Table 1 *(cont.)*

Organism	Relevant features	Source or reference*
pUA943	As pGem-T, but carrying a 1039 bp PCR fragment containing the *S. typhimurium cpdA* gene	This study
pUA944	As pUA943, but *cpdA*::ΩKm	This study
pUA945	As pGP704, but carrying a *cpdA*::ΩCm construction	This study
PUA948	pLV106 carrying a *pepE*::*lacZ* fusion; Km^R Gm^R	This study
pUA950	As pUA949, but carrying the 280 bp fragment containing the *S. typhimurium flhD* promoter	This study

* ATCC, American Type Culture Collection, Manassas, VA, USA; SGSC, Salmonella Genetic Stock Center, University of Calgary, Canada.

Table 2. Oligonucleotide primers used in this work

Primer	Sequence (5′–3′)*	Position†
FurA‡	CAACAGGACAGATCCGC	−16
FurB‡	GTCACGTCATCGTGCGC	+451
FlhD1§	*GAATTC*CTCCGTTGTATGTCACG	−253
FlhD2§	*GGATCC*GCAACTCGGATGTATGC	+27
FlgA1‖	*GAATTC*GGTTTCTTCCCGTCCACG	−333
FlgA2‖	*GGATCC*CGCCACGGCGAATCCTCG	+33
FliA1¶	*GAATTC*CACGGCGAAGATACAGG	−370
FliA2¶	*GGATCC*CCATTACACCTTCAGCGG	+33
FliC1#	*GAATTC*GTTATCGGCAATCTGGAGG	−219
FliC2#	*GGATCC*CAGACCGGAAGACAGACGC	+108
CpdA4**	CCCGGGGTATCCAGCGTAAAGTTCG	+693
CpdA6**	*GAATTC*GAAACGGTGAGCTATCAGG	−346
PepE1††	*GAATTC*TAATTTTTCAGGCGATAC	−155
PepE2††	*GGATCC*CAATTGATTCGCTATCAG	+81

* When present, added restriction sites are shown in italics.

† Position of the 5′ end of the oligonucleotide with respect to the translational start point of each *S. typhimurium* gene.

‡ Primers used to obtain the 467 bp fragment containing the *S. typhimurium fur* gene.

§ Primers used to obtain the 280 bp fragment containing the *S. typhimurium flhD* promoter.

‖ Primers used to obtain the 366 bp fragment containing the *S. typhimurium flgA* promoter.

¶ Primers used to obtain the 403 bp fragment containing the *S. typhimurium fliA* promoter.

Primers used to obtain the 327 bp fragment containing the *S. typhimurium fliC* promoter.

** Primers used to obtain the 1039 bp fragment containing the *S. typhimurium cpdA* gene.

†† Primers used to obtain the 248 bp fragment containing the *S. typhimurium pepE* promoter.

with T4 DNA polymerase to obtain blunt ends and inserted into the single *Sma*I cloning site of the low-copy-number pLV106 plasmid. To prevent any possible effect of the pLV106-*tet* promoter on the expression of the gene to be studied, only clones containing a pLV106 plasmid carrying the *lacZ* fusion in the opposite transcriptional direction to this promoter were selected for further work. Finally, plasmids containing the constructed fusions were introduced by biparental mating into desired *S. typhimurium* strains. The activity of *β*-galactosidase was assayed as described by Miller (1991). The enzyme units reported here were the means of at least three independent assays and all values were reproducible to within an error of ±10%.

cAMP determinations. The intracellular concentration of cAMP was determined using the cAMP enzyme immunoassay kit (Amersham Pharmacia Biotech), according to the instructions specified by the manufacturer. To do this, culture samples at different points during the exponential growth phase (OD_{550} of 0·2, 0·4 and 0·8 for cells growing in the absence of the chelating agent DPD, and 0·1, 0·2 and 0·4 for those growing in the presence of DPD) were taken. After boiling for 5 min in lysis buffer and centrifugation at 1500 ***g*** for 3 min at 4 °C, the supernatants were immediately frozen for use later in the assay. The intracellular concentration of cAMP obtained was in the range of values reported by Saier *et al*. (1975) in *S. typhimurium* cells. All cAMP determinations

were carried out independently at least three times and the standard deviation among each one of the triplicates was never higher or lower than 10%.

Protein analysis. Outer-membrane proteins from *S. typhimurium* wild-type or *fur* strains were extracted from cultures grown under the desired conditions as described by Ferreiros *et al.* (1990). Briefly, cultures were centrifuged at 48000 *g* and pellets were resuspended in 0·1 M acetate buffer/0·2 M lithium chloride at pH 5·8, incubated for 2 h at 45 °C in a shaking water bath and passed through a 21-gauge needle. These suspensions were then centrifuged at 10000 *g*, the pellets being discarded. Membrane fragments were obtained from the supernatant by centrifugation at 30000 *g* for 2·5 h, and the pellet was resuspended in distilled water. The protein concentration of outer-membrane samples was determined by the Lowry method and their profiles were examined by 12% PAGE in the presence of SDS (Laemmli, 1970).

To confirm the identity of the 52 kDa protein, SDS-PAGE gels were electroblotted onto polyvinylidene difluoride membranes (Bio-Rad) and stained with Coomassie blue. This protein was then recovered from the membrane and its N-terminal amino acid sequence was determined by Edman degradation using Protein Sequencer 477A (Applied Biosystems).

RESULTS AND DISCUSSION

Expression of cