		
E. coli		
DH5α	*recA1 endA1 hsdR17 supE44 thi-1 relA1 Δ(lacZYA-argF)U169 deoR*	Lab stock
S17.1 λ*pir*	*recA thi pro hsdR- M+RP4::2-Tc::Mu::Km Tn7 Tpr Smr Xpir*	[31]
P. aeruginosa		
PAO1	Wild-type (ATCC 15692 / CECT 4122)- Spanish Type Culture	Lab
PA14	Wild-type *P. aeruginosa* PA14	Lab
PAET1	*CF strain isolated from chronic patient*	Lab
ETS103 (Δ***nrdD***)	*P. aeruginosa* PAO1 *nrdD*::ΩTc; Tc^R	[15]
ETS102 (Δ***nrdJ***)	*P. aeruginosa* PAO1 *nrdJ*::ΩTc; Tc^R	[15]
ETS125	*P. aeruginosa* PAO1 *nrdD*::ΩTc; Tc^R ,*nrdJ*::ΩGm; Gm^R	[5]
ETS126 (Δ***cobN***)	*P. aeruginosa* PAO1 *cobN*::ΩGm, Gm^R	This work

Table S2. **Primers and probes used in this study.**

Name	**Sequence (5'→3')**	**Application**
pJET-rev	AAGAACATCGATTTTCCATGGCAG	Check-Cloning
pJET-up	CGACTCACTATAGGGAGAGCGGC	Check-Cloning
CobN1-HIIIup	AAGCTTATGCACCTGTTGCGCACCC	Cloning
CobN2BI-lw	GGATCCCAGAAGCGCTCGGCCTGCT	Cloning
CobN3BI-up	GGATCCTGAATCCGAAGTGGATCGC	Cloning
CobN4SI-lw	GAGCTCCTATTCCTCTTCCGACGTCCA	Cloning
CobN-5-low	CAGGCCAGGCCCTTGAAAC	Cloning
gapTaqM-low	GAGGTTCTGGTCGTTGGT	qRT-PCR
nrdATaqM2-low	TGTTCATGTCGTGGGTACG	qRT-PCR
nrdJTaqM2-low	GTAAACACCCGCACCACTTC	qRT-PCR
nrdDTaqM2-low	CCGAGTTGAGGAAGTTCTGG	qRT-PCR
Univ-Res-Gen-lw	AAGAATTCACGCGTCGCTCATGAGACAATA	Cloning
Univ-Res-Gen-up	AAGAATTCACGCGTATATATGAGTAAACTT	Cloning
nrdA-FAM	CTGGCACCTGGACATC	qRT-PCR probe
nrdJ-FAM	TCGGCTCGGTCAACCT	qRT-PCR probe
nrdD-FAM	CCCGACCTACAACATC	qRT-PCR probe
gap-FAM	CCTGCACCACCAACTG	PCR probe

Resum dels resultats i discussió

Les RNRs han sigut objecte d'un estudi profund i exhaustiu des del seu descobriment, ja que són enzims essencials per a la divisió cel·lular de tots els organismes que existeixen a la Terra. Inicialment, van ser una diana molt important per inhibir les malalties on hi predominava la divisió cel·lular, com ara el càncer en els organismes eucariotes [142, 226]. Aquesta inhibició de les RNRs se centrava en el bloqueig de l'activitat de l'única classe de RNR codificada pels organismes eucariotes, la RNR de classe Ia. En canvi, en els organismes procariotes, la inhibició de la reacció de la RNR és molt més complexa, ja que codifiquen per més d'una classe de RNR en el seu genoma. El descobriment de les diferents classes de RNR en procariotes va donar lloc a voler esbrinar les condicions ambientals específiques d'activitat enzimàtica i de transcripció de cadascuna de les classes de RNR, per tal d'inhibir-la. Codificar per més d'una classe de RNR en una mateixa cèl·lula permet mantenir un balanç equilibrat dels diferents dNTP per a la divisió o la reparació del material genètic en diferents condicions. Així doncs, els gens de cada classe de RNR han d'estar específicament regulats transcripcionalment segons les condicions ambientals de creixement del microorganisme. L'estudi d'aquests factors de transcripció permet conèixer i entendre les condicions de síntesi de cada classe de RNR.

En aquest treball, s'ha analitzat l'expressió de cadascuna de les classes de RNR al bacteri *P. aeruginosa,* ja que és un bacteri capaç de formar biofilm, resistent a la majoria d'antibiòtics actuals i a més, codifica per a les tres classes de RNR descrites (la classe Ia, II i III). S'ha estudiat específicament el factor de transcripció NrdR de *P. aeruginosa*, com a principal regulador dels gens *nrd*. S'ha aprofundit en la recerca de nous factors transcripcionals específics de diferents condicions de creixement de *P. aeruginosa,* com ara en la formació de biofilm o durant el procés d'infecció. A partir de la realització d'una anàlisi bioinformàtic de cadascun dels promotors s'han trobat factors de transcripció reguladors dels gens *nrd,* com ara; factors de transcripció involucrats en el metabolisme anaeròbic i relacionats amb la virulència o la formació de biofilm.

A continuació es mostren els factors de transcripció identificats en cadascun dels promotors estudiats com a resultat dels objectius proposats en la tesi i que han estat publicats en 2 articles, 2 manuscrits sotmesos per publicar i 1 manuscrit en preparació. Aquests factors de transcripció han sigut objecte d'estudi al llarg de la tesi, i per tant es discutiran a continuació.

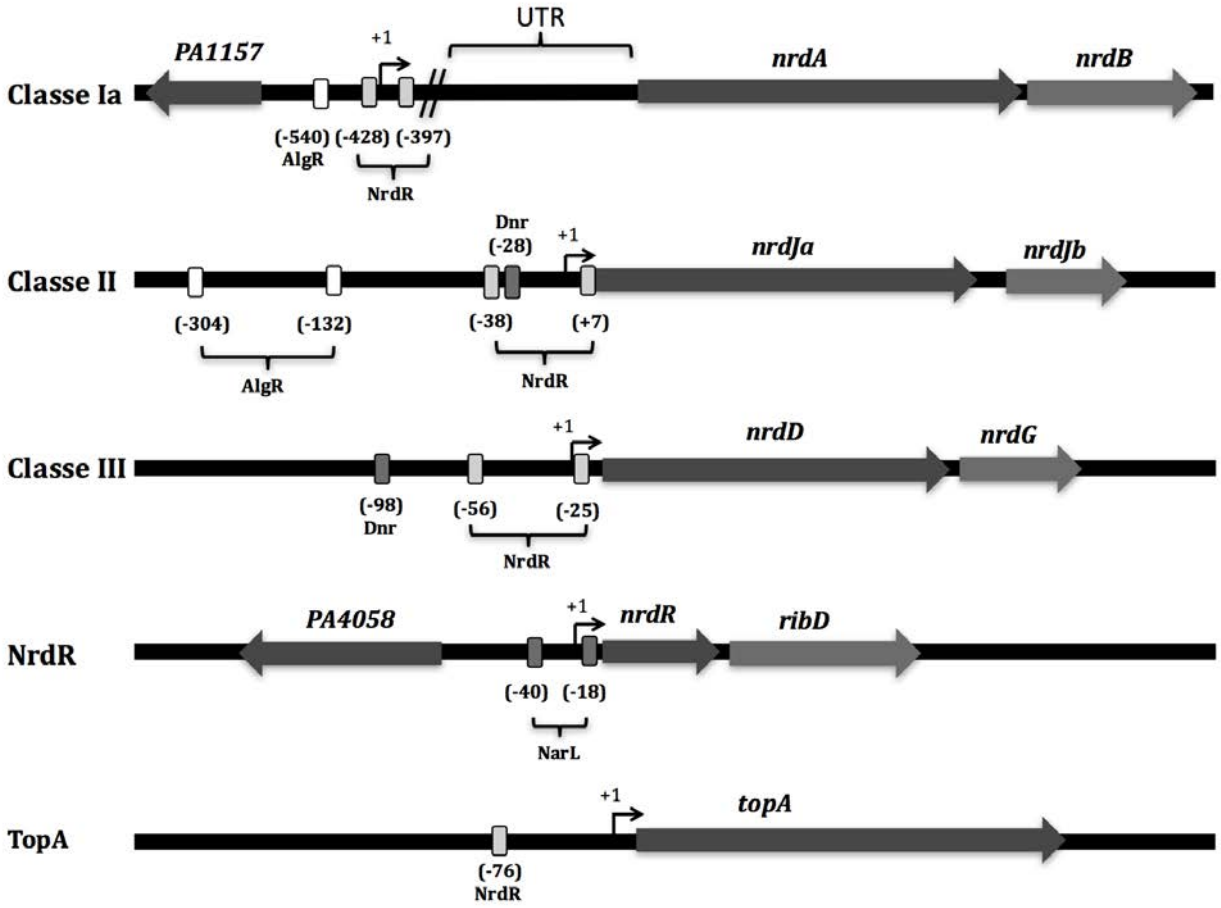


Figura 12: Esquema general dels factors transcripcionals identificats reguladors dels gens de les RNRs de *P. aeruginosa*. Promotors dels gens *nrdAB, nrdJab, nrdDG, nrdR-ribD* i *topA* de *P. aeruginosa* i la posició de les caixes d'unió dels factors de transcripció des de l'inici de traducció descrits en aquest treball (la representació no es troba a escala).

Capítol 1: El factor de transcripció NrdR

Tal com s'ha detallat anteriorment, les cèl·lules procariotes tenen la capacitat de codificar per a més d'una classe de RNR en el seu genoma [121], per tant, ha d'existir una forta regulació transcripcional per regular específicament cadascun dels gens de les RNRs. NrdR és un factor transcripcional, estudiat en profunditat, que té la capacitat d'unir-se a les regions promotores dels gens *nrd* tot i així, no es coneix si regula de manera diferencial els diferents gens *nrd.*

NrdR inhibeix l'expressió dels gens de les classes de RNR en diferents microorganismes com ara *E. coli* (classe Ia, Ib i III) [96], *Chlamydia sp* (classe Ic) [126], *M. tuberculosi* (classe Ib i III) [227], *S. typhimurium* [127] o *S. coelicolor* (classe Ia i II) [123]. Tot i així, fins ara tan sols s'ha estudiat el factor de transcripció NrdR en microorganismes on codifiquen, com a màxim, dues

classes diferents de RNR. Conseqüentment, en aquest capítol s'ha estudiat el factor de transcripció NrdR de *P. aeruginosa* en profunditat, per tal de determinar exactament quin és el seu paper en la regulació diferencial per a cada classe de RNR (la classe Ia (*nrdA*), II (*nrdJ*) i III (*nrdD*)).

A *P. aeruginosa,* el factor de transcripció NrdR es troba codificat en l'operó *nrdR* (PA4057)-*ribD* (PA4056) donant lloc a una proteïna de 154 aminoàcids d'un pes molecular de 17.9 kDa (Figura 8 i Figura 1 de l'article 1). Manté un 70% d'identitat i 82% de similaritat amb el NrdR d'*E. coli*. L'estructura de NrdR de *P. aeruginosa* revela dos dominis conservats: el domini N-terminal o *Zinc-finger* d'unió a l'ADN (3-34 aa) i el domini C-terminal o *ATP-cone* (49-139 aa) amb la possibilitat d'unir nucleòtids.

En aquest treball, per poder estudiar la funció de NrdR a *P. aeruginosa* es va utilitzar una soca mutant isogènica de *nrdR* cromosòmica (Δ*nrdR* o PW7855) sense afectació polar sobre la transcripció del gen *ribD*. S'ha vist que aquesta soca mutant *nrdR* té la mateixa taxa de creixement que una soca salvatge de *P. aeruginosa* PAO1 tant en condicions de creixement aeròbies com anaeròbies, per tant, NrdR no és essencial pel creixement de *P. aeruginosa* (Figura 1 de l'article 1), tal com s'havia vist pel creixement d'*E. coli* [128].

A més, es va comprovar si la deleció del factor de transcripció NrdR afecta la virulència de *P. aeuginosa* PAO1, ja que, l'absència de *nrdR*, en una soca d'*E. coli* adherent-invasiva, produeix desregulació dels gens que participen en la virulència [128]. Per determinar la virulència de *P. aeruginosa* PAO1 salvatge i de la soca mutant *nrdR* es van infectar dos models d'animals; *D. melanogaster* i *Danio rerio* (aquest últim, en col·laboració del grup del Prof. Angel Raya, del Centre de Medicina Regenerativa de Barcelona (CMRB)). En els dos models animals es va obtenir el mateix resultat, NrdR no afecta la virulència de *P. aeruginosa* de manera significativa (Figura 13, i Fig. 6 de l'article 1). Tant en la soca Δ*nrdR* com en la soca salvatge, abans de les 20 h post-infecció el 50% de les mosques i el 80% els peixos van morir.

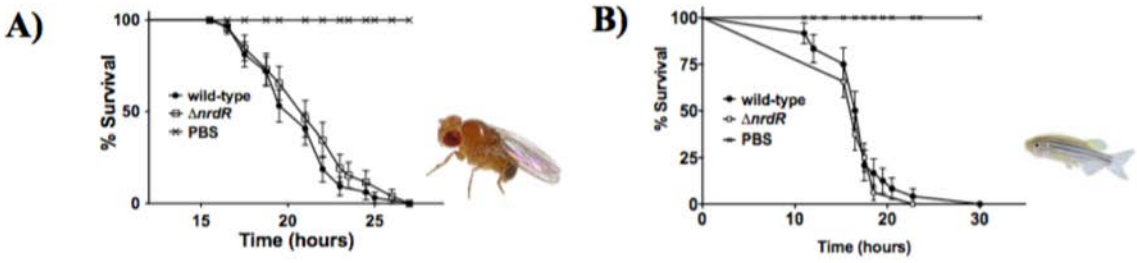


Figura 13: Taxa de mortalitat de *D. melanogaster* i *D. rerio* per la infecció de *P. aeruginosa* salvatge i Δ*nrdR* (A) correspon a la Figura 6 de l'article 1).

Per una altra banda, una vegada descartat l'afectació de NrdR al creixement i la virulència de *P. aeruginosa,* es va analitzar el patró d'expressió de *nrdR* a *P. aeruginosa* PAO1 per poder

entendre el paper regulador de NrdR. Es va construir una fusió transcripcional plasmídica del promotor *nrdR* a la GFP (P*nrdR::gfp*) i es va analitzar l'expressió de *nrdR* en una soca salvatge de *P. aeruginosa* PAO1. Els resultats obtinguts mostren que l'expressió de *nrdR* augmenta en fase exponencial tant en condicions aeròbiques com anaeròbiques (també observat en [96]), sent en condicions anaeròbiques quan s'esdevé la màxima expressió (Figura 1 de l'article 1). Per tant, es va estudiar el mecanisme transcripcional que induïa aquest augment d'expressió en condicions anaeròbies. Bioinformàticament, es va analitzar el promotor de *nrdR* utilitzant l'algoritme Virtual Footprint de la base de dades PRODORIC [228] i es van identificar dues caixes d'unió del factor transcripcional NarL. NarL és un dels principals factors de transcripció que regulen els gens responsables del metabolisme anaeròbic de *P. aeruginosa* [229] (Apartat 1.2.2.). Les dues caixes identificades en el promotor de *nrdR* es van anomenar caixa NarL1 (CTACCAT) i caixa NarL2 (TACGCCT), situades a 40 bp i 18 bp de l'inici de traducció, respectivament.

Per tal de corroborar la unió del factor transcripcional NarL en el promotor del gen *nrdR*, es van realitzar diferents mutacions puntuals dirigides a les dues caixes d'unió de NarL. El resultat obtingut va mostrar una disminució de l'expressió de *nrdR* en condicions anaeròbiques, tant en una soca mutant cromosòmica pel gen *narL* (PW7549), com per la mutació de les caixes NarL en la fusió plasmídica de la soca salvatge (*wild-type)* de *P. aeruginosa* PAO1 (Figura 2 de l'article 1). Aquests resultats demostren que **el factor de transcripció NarL s'uneix al promotor del gen *nrdR*** en condicions anaeròbiques regulant la seva expressió en aquestes condicions.

Posteriorment, s'ha aprofundit en l'estudi de NrdR com un factor de transcripció encarregat de regular l'expressió diferencial dels gens de les classes de RNR, tant en condicions de creixement aeròbies, com anaeròbies. Així doncs, per estudiar la regulació de NrdR sobre els gens *nrd,* es va utilitzar plasmidis que contenien fusions transcripcionals de les regions promotores a la GFP (P*nrdA::gfp*, P*nrdJ::gfp* i P*nrdD::gfp**[1]) i es va mesurar la fluorescència relativa produïda pel gen reporter *gfp* en una soca salvatge (*wild-type*) i una soca mutant *nrdR* (Figura 3 de l'article 1). Els resultats es van corroborar mesurant els nivells de mRNA de cadascun dels gens mitjançant qRT-PCR en fase exponencial (DO_{550} = 0.6). Els resultats obtinguts, utilitzant les diferents tècniques, mostren que en aerobiosi hi ha un augment de l'expressió de les tres classes de RNR en una soca deficient pel gen *nrdR* comparat amb una soca salvatge (*wild-type)*. Aquest resultat indica, com estudis anteriors, que NrdR actua com un repressor en condicions aeròbies [96]. En canvi, en condicions anaeròbies la situació és diferent. NrdR tan sols reprimeix el gen *nrdA* i sorprenentment, l'expressió de *nrdJ* i *nrdD* no es troba significativament reprimida per NrdR. Possiblement, el

[1] ***Els gens de cadascuna de les classes de RNR (*nrdAB*, *nrdJab* i *nrdDG*) es nombraran tan sols amb el primer gen de l'operó, referint-se al promotor estudiat en cada cas.

factor de transcripció NrdR permet l'expressió dels gens *nrdJ i nrdD* en aquestes condicions, ja que les RNRs de classe II i III són responsables de la síntesi dels dNTP en absència d'oxigen [110]. Així doncs, segurament NrdR s'uneixi de manera molt dèbil o no s'uneixi al promotor de *nrdD i nrdJ*, sinó que s'uneixen factors de transcripció que regulen gens del metabolisme anaeròbic per tal d'induir la seva expressió. S'han identificat caixes d'unió dels factors de transcripció Anr o Dnr ambdós promotors compartint part de la caixa NrdR-box (analitzat en els capítols següents). Per tant, en condicions anaeròbies Anr o Dnr es podrien unir al promotor de *nrdJ* o *nrdD* permetent la seva expressió i evitant la repressió per NrdR. Tot i així, s'ha observat que **el factor de transcripció NrdR és capaç de regular les tres classes de RNR codificades a *P. aeruginosa***.

A més, l'activitat enzimàtica de les RNRs *in vivo* es va analitzar mesurant els nivells de dNTP produïts en la cèl·lula en absència i en presència de NrdR (Figura 3 de l'article 1). Es va realitzar un assaig per mesurar els nivells de dNTP (assaig DPA) en una soca salvatge i una soca Δ*nrdR*. En absència de NrdR, el *pool* de dNTP obtingut va augmentar en un 25% comparat amb una soca salvatge (*wild-type)* de *P. aeruginosa* PAO1. Per tant, l'absència del factor de transcripció Δ*nrdR* indueix un augment de l'expressió dels gens *nrd* en condicions aeròbies que resulta en un augment de la seva activitat global, augmentant la concentració de dNTP. En presència de hidroxiurea (HU) (molècula radical *scavenger*, apartat 1.1.8.) la RNR de classe Ia s'inhibeix disminuint els nivells de dNTP. En altres organismes com *S. typhimurium* o *Chlamydia sp* també s'ha vist que el tractament amb hidroxiurea disminueix els nivells de dNTP [126, 127]. Tot i així, en absència de NrdR i presència de HU, els nivells de dNTP es mantenen com una soca salvatge (*wild-type)*. Conseqüentment, s'ha demostrat que NrdR pot modular els nivells de dNTP de *P. aeruginosa* via la regulació transcripcional dels gens *nrd*.

Per determinar la unió directa de NrdR a les caixes NrdR-box de cadascun dels promotors dels gens *nrd* es va intentar sobreexpressar NrdR amb l'objectiu de realitzar, mitjançant la unió directa de NrdR sobre els promotors analitzats, un gel de mobilitat electroforètica (EMSA). Tot i així, no es va poder purificar la proteïna degut a que la sobreproducció de NrdR formava cossos d'inclusió totalment insolubles. Per tant, la unió de NrdR als diferents promotors es va estudiar mutant la seqüència de les caixes NrdR en cadascun dels promotors de les diferents classes de RNR. En tots els casos es va mutar la caixa NrdR2, ja que la caixa NrdR1 afectava la posició -10 del promotor. Es va mesurar l'activitat dels promotors amb les caixes NrdR mutades i dels promotors salvatges (*wild-type)* per a cada classe de RNR (Figura 3 de l'article 1). Els resultats corroboren l'experiment anterior, NrdR s'uneix de manera específica als promotors dels gens *nrd*. A més, tan sols la mutació d'una de les caixes NrdR dels promotors dels gens *nrd* evita la regulació d'aquests per NrdR. Possiblement, la presència de dues caixes NrdR-box en cadascun dels promotors és deguda a la

dimerització de NrdR, regulat o no, pels nivells de dNTP de la cèl·lula [122, 130]. Els nivells de dNTP poden regular l'estat d'oligomerització, com ara en la RNR de classe Ia (regulació al·lostèrica d'activitat). Actualment, la regulació de l'activitat de NrdR per la unió de dNTP sobre la regulació dels gens *nrd* està sent estudiada en el laboratori.

Un estudi l'any 2005 va identificar possibles caixes d'unió de NrdR a diversos promotors de diferents organismes [125]. Concretament a *P. aeruginosa* es va identificar totes les caixes d'unió de NrdR als gens *nrd* i a més, una caixa d'unió de NrdR al promotor de la topoisomerasa I (P*topA).* La topoisomerasa I junt amb l'ADN girasa són els enzims encarregats de mantenir la topologia de l'ADN, donant un balanç equilibrat entre l'ADN relaxat i l'ADN superenrotllat. La topoisomerasa I, en els organismes procariotes, tan sols pot relaxar l'ADN superenrotllat de manera negativa [230]. Així doncs, en aquest capítol, es va analitzar la regulació de l'expressió de *topA* per la unió de NrdR al seu promotor. Es va clonar el promotor de *topA* en el plasmidi pETS130 per construir una fusió transcripcional a la GFP. Sorprenentment, el resultat va ser el contrari a l'obtingut amb els gens *nrd* (Figura 4 de l'article 1). Tant en condicions aeròbies com anaeròbies, el promotor del gen *topA* es reprimia en una soca mutant per *nrdR*. Utilitzant la complementació del gen *nrdR* i mutant la caixa d'unió de NrdR al promotor es va determinar la unió directa de NrdR al promotor. Una possible causa que NrdR actuï com inductor en lloc de repressor, pot ser el nombre de caixes NrdR en el promotor. En el promotor de *topA* tan sols s'ha identificat una sola caixa d'unió de NrdR i en canvi, en els promotors dels gens *nrd* s'han identificat dues. Tot i així, la unió de dNTP a NrdR també podria estar implicat, ja que determinaria l'estat d'oligomerització de la proteïna o la seva capacitat d'unir-se a la caixa NrdR (Figura 7 de l'article 1). Per tant, la inducció de l'expressió de *topA* obre un nou camí per estudiar el factor de transcripció NrdR com a repressor i com a activador.

Complementàriament, es va analitzar el grau de superenrotllament de l'ADN plasmídic (ADN relaxat o superenrotllat) determinat pel balanç d'activitat entre TopA i GyrA. Es va utilitzar el plasmidi (pUCP20T) extret d'una soca Δ*nrdR* i d'una soca salvatge (*wild-type)* en fase exponencial i estacionària de creixement de *P. aeruginosa* (DO_{550} 0.5 i 2) (Figura 4 de l'article 1). En fase exponencial, el plasmidi extret de la soca Δ*nrdR* mostra més ADN superenrotllat negativament que una soca salvatge (*wild-type)*. En fase estacionària, en canvi, la diferència no és significativa. Aquests resultats corroboren els experiments obtinguts en l'assaig transcripcional anterior on **NrdR regula l'expressió de *topA*.**

Així doncs, per primera vegada el factor de transcripció NrdR, descrit anteriorment com a regulador específic dels gens *nrd*, és capaç de regular de manera directa un altre gen diferent de *nrd* i per tant, NrdR podria regular altres gens diferents dels gens *nrd*. Conseqüentment, es va realitzar

una anàlisi transcripcional global o *microarray* (Affymetrix *P. aeruginosa* GeneChip microarray platform) en condicions aeròbies i anaeròbies, per determinar els canvis transcripcionals directes o indirectes produïts al genoma de *P. aeruginosa* en absència o presència de *nrdR* (Figura 5 de l'article 1). L'ARN extret en fase exponencial (DO_{550} = 0.5) es va comparar entre una soca salvatge (*wild-type)* PAO1 i una soca Δ*nrdR* (*log-fold change* >1.5*).* Els resultats mostren que aeròbiament, en una soca Δ*nrdR* es van desregular 47 gens, dels quals 31 es trobaven induïts i 16 reprimits. En canvi, en anaerobiosi dels 111 gens desregulats, 85 gens estaven reprimits mentre que tan sols 26 induïts. Anaeròbiament es van desregular 2.5 vegades més gens que en condicions aeròbies, possiblement degut a l'augment de l'expressió de NrdR en condicions anaeròbies. Els gens principalment desregulats aeròbiament, van ser *cupA1* (subunitat de les fímbries), proteïnes hipotètiques (PA4139, PA1383 i PA2223) i alguns gens relacionats amb la resistència a antibiòtics (*mexA-oprM*) a més dels gens de les RNRs. Anaeròbiament, els gens principalment desregulats van ser els gens *mexEF-oprN* (resistència a antibiòtics), proteïnes hipotètiques (PA3283, PA3281 i PA0565), gens del sistema SOS i *pyoS5*. Possiblement, aquesta desregulació transcripcional identificada en una soca mutant pel gen *nrdR* és conseqüència de la desregulació provocada en el gen *topA,* ja que no s'han identificat caixes d'unió de NrdR en cap dels promotors dels gens desregulats. Aquesta desregulació del gen *topA* que afecta la topologia d'ADN pot també afectar de manera indirecta a l'expressió de tots aquests gens. Un estudi de Lawrence G. Rahme, mostra que alguns dels gens desregulats en absència de NrdR també estan desregulats en absència de *topA* (dades no publicades, Gene Expression Omnibus GSE24038) com ara, els gens del sistema SOS [231].

Per tant en aquest capítol, s'ha conclòs que **el paper del factor de transcripció NrdR és un regulador de l'expressió dels gens *nrd* i d'altres gens, com *topA*.** Tot i així, els gens de les diferents classes de RNR de *P. aeruginosa* estan regulats també per altres factors de transcripció específics per les diferents condicions ambientals.

Capítol 2: El factor de transcripció AlgR

P. aeruginosa utilitza diferents sistemes de dos components sensibles a canvis ambientals per expressar factors de virulència en un procés d'infecció aguda o crònica. Un d'aquests sistemes de dos components és el sistema AlgZR. Aquest sistema es troba associat a l'expressió dels gens del sistema *quorum sensing*, del pili tipus IV, del sistema de secreció III, del metabolisme anaeròbic, de la producció de rammolípids o de cianina. L'AlgR va ser el primer factor de transcripció descobert

implicat en la via de síntesi de l'alginat (12 gens; *algD, alg8, alg44, algK, algE, algG, algX, algL, algI, algJ, algF i algA)*, sent *algD* el primer gen de l'operó [232].

La proteïna AlgR (27.6 kDa) conté un domini N-terminal semblant a altres factors de transcripció de la família CheY-like (domini REC), com OmpR o NtrC, i un domini C-terminal d'unió a l'ADN homòleg a la família de reguladors LyR/YehT/AgrA, que conté *loops* sense estructura definida per reconèixer la unió de l'ADN. El domini AlgR-REC conté residus d'aspartat (Asp7, Asp8 i Asp54) formant un *pocket* acídic que permet la fosforilació del residu Asp54 el qual induirà un canvi de conformació de la proteïna [233, 234]. La substitució del residu Asp54 per una asparagina (AspD54N) elimina la possibilitat de ser fosforilat modificant així la conformació d'unió d'AlgR a l'ADN i la regulació transcripcional [233]. El domini C-terminal d'unió a l'ADN de l'AlgR és poc comú, ja que només el 3 % dels factors de transcripció procariotes contenen aquest domini [235], com ara AgrA de *S. aureus* o *Bacillus cereus* sent un factor important per a l'expressió de factors de virulència en aquests organismes [235].

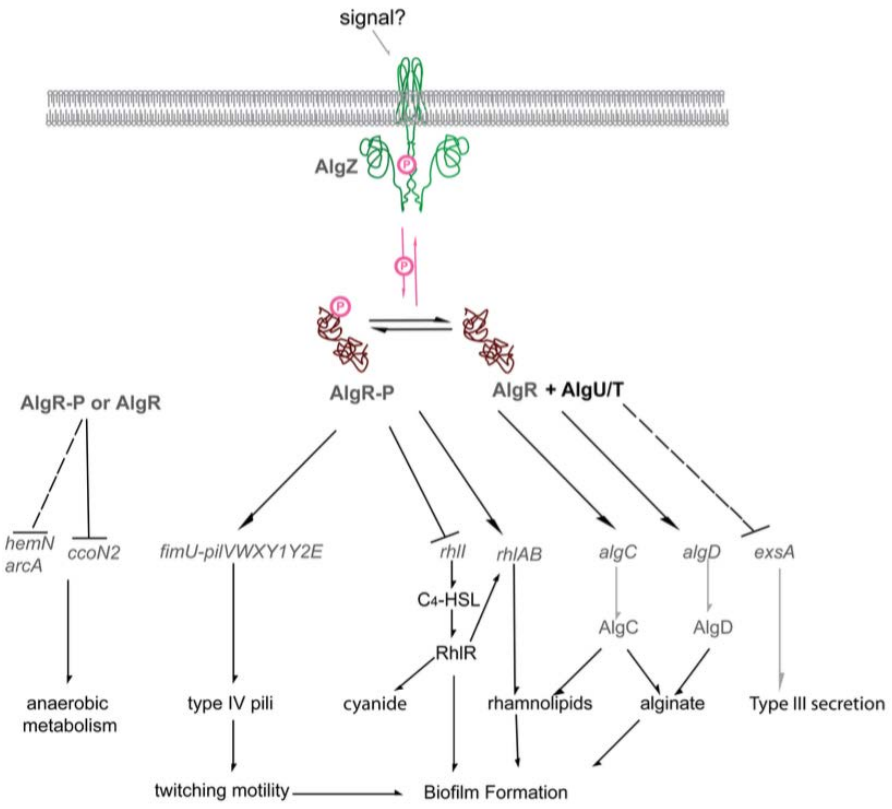


Figura 14: Representació de la regulació gènica del sistema de dos components AlgZR. (Imatge extreta de [234]).

L'AlgZ (o FimS), el segon component del sistema, conté quatre hèlixs transmembrana molt semblants als que es troben a les histidines *quinases* situades a la membrana, doncs és considerat el sensor del sistema de dos components AlgZR capaç de fosforitzar AlgR sota condicions ambientals

específiques [236]. Per exemple, l'AlgR fosforilat indueix els promotors *fimU i rhlAB* permetent el moviment *twitching* i la formació de rammolípids [233, 237, 238].

Aproximadament, existeixen 157 gens que presenten alta afinitat a la unió d'AlgR en el seu promotor, com ara els gens de la síntesi d'alginat, de resistència antibiòtics, de motilitat, del metabolisme de les pirimidines o purines, del metabolisme del carboni, d'adquisició del ferro o d'estrès cel·lular [113, 239, 240]. L'any 2004 es va observar mitjançant experiments de transcripció global (*microarray*), una desregulació de l'expressió de la RNR de classe II (*nrdJa)* en absència del gen *algR* [113] i l'any 2015, una possible caixa d'unió d'AlgR en la regió intergénica de PA1157-*nrdA (PA1156),* tot i que els autors indicaven la regulació d'AlgR cap al gen PA1157 [240]. Per tant, el segon capítol de la tesi es va centrar en l'estudi de la regulació del sistema de dos components AlgRZ sobre els gens de les RNRs.

A partir d'una cerca bioinformàtica es van determinar les caixes d'unió d'AlgR als promotors *nrd.* Es van identificar possibles caixes AlgR; en el promotor de *nrdA,* la qual coincideix amb la identificada per ChIP-seq [240], i en el promotor *nrdJ* (descrites en la figura S2 de l'article 2), no identificat anteriorment, en canvi, cap caixa AlgR en el promotor del gen *nrdD.*

Específicament, els factors de transcripció de la família AgrA (com AlgR) s'uneixen a dues seqüències repetides, encara que l'AlgR pot regular gens que contenen un nombre diferent de caixes d'unió en el promotor; *fimU* (2 caixes AlgR), *hcnA* (1 caixa AlgR), *rhlI* (1 caixa AlgR) o *algD* (3 caixes AlgR). En els promotors *nrd* s'han identificat dues caixes d'unió d'AlgR en el promotor *nrdJ* (anomenades AlgR box 1 i AlgR box 2) i una caixa AlgR en el promotor de *nrdA* (anomenada AlgR box 1). La situació d'aquestes caixes, allunyades de l'inici de transcripció (entre 100-300 bp), suggereix que a més d'AlgR, existeixen altres factors de transcripció que interaccionen (amb l'AlgR, o no) per regular l'expressió de *nrdJ* i *nrdA.* A més, una anàlisi en profunditat de les caixes AlgR en diferents promotors, mostra la presència d'un nucleòtid cisteïna (C) en la posició 7 (5→3), que determina una unió forta de AlgR a l'ADN [241-243], com ara en les caixes 1 i 2 del promotor d'*algD,* 1 i 3 del promotor d'*algC,* en el promotor *rhlI* o de *rhlA,* o en la caixa identificada en el promotor de *nrdA.* Així doncs, la unió d'AlgR al promotor de *nrdA* és forta, mentre que al promotor de *nrdJ* es considera feble (Figura S4 de l'article 2).

Per tal de comprovar la unió directa de l'AlgR sobre els promotors dels gens *nrdA* i *nrdJ* es va sobreexpressar la proteïna AlgR i realitzar assajos d'unió d'EMSA (*electrophoretic mobility shift assay).* Inicialment, es van amplificar diversos fragments dels promotors per acotar la zona d'interacció i assegurar l'especificitat de la unió. La mutació de cadascuna de les caixes AlgR identificades en els promotors *nrdA* i *nrdJ* comporta la no unió d'AlgR a l'ADN, per tant, **AlgR**

s'uneix de manera específica als promotors de *nrdA* i *nrdJ*. Tanmateix, la unió d'AlgR als promotors de *nrdA* i *nrdJ* es va corroborar analitzant els complexes d'AlgR-ADN mitjançant Microscòpia de Força Atòmica (AFM). Utilitzant aquesta tècnica es va demostrar que la unió d'AlgR en el promotor produeix una curvatura en l'ADN indicant que l'AlgR modula la topologia de l'ADN en el lloc en el qual s'hi uneix. Aquesta curvatura podria permetre la unió o la interacció d'altres factors de transcripció que induirien o reprimirien l'expressió dels gens en presència d'AlgR, com ara Anr (factor de transcripció anaeròbic). S'ha observat que molts gens regulats per AlgR també ho estan per Anr (*arcDABC*, *ccoP2*, *hcnB*, *oprG* o *hemN*) [113, 163, 234, 238, 244-246], inclús *nrdJ* (Figura S7 de l'article 2 i capítol 3).

Segons a les observacions descrites *in vitro*, es va analitzar la regulació d'AlgR sobre els gens *nrd* de manera *in vivo*. Es va utilitzar una soca de *P. aeruginosa* PAO1 mutant isogènica pel factor de transcripció AlgR (Δ*algR*) i una soca PAO1 *salvatge* (*wild-type*) que contenien les fusions transcripcionals dels diferents promotors (*PnrdA*, P*nrdJ*, P*nrdD* i PPA1157). A més, es va utilitzar el promotor del gen *algD (PalgD*) com a control de la regulació de l'expressió a través d'AlgR. També, es va complementar la mutació cromosòmica Δ*algR* utilitzant la sobreexpressió d'AlgR salvatge o *wild-type* (pUCP20T-P*fimS-algR*) i d'AlgR D54N (sense capacitat de ser fosforilat) (pUCP20T-AlgRD54N). Els resultats van mostrar que el gen PA1157 no es troba regulat per l'AlgR, per tant, la unió d'AlgR a la regió intergènica (PA1157-*nrdA*), identificada l'any 2015 [240], regula específicament l'expressió del gen *nrdA*, en lloc del gen PA1157 (Figura 1 de l'article 2). Tampoc es va detectar diferències d'expressió sobre el promotor P*nrdD* ni en condicions aeròbies ni anaeròbies. En canvi, es va observar una disminució de l'expressió dels gens *nrdA* i *nrdJ* en absència del gen *algR*. Així doncs, es va identificar la regulació transcripcional per AlgR en aquells gens on s'havia identificat les caixes d'unió d'AlgR en els seus promotors.

Per una banda, la regulació d'AlgR sobre el gen *nrdA* es va demostrar *in vivo* mutant la caixa d'unió al promotor, tant en un cultiu líquid (Figura 2 de l'article 2), com durant la formació de colònies (Figura 4 de l'article 2) o de biofilm (Figura 5 de l'article 2). A més, es va identificar una regulació diferencial sobre el gen *nrdA* segons si l'AlgR es troba fosforilat o no (Figura 1, 4 i 5 de l'article 2). L'AlgR salvatge provoca la inducció de *nrdA* en totes les condicions de creixement analitzades, en canvi, l'AlgR D54N (no fosforilat) no l'indueix, a diferència del gen *algD*, que s'indueix específicament per l'AlgR D54N. No obstant, no es van observar diferències en la unió de l'AlgR salvatge o l'AlgR D54N en la unió al promotor de *nrdA* de manera *in vitro* (Figura S3 de l'article 2). Cal destacar que la fosforilació d'AlgR és deguda per l'activació del sistema de dos components AlgZR sensible a condicions ambientals, com ara en condicions d'estrès cel·lular (p.e. estrès oxidatiu) [101, 247] per tal d'induir els gens d'unió a superfícies i de formació de biofilm

[113, 248, 249]. De manera que es va comprovar si AlgZR participava en la inducció dels gens *nrd* sota la inducció d'estrès oxidatiu. Es va observar que l'expressió de *nrdA* es disminuïa en absència d'*algR* així com, mutant la caixa d'unió d'AlgR del promotor (Figura 6 de l'article 2). Per tant, **el sistema AlgZR és el responsable de la inducció dels gens *nrd* sota condicions d'estrès oxidatiu**, sent la primera vegada que s'identifica el factor de transcripció implicat en induir les RNRs sota aquestes condicions.

Per una altra banda, la regulació d'AlgR sobre el gen *nrdJ* és més complexa que en *nrdA,* ja que s'ha observat una regulació diferencial, independent de fosforilació, entre la unió d'AlgR a la caixa AlgR 1 o a la caixa AlgR 2 del promotor *nrdJ*. Sorprenentment, la mutació del gen *algR* produeix la disminució de l'expressió de *nrdJ,* indicant que l'AlgR actua com un inductor del gen *nrdJ,* en canvi, la doble mutació de les caixes AlgR en el promotor de *nrdJ* produeix un augment de l'expressió de *nrdJ* durant la formació de biofilms o de colònies (Figura 4C i 5C de l'article 2). Contràriament, la inducció d'estrès cel·lular mitjançant estrès oxidatiu provoca la disminució de l'expressió de *nrdJ* tant en absència d'*algR* com per la mutació de les dues caixes AlgR (Figura 6 de l'article 2). Aquesta diferència de regulació d'AlgR es va analitzar en detall mutant individualment la caixa 1 i la caixa 2 d'AlgR. Aquest resultat va indicar que la mutació de la caixa 1 provoca un clar augment de l'expressió de *nrdJ,* mentre que la mutació de la caixa AlgR 2, una reducció. Així doncs, es va suggerir que la regulació d'inducció de l'expressió *nrdJ* es produïa principalment per la unió d'AlgR a la caixa AlgR 2, i que la unió d'AlgR a la caixa AlgR 1, inhibeix l'expressió de *nrdJ,* tan sols en determinades condicions. Concretament, es va observar una reducció de l'expressió de *nrdJ* sota un fenotip mucoide (PAO1 Δ*mucA),* on hi predomina l'AlgR no fosforilat [193, 250, 251], durant la formació de colònies i de biofilm (Figura 4D i 5D de l'article 2). Conseqüentment, s'ha proposat que la caixa AlgR 2 és la responsable d'induir l'expressió de *nrdJ* en condicions d'estrès i la caixa AlgR 1 inhibeix l'expressió de *nrdJ* sota un fenotip mucoide afavorint probablement, l'expressió de la RNR de classe III degut a una disminució d'oxigen en aquestes condicions.

Basat en tots els resultats obtinguts en aquest capítol es va dissenyar un model de regulació dels gens *nrd* per AlgZR (Figura 7 de l'article 2). Aquest model de regulació consisteix en què sota un senyal d'estrès o d'un tractament d'antibiòtic d'un cultiu de *P. aeruginosa* (planctònic), el sistema de dos components AlgZR, s'activa i permet la fosforilació d'AlgR. Una vegada fosforilat, s'indueix la transcripció de *nrdAB,* per tal de permetre la replicació de l'ADN, així com la inducció de l'expressió de l'operó *fimUpilVWXY1Y2E* i *rhlAB.* Un augment de fímbries i pilis, permet a *P. aeruginosa* adherir-se a la superfície i formar microcolònies, fins a obtenir un cultiu madur en fase

estacionària en el qual es comencen a expressar metabòlits derivats del metabolisme primari per protegir-se. Un dels metabòlits importants per a la cèl·lula és la síntesi de la vitamina B_{12}, sintetitzada a partir de la molècula *uroporphyrinogen III* pels gens *hemCD*, localitzats en l'operó *fimS-algR-hemC-hemD* [252, 253]. Aquest operó es troba regulat sota dos promotors (ZT1 i ZT2) activats per Vfr en condicions planctòniques d'una soca no mucoide [193]. Però, una vegada s'hagin establert les microcolònies en la superfície, *P. aeruginosa* patirà una sèrie de canvis que induiran a un fenotip generador de biofilm, on hi predominarà la producció d'exopolisacàrids com l'alginat o diferents metabòlits, i de la vitamina B_{12} (Capítol 5), la qual actuarà com a cofactor de la RNR de classe II (induïda també durant la formació de biofilms [254]). A més dels canvis fenotípics, també es produeixen alteracions genètiques com ara la mutació del gen *mucA*, generant el fenotip conegut com a mucoide. Sota aquesta mutació genètica, en absència de MucA, el factor sigma AlgU/T induirà l'expressió dels gens de l'alginat (*algD-8-44-KEGXLIJFA* i *algC-argB*), així com, l'expressió d'*algR*, a través de la unió d'AlgU/T, a un altre promotor situat en el gen *algZ o fimS* (RT1 i RT2). Aquest promotor regularà l'expressió tan sols d'*algR-hemCD* (i no de *fimS*) per permetre que l'AlgR reguli el gens específics de la formació de biofilm, independents de fosforilació, com ara *nrdJ* o *algD*. A més, una vegada el biofilm es va fent madur, augmenta el gruix i també, disminueix la concentració d'oxigen en l'interior, per tant, s'induiran els gens de l'anaerobiosi regulats principalment per Anr i Dnr que permetran l'expressió, tant de *nrdJ*, com de *nrdD* (Capítol 3 i 4).

Capítol 3: Les RNRs durant la formació de biofilm

P. aeruginosa és un bacteri capaç de produir infeccions cròniques en diferents teixits, com ara en el pulmó de pacients immunodeprimits, en els pacients amb la malaltia pulmonar obstructiva crònica (MPOC) o en pacients de fibrosi quística (FQ). L'establiment de les infeccions cròniques en el pacient es troba relacionat amb la capacitat del bacteri per formar biofilm (biopel·lícula) en el teixit afectat (Apartat 1.2.3.). En un creixement en forma de biofilm, *P. aeruginosa* indueix els gens encarregats de la síntesi d'exopolisacàrids, augmenta la comunicació entre cèl·lula i cèl·lula i la resistència a antibiòtics, produint un patró d'expressió genètic diferent [201, 255]. Principalment, la síntesi d'exopolisacàrids com l'alginat, provoca un augment de la matriu extracel·lular polimèrica que produeix un gradient en la concentració de nutrients i d'oxigen al llarg del biofilm, fins a arribar a condicions estrictes d'anaerobiosi en la part més profunda del biofilm [201, 256, 257]. Les condicions anaeròbiques són predominants en els biofilms madurs com també en els esputs dels

pacients de fibrosi quística [174]. Per aquesta raó, una vegada determinada la regulació de les RNRs en cultiu planctònic a través de NrdR i AlgR, en aquest capítol es va voler estudiar en profunditat el paper de les diferents classes de RNR durant el procés de la formació d'un biofilm madur on les condicions anaeròbies tenen una rellevància important.

En condicions anaeròbies, les RNRs de classe II i III de *P. aeruginosa* poden tenir activitat enzimàtica, en canvi, la RNR de classe Ia només és activa en condicions d'aerobiosi [24, 110]. Per tant, tenint en compte que al llarg de la formació un biofilm madur es disminueix la concentració d'oxigen, ens vam centrar a avaluar la importància de les RNRs de classe II i III en un biofilm.

Inicialment, es va analitzar el creixement de *P. aeruginosa* en un cultiu planctònic anaeròbic per caracteritzar la importància de les RNRs en condicions d'absència o presència d'oxigen. Es van utilitzar les soques mutants per a cada classe de RNR (classe II o Δ*nrdJ* i classe III o Δ*nrdD*) i es va construir una soca mutant doble per a les dues classes de RNR (Δ*nrdJ*Δ*nrdD*). La deleció de les RNRs de classe II o III va provocar una disminució del creixement, i tan sols l'addició de vitamina B_{12} en el medi restableix el creixement planctònic anaeròbic en la soca Δ*nrdD,* quan NrdJ és activa [110, 116] (Taula S3 de l'article 3). En canvi, la soca doble mutant (Δ*nrdJ*Δ*nrdD*) no pot créixer en absència d'oxigen, tot i afegir vitamina B_{12} al medi, ja que la RNR de classe Ia no és activa. Així doncs, és necessari la presència de les dues classes de RNR per suportar el màxim creixement anaeròbic de *P. aeruginosa,* tot i que la RNR de classe II dependrà de la disponibilitat de vitamina B_{12} en el medi (Apartat de resultats 5 i discutit més endavant).

Posteriorment, es va realitzar un biofilm sota condicions de creixement aeròbies i anaeròbies amb un flux de nutrients estàtic en plaques de micropouets, utilitzant les soques de *P. aeruginosa*; PAO1 salvatge, Δ*nrdJ*, Δ*nrdD*, Δ*nrdJ*Δ*nrdD*, Δ*nrdJ* + pETS159 (NrdJ) *i* Δ*nrdD* + pETS60 (NrdDG). A més, es va afegir una soca control, la soca mutant Δ*dnr*, ja que el gen *dnr* codifica per un factor de transcripció essencial (Dnr) que regula els gens encarregats del metabolisme anaeròbic [164]. Els resultats obtinguts van mostrar una disminució de la biomassa del biofilm en absència de les RNRs de classe II i III, i de la soca Δ*dnr* (factor de transcripció anaeròbic) (Figura 1 de l'article 3). Aquesta disminució la trobem tant en presència com en absència d'oxigen, per tant, aquest resultat indica una disminució de biofilm degut a la disminució de síntesi de dNTP en les parts del biofilm on hi ha condicions anaeròbies. Aquest resultat és més significatiu en condicions estrictament anaeròbies, ja que no és funcional la RNR de classe Ia que depèn d'oxigen. Tanmateix, la complementació de les dues classes de RNR (II i III) restableix el biofilm i per tant, les RNRs de classe II i III són necessàries per a la formació d'un biofilm robust on les condicions anaeròbies hi són presents.

Altrament, es va procedir a formar un biofilm amb aportació continu de nutrients (flux continu), semblant a un biofilm produït en condicions *in vivo* com el que es forma en els catèters dels hospitals o en els pulmons de pacients infectats, utilitzant les soques de *P. aeruginosa* PAO1 salvatge, Δ*nrdJ*, Δ*nrdD i* Δ*nrdJ*Δ*nrdD*. Després de 6 dies de flux continu es va analitzar el biofilm format mesurant la biomassa i el gruix del biofilm utilitzant un microscopi confocal (CLSM), el software Image J i COMSTAT (Figura 1B de l'article 3). Es va observar una disminució de la formació de biofilm (corresponent a la biomassa i al gruix) en les soques mutants per les RNRs de classe II i III respecte al biofilm format per la soca salvatge PAO1 (*wild-type)*, tal com s'havia observat en un biofilm de flux estàtic. Per tant, **les diferents classes de RNR de classe II i III són importants per la formació de biofilms de *P. aeruginosa.***

La concentració d'oxigen al llarg del biofilm és un paràmetre crític pel creixement bacterià de *P. aeruginosa* i defineix un patró d'expressió genètic, així com l'estructura i la morfologia cel·lular [201, 257, 258]. En absència d'oxigen, les cèl·lules de *P. aeruginosa* PAO1 no es poden dividir correctament i es troben filamentades, tot i així, si s'afegeix vitamina B_{12} al medi o es redueix els nivells d'òxid nítric, les cèl·lules es deixen d'allargar i poden dividir-se correctament [194, 259]. Un estudi l'any 2012 associava filamentació cel·lular amb una disminució de l'activitat de la RNR de classe II en condicions anaeròbies [116]. Per tant, en aquest capítol també es va analitzar els canvis de la morfologia cel·lular de les diferents soques de *P. aeruginosa* PAO1 (salvatge, Δ*nrdJ*, Δ*nrdD* i Δ*nrdJ*Δ*nrdD*) crescudes en un biofilm de flux continuo a causa de la presència de condicions anaeròbies (Figura 2 de l'article 3). Es van realitzar diferents imatges en pla longitudinal per veure les diferències estructurals en la part superior i inferior del biofilm. Es va observar que les cèl·lules de les soques mutants per a les RNRs de classe II i III es troben filamentades a diferència de les cèl·lules de la soca salvatge de *P. aeruginosa* PAO1 (*wild-type)*. Aquest resultat indica una alteració de la divisió cel·lular per l'absència tant de la RNR de classe II, com de la classe III, provocant una disminució de la síntesi de dNTP que evita la correcta formació de biofilm en la part més anaeròbia del biofilm. Específicament, s'ha vist que les cèl·lules de la part superior del biofilm no es troben filamentades, excepte en absència de la RNR de classe II, possiblement a causa que l'activitat de la RNR de classe Ia es troba activa. En canvi, en la part inferior del biofilm (corresponen a la part més anaeròbia) totes les soques mutants per RNR tenen les cèl·lules filamentades. En la RNR de classe II totes les cèl·lules es troben filamentades, indicant que la RNR de classe III no pot suportar la síntesi dels dNTP requerida. Però, en la soca mutant per a la RNR de classe III es poden trobar tant cèl·lules filamentades com no, segurament, a causa que en un biofilm Δ*nrdD*, la RNR de la classe II pot suportar la síntesi de dNTP en presència de vitamina B_{12}.

Possiblement, en un biofilm madur hi ha síntesi de vitamina B_{12} i una difusió d'aquesta al llarg del biofilm [116, 260] (Capítol 5).

Resumidament, en un biofilm la RNR de classe Ia de *P. aeruginosa* suporta la síntesi de dNTP pel creixement en les capes superiors del biofilm (en contacte amb l'oxigen), en les parts del biofilm on hi ha condicions de microaerofilia i vitamina B_{12}, la RNR de classe II és la responsable de sintetitzar els dNTP i, en la part estrictament anaeròbia d'un biofilm, s'encarrega la RNR de classe III (Figura 6 de l'article 3). Per tant, les RNRs permeten la divisió cel·lular en un creixement en forma de biofilm.

En un biofilm madur respecte a un creixement planctònic, *P. aeruginosa* expressa diferencialment la meitat del seu genoma, com ara els gens del metabolisme anaeròbic [168]. Alguns estudis també han detectat un augment de l'expressió de la RNR de classe II sota condicions anaeròbies respecte a les aeròbies (3.2 vegades més) [112] o en un esput anaeròbic [114]. A més, en una anàlisi proteòmic s'ha identificat un augment de les proteïnes NrdJ i NrdD en condicions anaeròbies respecte a les aeròbies [172]. Per tant, a partir d'aquests estudis es va determinar l'expressió de cadascuna de les classes de RNR (*nrdA*, *nrdJ* i *nrdD)* tant en condicions de creixement d'anaerobiosi/aerobiosi com en un creixement en forma de biofilm/planctònic (en fase estacionària) mitjançant *real-time* PCR (Taula 1 de l'article 3). Per una banda, els resultats obtinguts mostren que els gens de les tres classes de RNR (*nrdA*, *nrdJ* i *nrdD)* s'indueixen en condicions planctòniques anaeròbies (2.1, 85.1 i 110, respectivament) respecte a un cultiu planctònic aeròbic. La classe de RNR més induïda en condicions anaeròbies estrictes és la RNR de classe III, mentre que la classe menys induïda és la RNR de classe Ia. Sorprenentment, s'ha vist una inducció de la RNR de classe Ia en condicions anaeròbies respecte a les aeròbies, possiblement a causa de l'estrès provocat per la dificultat de *P. aeruginosa* per dividir-se en condicions anaeròbies [221]. Per una altra banda, en un biofilm l'expressió dels gens de les tres classes de RNR també es troben induïts respecte a un cultiu planctònic. La classe de RNR que més s'indueix en un biofilm és la RNR de classe II, unes 1500 vegades, a diferència d'un cultiu planctònic. En canvi, *nrdD* es troba més expressat en condicions planctòniques anaeròbies estrictes que en condicions de biofilm, possiblement a causa que en un biofilm pot haver-hi condicions de microaerofilia. Tot i així, **tant l'expressió de *nrdJ* com de *nrdD* augmenten durant la formació de biofilm.** Aquesta inducció dels gens *nrdJ i nrdD* en absència d'oxigen mostra que el factor de transcripció NrdR no reprimeix l'expressió de les RNRs de la classe II i la classe III, tal com s'havia descrit en un dels capítols anteriors, i per tant, els gens *nrdJa* i *nrdD* s'indueixen per un altre factor de transcripció en condicions anaeròbies [261].

Existeixen un seguit de factors de transcripció que regulen els gens que participen en el procés de la desnitrificació i altres gens induïts en condicions anaeròbies. Aquests factors de transcripció són l'Anr, el Dnr i el NarL (Apartat 1.2.2.), sent l'Anr el factor de transcripció que inicia la cascada de regulació. Resumidament, en absència d'oxigen el factor de transcripció Anr regula tant NarL com Dnr, mentre que Dnr és un sensor de l'òxid nítric (ON) i, NarL és un regulador dels gens que inicien la via de la desnitrificació [157]. Per tant, es va avaluar la inducció de *nrdJ* i *nrdD* en condicions anaeròbies degut a aquests factors de transcripció. Inicialment, es va comparar l'activitat del promotor de *nrdJ* (P*nrdJ*) i *nrdD* (P*nrdD*), clonats en el plasmidi pETS130, en les diferents soques de *P. aeruginosa* PAO1; *salvatge*, Δ*anr*, Δ*dnr* i Δ*narL* (Figura 3 de l'article 3). El medi de cultiu utilitzat contenia nitrat (KNO_3) o òxid nítric (ON) com a donadors d'electrons pel metabolisme anaeròbic de *P. aeruginosa*. Els resultats obtinguts no van mostrar cap afecte de l'expressió de P*nrdJ* i P*nrdD* en absència de *narL*. S'ha vist que en una soca mutant *narL* (on no hi ha inducció de l'expressió de *nrdR*) es manté l'expressió de *nrdJ* i *nrdD* com una soca salvatge, aquest resultat corrobora que NrdR no reguli *nrdJ* ni *nrdD* en condicions anaeròbies [261]. En canvi, es va observar una disminució de l'expressió de la RNR de classe II en les soques mutants Δ*anr* i Δ*dnr* comparant amb una soca salvatge de PAO1. Al contrari, no hi va haver diferències significatives en l'expressió de la RNR de classe III en les soques mutants Δ*anr* i Δ*dnr* (discutit en el capítol 4). Per tant, una vegada descartat un dels factors transcripcionals (NarL), l'estudi es va centrar en la inducció dels gens *nrdJ* i *nrdD* en anaerobiosi via Anr o Dnr.

Tenint en compte que el factor de transcripció Anr regula Dnr i que s'observa una desregulació de l'expressió de *nrdJ* en absència d'ambdós, *dnr i anr*, doncs, el factor de transcripció responsable de la inducció de *nrdJ* és Dnr. Aquest resultat s'ha corroborat mitjançant *western blot*, i s'ha vist que NrdJ s'indueix en anaerobiosi després de 3 h d'anaerobiosi en una soca PAO1 salvatge, però en absència de *dnr*, NrdJ no es troba induït (Figura 3 de l'article 3). A més, si es complementa la soca Δ*dnr* amb una còpia extra de Dnr plasmídica, els nivells de proteïna NrdJ s'esdevenen com una soca salvatge. Per tant, **Dnr indueix l'expressió de *nrdJ* en condicions anaeròbies**.

A partir d'una cerca bioinformàtica es va trobar una possible caixa d'unió dels factors de transcripció Anr o Dnr en tots dos promotors de les dues classes de RNRs (II i III). Actualment, no s'han trobat diferències entre les caixes d'unió d'Anr o de Dnr als promotors dels gens els quals s'han descrit com a regulats per Anr o Dnr [164]. Tot i així, es van localitzar caixes d'unió Anr i Dnr seguint la seqüència consens $TTGA^{T}/_{C}NNNN^{A}/_{G}TCAA$, en la posició -28 i -98 bp de l'inici de traducció dels promotors *nrdJ* i *nrdD*, respectivament. Per determinar la unió directa d'aquest factor de transcripció al promotor de *nrdJ* i *nrdD* es va realitzar una mutació dirigida de les possibles caixes d'unió de Dnr en els promotors plasmídics. Tot i no veure diferències en l'activitat del

promotor de *nrdD* en absència de *dnr* o en la mutació de la possible caixa Anr-Dnr, s'ha vist que *nrdD* s'indueix en condicions anaeròbies, doncs aquesta inducció a de ser deguda a algun altre factor de transcripció fins ara desconegut (Capítol 4). En canvi, l'expressió del promotor de *nrdJ* amb la mutació de la caixa Dnr-box és comparable amb l'obtinguda en les soques mutants per Δ*anr* i Δ*dnr* (Figura 4 de l'article 3). Per tant, Dnr s'uneix específicament en el promotor del gen *nrdJ*.

Posteriorment, es va voler demostrar si aquesta inducció de *nrdJ* per Dnr en condicions anaeròbiques també es produïa en condicions de biofilm. Es va analitzar l'activitat del promotor de cadascuna de les RNRs durant la formació de biofilm mesurant *gfp* i es va comparar amb l'expressió obtinguda en un cultiu planctònic (3h) (Figura 5 de l'article 3). Es va afegir el promotor del gen *oprF* com a control positiu, ja que es va veure que la seva expressió s'induïa en biofilms [175, 262]. L'expressió de *nrdJ* i d'*oprF* es va induir al llarg de la formació de biofilm. Tot i així, si la caixa d'unió de Dnr en el promotor de *nrdJ* es troba mutada, la inducció de l'expressió de *nrdJ* es disminueix. El resultat es va corroborar realitzant una *real-time* PCR, on es va veure una disminució de l'expressió de *nrdJ* en absència de Δ*dnr* durant la formació de biofilm (*fold-change* de 1500 en soca salvatge v.s. 25 en soca mutant Δ*dnr*). Per tant, **NrdJ s'indueix en biofilm a través de Dnr quan l'oxigen és limitant.**

Capítol 4: NrdD durant el creixement anaeròbic i la infecció de *P. aeruginosa*

En els capítols anteriors s'ha determinat l'expressió de *nrdA* i *nrdJ* en diferents condicions, com ara estrès oxidatiu, biofilm o creixement anaeròbic. Tot i així, no s'ha determinat cap factor de transcripció específic per la regulació del gen de la RNR de classe III, *nrdDG*. Tenint en compte que la majoria d'infeccions es consideren anaeròbies es va analitzar l'expressió dels gens de les diferents RNRs en diferents soques; tant a la soca *P. aeruginosa* PAO1 com a altres soques més recentment aïllades de pacients clínics, per tal de trobar diferències d'expressió dels gens RNR entre soques, així com, per analitzar específicament l'expressió de *nrdD*. Les soques de *P. aeruginosa* utilitzades en aquest capítol són les següents: PAO1, PA14, soques aïllades d'infeccions cròniques de pacients de FQ de l'Hospital Vall d'Hebron de Barcelona (PAET1, PAET2, PAET4, PAET6) i soques XDR (*Extensively Drug Resistant*) aïllades de pacients amb una infecció aguda (PA166, PA54, PA1016) [263].

En un estudi de l'any 2011 es va observar que les cèl·lules de la soca de *P. aeruginosa* PAO1 en condicions anaeròbies filamentaven [177]. Aquest estudi va suggerir que durant el procés de

desnitrificació *P. aeruginosa* produeix òxid nítric (ON) al medi com a producte intermediari, el qual inhibeix la síntesi de vitamina B_{12} (cofactor de la RNR de classe II) i per tant, afecta l'activitat de la RNR de classe II i provoca filamentació cel·lular degut a l'estrès cel·lular per la disminució dels nivells de dNTP dins la cèl·lula que no permet formar el *septum* divisiori [177, 179]. Sorprenentment, en aquest capítol, l'anàlisi de la morfologia cel·lular de les diferents soques aïllades de pacients clínics va demostrar, a diferència de la soca de *P. aeruginosa* PAO1, que les cèl·lules no es trobaven filamentades sota condicions d'anaerobiosi (Figura 1 de l'article 4). Es va demostrar que la filamentació cel·lular de la soca de *P. aeruginosa* PAO1 és degut a la falta general d'activitat de les RNRs que bloqueja la síntesi de l'ADN en condicions anaeròbies. Per tant, l'addició de vitamina B_{12} en un cultiu de *P. aeruginosa* PAO1 evita la filamentació cel·lular augmentant l'activitat d'una de les classes de RNR que poden ser actives en condicions anaeròbies, la RNR de classe II (Figura 1 de l'article 4, i posteriorment detallat en el capítol 5). A més, la sobreexpressió de la RNR de classe III, permet a *P. aeruginosa* PAO1, sintetitzar suficients nivells de dNTP per créixer en condicions anaeròbies sense estrès cel·lular, i doncs, sense filamentació cel·lular. Assumint que la síntesi de la vitamina B_{12} tan sols es produeix en condicions aeròbies [116] i per tant, la RNR de classe II no pot sintetitzar dNTP en absència del cofactor vitamina B_{12} (Capítol 5), s'ha suggerit que la RNR de classe III és la responsable de suportar la síntesi de dNTP durant el creixement anaeròbic de *P. aeruginosa* PAO1. Així doncs, la filamentació cel·lular de *P. aeruginosa* PAO1 és degut a la falta d'activitat de la RNR de classe III.

Es va analitzar si existien diferències d'expressió de cadascuna de les classes de RNR que expliqués la disminució d'activitat de la RNR de classe III de la soca de *P. aeruginosa* PAO1 en absència d'oxigen a diferència de les soques aïllades clíniques (Figura 2 de l'article 4). En condicions anaeròbies, es va observar una disminució de l'expressió de *nrdA* i *nrdJ* a les soques d'origen clínic respecte a la soca referent de *P. aeruginosa* PAO1, en canvi, també es va observar un **augment de l'expressió de *nrdD* en totes les soques aïllades clíniques** (9.69 vegades més a la soca PAET1, 9.25 vegades més a la soca PAET2, 3.04 vegades més a la soca PA166, 2.52 vegades més a la soca PA54 i 1.51 vegades més a la soca PA14). De la mateixa manera, es va observar un augment significatiu de la diferència d'expressió de *nrdD* entre condicions anaeròbies i aeròbies en les soques aïllades clíniques, menys significatiu a la soca de *P. aeruginosa* PAO1.

P. aeruginosa és un bacteri que s'adapta al medi produint una sèrie de mutacions genètiques que li permet expressar diferencialment les proteïnes adequades a la condició de creixement requerida [264, 265]. Per aquest motiu es va analitzar la seqüència dels promotors del gen *nrdD* de les diferents soques de *P. aeruginosa* obtingudes (PAO1, PA14, PAET1, PAET2, PAET4, PAET6, PA54, PA166 i PA1016) per si s'observava mutacions puntuals que expliquessin aquest augment de

l'expressió en les soques aïllades clíniques. Seqüenciant les regions promotores dels gens *nrd* es va observar un ***mismatch* d'un nucleòtid en la caixa d'unió de Anr-Dnr del promotor del gen *nrdD* de la soca de *P. aeruginosa* PAO1**. Aquests factors de transcripció (Anr i Dnr) participen en la regulació de gens essencials pel metabolisme anaeròbic mitjançant la unió a la seqüència consens **T**TGA$^{T}/_{C}$NNNN$^{A}/_{G}$TCAA del promotor del qual regulen [164]. En l'article 3, es va determinar que Dnr regulava l'expressió de la RNR de classe II en condicions d'anaerobiosi i biofilm [254] i l'any 2003 a *E. coli*, es va determinar que el factor de transcripció FNR (homòleg a Anr-Dnr *d'E. coli*) regulava el gen *nrdDG* a través de la unió directa a la caixa FNR del promotor [117].

En el promotor del gen *nrdD* de la soca de *P. aeruginosa* PAO1, la caixa d'unió de Anr-Dnr segueix la seqüència **C**TGACGCAGATCAA, mentre que la seqüència de totes les soques aïllades clíniques i PA14, segueixen la seqüència **T**TGACGCAGATCAA (Figura 3 de l'article 4). Així doncs, la caixa d'unió de Anr/Dnr al promotor de *nrdD* en la soca de *P. aeruginosa* PAO1 conté una mutació puntual d'una cisteïna (C) situada en la primera posició, en lloc d'una timina (T), com el promotor de les soques aïllades clíniques. Un estudi l'any 1996 a *P. aeruginosa*, va determinar que la unió d'Anr al promotor de l'operó *arcDABC* es perdia si es modificava el primer nucleòtid (C) de la caixa consens de FNR per una timina (T) [266], tal com succeeix en el promotor de *nrdD* de *P. aeruginosa* PAO1. La modificació d'aquest nucleòtid (C>T) en el promotor de *nrdD* de *P. aeruginosa* PAO1 retorna a l'expressió de *nrdD* a l'expressió observada en les soques aïllades clíniques augmentant l'expressió d'aquest. A més, la mutació cromosòmica d'ambdós factors de transcripció (Δ*anr* i Δ*dnr)* produeix la mateixa expressió de *nrdD* que la soca de *P. aeruginosa* PAO1 (pETS136-C). Aquest resultat indica que ni Anr ni Dnr es poden unir correctament en el promotor de *nrdD* de la soca de *P. aeruginosa* PAO1. A més, al complementar la mutació cromosòmica de les soques mutants pels factors de transcripció (Δ*anr* i Δ*dnr)* amb un vector que conté clonat el factor de transcripció Dnr, l'expressió de *nrdD* (pET196-T) és similar a l'obtinguda en les soques aïllades clíniques. Així doncs, **existeix una caixa Anr-Dnr activa en promotor de *nrdD* específica pel factor de transcripció Dnr**. Tot i així, l'expressió de *nrdD* es veu afectada si hi ha una mutació puntual del nucleòtid en la posició 1 (C) com s'observa en la soca de *P. aeruginosa* PAO1 (Figura 3 de l'article 4; creixement planctònic i Figura S2 de l'article 4; creixement en forma de biofilm). La disminució de l'expressió de *nrdD* en la soca de *P. aeruginosa* PAO1 en condicions d'anaerobiosi explica l'estrès cel·lular produït per la disminució de síntesi de dNTP en aquestes condicions provocant filamentació cel·lular i la inducció d'expressió dels gens *nrdA* i *nrdJ* (Figura S1 de l'article 4).

D'altra banda, la soca de *P. aeruginosa* PAO1 és la soca de referència de *P. aeruginosa* en la majoria de laboratoris. Va ser aïllada d'una infecció en una ferida l'any 1954 a Melbourne

(Australia: American Type Culture Collection ATCC 15692) [267]. El genoma de *P. aeruginosa* PAO1 és gran (6.3 Mb) i té capacitat d'adaptar-se en diferents condicions ambientals, tot i tenir la majoria de mecanismes endògens patogènics suprimits. Altres soques de *P. aeruginosa*, aïllades més recentment, com ara la soca PA14, són més virulentes que la soca referent PAO1 [268, 269]. Específicament, *P. aeruginosa* PA14 conserva dues illes de patogenicitat (PAPI i PAPII) en el seu genoma [270]. Per tant, les soques aïllades de pacients clínics estan més adaptades a condicions de creixement anaeròbies, com ara les infeccions cròniques o formació de biofilms. Alguns estudis han demostrat la implicació de NrdD durant el procés d'infecció, tant a *P. aeruginosa* [110], com en altres microorganismes; *E. coli* LF82, *Porphyromonas gingivalis, Staphylococcus aureus* i *Streptococcus sanguinis* [128, 271-274]. Per tant, en aquest treball es va analitzar com afecta la modificació d'aquest nucleòtid en l'expressió de *nrdD* i en la virulència de *P. aeruginosa* PAO1 durant la infecció del peix zebra (*Danio rerio*). En primer lloc, es va observar que la classe de RNR més expressada durant el procés d'infecció *in vivo* era *nrdD* (Figura 4 de l'article 4), augmentant encara més la seva expressió sota el promotor P*nrdD* (C>T) (com el promotor de les soques aïllades clíniques) (Figura 4 de l'article 4). En segon lloc, es va analitzar la virulència de diferents soques de *P. aeruginosa* PAO1 que indueixen l'expressió de *nrdD* i que presenten una morfologia cel·lular no filamentada (Figura S1 de l'article 4). La inducció de *nrdD* en aquestes soques de *P. aeruginosa* PAO1 va provocar la disminució de l'expressió de *nrdA* i *nrdJ* en condicions anaeròbies, tal com s'havia observat en les soques aïllades clíniques (Figura 2 de l'article 4). Els resultats de mortalitat van mostrar un augment de la supervivència de *D. rerio* si s'infectaven amb la soca mutant Δ*nrdD*, i una disminució de la supervivència si es complementa la mutació Δ*nrdD*. En canvi, la virulència de *P. aeruginosa* PAO1 va augmentar en les soques que sobreexpressaven el gen *nrdD* (Figura 4 de l'article 4). Per tant, **la RNR de classe III és important durant el procés d'infecció**, i **la disminució de l'expressió de *nrdD* afecta la virulència de *P. aeruginosa***, tal com s'observa en la soca de *P. aeruginosa* PAO1. Així doncs, la soca de *P. aeruginosa* PAO1 no és la millor soca de referència per l'estudi de la virulència i el creixement anaeròbic de *P. aeruginosa.*

Capítol 5: La vitamina B_{12} i les RNRs de *P. aeruginosa*

L'expressió dels gens de cadascuna de les classes de RNR de *P. aeruginosa* es troba regulada per factors de transcripció dependents de les condicions ambientals. Aquestes condicions ambientals també determinaran l'activitat de cadascuna de les classes de RNR, com ara; l'activitat

de la RNR de classe Ia (NrdA) depèn de la presència d'oxigen, l'activitat de la RNR de classe III (NrdD) depèn de l'absència d'oxigen i l'activitat de la RNR de classe II (NrdJ) del cofactor adenosilcobalamina o vitamina B_{12} [124].

El cofactor vitamina B_{12} de l'enzim NrdJ sovint és sintetitzat *de novo* per bacteris. Tot i ser essencial pel metabolisme dels animals (i humans) i d'algunes bacteris, no té cap funció en plantes, fongs o altres bacteris [260]. Existeixen dues vies de síntesi de la vitamina B_{12} diferenciades pel punt d'inserció del cobalt; la ruta aeròbia o d'inserció de cobalt posterior (estudiada principalment a *P. denitrificans*) i la ruta anaeròbia o ruta d'inserció de cobalt inicial (estudiada a *S. typhimurium*) [260]. Durant la síntesi de la vitamina B_{12} hi participen almenys 25 gens específics de cada ruta (*cbi* o *cob*) per convertir l'Uropirinògen (uroIII) a Adenosilcobalamina (AdoCbl).

Inicialment, la majoria de reaccions dependents de vitamina B_{12} en bacteris participaven en la fermentació anaeròbica de molècules petites. Posteriorment, l'aparició d'oxigen a la terra i la respiració aeròbia va provocar que molts microorganismes perdessin la capacitat de sintetitzar vitamina B_{12}, tot i així, actualment, s'han mantingut reaccions independents d'oxigen però dependents de vitamina B_{12}, com ara la síntesi de la metionina o la reducció de nucleòtids (RNR de classe II). A més, en alguns organismes, la vitamina B_{12} no només actua com a cofactor per diferents enzims sinó que també, pot actuar unint-se a una seqüència conservada anomenada *riboswitch*, per regular l'expressió post-transcripcional o post-traduccional de diferents gens [222].

Específicament en la RNR de classe II de *P. aeruginosa*, la vitamina B_{12} s'uneix a la subunitat NrdJa formant el complex NrdJa-NrdJb i protegint el radical essencial per a la reacció [223]. Per tant, en absència del cofactor vitamina B_{12} la RNR de classe II no tindrà activitat enzimàtica. Paradoxalment, la síntesi de vitamina B_{12} a *P. aeruginosa* ha sigut observada tan sols en condicions aeròbies [116], com a *P. denitrificans*, tot i així, l'expressió de *nrdJ* s'ha vist induïda en condicions anaeròbies, en fase estacionària i durant la formació de biofilm [24, 110, 254]. Per tant, a *P. aeruginosa* l'expressió de la RNR de classe II i la síntesi de vitamina B_{12} es produeix en condicions diferents. Per tal de resoldre aquesta incoherència es van analitzar les condicions de síntesi de la vitamina B_{12} i d'activitat de NrdJ.

P. aeruginosa utilitza principalment la RNR de classe Ia i la RNR de classe III, en condicions aeròbiques i anaeròbiques, respectivament, per sintetitzar els dNTP per a la divisió cel·lular [24, 116] (Capítol 4), en canvi, s'ha vist que la RNR de classe II no és essencial en aquestes dues condicions (Figura 1 de l'article 5). Aeròbiament, s'ha observat que l'absència de *nrdJ* no afecta el creixement de *P. aeruginosa* PAO1. Però, en condicions anaeròbies, *P. aeruginosa*, tot i expressar *nrdJ*, utilitza NrdD per sintetitzar dNTP. La mutació de *nrdJ* provoca una disminució del creixement anaeròbic de *P. aeruginosa* PAO1 degut l'expressió deficient de *nrdD* produint

filamentació cel·lular (Capítol 4) [116, 177]. Tot i així, en condicions de microaeròfila, com ara en un biofilm, NrdJ pot ser actiu en presència de vitamina B_{12}. En aquest capítol, s'ha observat que l'activitat de NrdJ és independent d'oxigen i a més, que la via aeròbica d'inserció del cobalt (utilitzant CobN) és important per el creixement de *P. aeruginosa* en condicions aeròbies i en un biofilm (Figura 1 i 2 de l'article 5). Tenint en compte que les parts més externes d'un biofilm estan en contacte amb l'oxigen, hem suggerit que la síntesi de vitamina B_{12} es produeix en la part aeròbia del biofilm i posteriorment es difon a través de l'estratificació del biofilm. A més, s'ha vist que l'addició de vitamina B_{12} en un biofilm comporta un augment del seu gruix, degut a que l'aport extern de vitamina B_{12} permet activar NrdJ, sintetitzant dNTPs en les parts més anaeròbies o microaeròbies d'un biofilm de *P. aeruginosa* PAO1 on no hi ha suficient activitat de NrdD (Figura 2 de l'article 5).

També, es va observar un augment de l'expressió dels gens *nrd* degut a la inhibició o disminució de l'activitat de les RNRs de classe Ia i III, aeròbiament i anaeròbiament, respectivament. Tot i així, mitjançant l'addició de vitamina B_{12}, NrdJ és actiu i permet la síntesi de dNTP, fet que provoca la disminució de l'expressió dels gens *nrd* (Figura S1 de l'article 5 i Capítol 4). Tot i així, no es va observar cap regulació via B_{12}-*riboswitch*, a diferència de l'observada en els gens de la síntesi de la metionina o cobalamina de *P. aeruginosa* [123, 222] o de les RNRs (*nrdAB* i *nrdDG*) de *S. coelicolor* [97]. Així doncs, l'augment de *nrdJ* en condicions anaeròbies observat en diferents estudis s'explicaria per la disminució d'oxigen en un biofilm, així com, per la disminució d'activitat de la RNR de classe III degut a la deficiència d'expressió de *nrdD* en la soca PAO1 (Capítol 4).

Per una altra banda, per corroborar la disponibilitat de vitamina B_{12} durant el creixement de *P. aeruginosa* es va quantificar específicament els nivells de vitamina B_{12} en condicions aeròbies, anaeròbies i de formació de biofilm a diferents soques de *P. aeruginosa*. Es va observar vitamina B_{12} tan sols en condicions aeròbies i biofilm, tant en la soca PAO1 com a les dues soques més recentment aïllades de pacients clínics (PAET1 i PA14) (Taula 1 de l'article 5). En canvi, no es van detectar nivells de vitamina B_{12} en un cultiu crescut en condicions anaeròbies. Sorprenentment, es va observar un augment dels nivells de vitamina B_{12} en condicions de creixement en biofilm respecte a un cultiu planctònic en totes les soques. Diferents anàlisis transcriptòmics han observat una inducció dels gens de la síntesi de la vitamina B_{12} (*cob*) en la formació de biofilm [275], en soques mucoides [276] o en fase estacionària [277], en canvi, una disminució en condicions d'anaerobiosi [171]. Tot i així, es va observar més concentració de vitamina B_{12} en les soques PA14 i PAET1 que en PAO1, suggerint que en condicions aeròbies aquestes soques indueixen més la síntesi de vitamina B_{12} (Figura 3 de l'article 5). En canvi, en un biofilm, es va quantificar més

concentració de vitamina B_{12} en la soca PAO1 que en les soques PA14 i PAET1. En el capítol 4, es va demostrar que aquestes soques expressen els gens de les RNRs diferencialment. Per tant, la concentració diferencial de vitamina B_{12} explicaria una diferència d'activitat de les RNRs en les soques PA14 i PAET1, com ara, la resistència al tractament per HU (Radical *scavenger*, apartat 1.1.8.) degut a un augment d'activitat de NrdJ [24]. Tot i així, es necessiten més experiments que corroborin el mecanisme de resistència d'aquestes soques clíniques al tractament per HU.

Per tant en aquest capítol, s'ha demostrat que la RNR de classe II (NrdJ) és activa en condicions aeròbies i durant la formació de biofilm, degut a la síntesi de vitamina B_{12} en aquestes condicions, la qual es pot difondre pel biofilm permetent la síntesi de dNTP a través de NrdJ, tal com s'havia suggerit en el capítol 3. Però, en canvi, NrdJ no és activa en condicions d'anaerobiosi, ja que no s'han detectat nivells de vitamina B_{12} ni cap evidència de síntesi sota aquestes condicions (Figura 4 de l'article 5). Tot i així, NrdJ té activitat en absència d'oxigen si la vitamina B_{12} és captada del medi.

Conclusions

Tenint en compte les dades explicades en els apartats anteriors, en aquesta tesi s'ha donat resposta als objectius plantejats anteriorment, i s'han aportat dades que corroboren i amplien les dades bibliogràfiques inicials amb les quals contàvem, oferint nova informació sobre les RNRs bacterianes. Així doncs, les conclusions de la tesi són les següents:

1.- El factor de transcripció NrdR s'indueix en fase exponencial, i a través del factor de transcripció NarL s'expressa sota condicions anaeròbies.

2.- El factor transcripcional NrdR reprimeix l'expressió dels gens *nrd* (*nrdA, nrdJ i nrdD)* de *P. aeruginosa* regulant els seus nivells d'expressió sota diferents condicions ambientals. En condicions aeròbiques, NrdR reprimeix l'expressió dels gens de les tres classes de RNR, mentre que en condicions anaeròbies, NrdR només reprimeix significativament l'expressió del gen *nrdA.*

3.- El gen de la topoisomerasa I (*topA*) es troba regulat per NrdR a través de la unió d'aquest a una sola caixa NrdR en el seu promotor. A diferència dels gens *nrd*, NrdR indueix l'expressió de *topA.*

4.- La deleció del gen *nrdR* provoca la desregulació de l'expressió d'altres gens diferents de *nrd* (segurament de manera indirecta), tant en condicions aeròbies (47 gens) com anaeròbies (111 gens).

5.- El factor de transcripció AlgR regula l'expressió de les RNRs de classe Ia i II durant el procés de formació de microcolònies i de biofilm, en canvi, no regula l'expressió del gen de la RNR de classe III ni del gen PA1157. AlgR s'uneix a una sola caixa AlgR en el promotor del gen *nrdA* induint la seva expressió. En canvi, en el promotor del gen *nrdJ*, AlgR s'uneix a dues caixes AlgR diferents; la caixa AlgR 1 reprimeix l'expressió de *nrdJ*, mentre que la caixa AlgR 2 l'indueix.

6.- El factor de transcripció AlgR fosforilat, a través d'AlgZ, indueix els gens de les RNRs de classe Ia i II (*nrdA* i *nrdJ*) en condicions d'estrès oxidatiu cel·lular.

7.- Les RNRs de classe II i III (NrdJ i NrdD) són importants per a la formació d'un biofilm madur de *P. aeruginosa* on l'oxigen és limitant.

8.- Els gens de les RNRs de classe II i III (*nrdJ* i *nrdD*) s'indueixen en condicions anaeròbies i durant la formació de biofilm a través del factor de transcripció que regula els gens expressats en anaerobiosi, el Dnr. La RNR de classe II és la RNR més induïda en condicions de formació de biofilm, mentre que la RNR de classe III, en condicions estrictes d'anaerobiosi.

9.- La soca de *P. aeruginosa* PAO1 filamenta en condicions d'anaerobiosi, a diferència de les soques aïllades clíniques, degut a l'estrès produït per la reducció de l'activitat o de l'expressió de la RNR de classe III.

10.- Existeix una mutació d'un nucleòtid en el promotor del gen *nrdD* a la soca de laboratori *P. aeruginosa* PAO1 que altera la unió del factor de transcripció Dnr. Aquesta mutació redueix l'expressió de *nrdD* en la soca *P. aeruginosa* PAO1, en comparació amb l'expressió de *nrdD* en les soques de *P. aeruginosa* aïllades clíniques. L'estrès produït per la reducció de l'expressió de *nrdD* en la soca *de P. aeruginosa* PAO1 provoca la inducció l'expressió de *nrdA* i *nrdJ* sota condicions anaeròbies.

11.- La RNR de classe III és essencial pel creixement en condicions anaeròbies de *P. aeruginosa*, així com també durant el procés d'infecció. La reducció de l'activitat de la RNR de classe III provoca una disminució de la virulència de *P. aeruginosa* PAO1.

12.- *P. aeruginosa* sintetitza vitamina B_{12} en fase estacionària sota condicions aeròbiques a través de la via de biosíntesi d'incorporació del cobalt posterior, on hi participa el gen *cobN*. No hi ha síntesi de vitamina B_{12} en condicions anaeròbiques, per tant, la RNR de classe II, tot i ser expressada, no és activa enzimàticament.

13.- La síntesi de vitamina B_{12} a *P. aeruginosa* es troba induïda en condicions de creixement en forma de biofilm. Els nivells de vitamina B_{12} es difondran al llarg de l'estratificació del biofilm permetent la síntesi de dNTP a través de NrdJ.

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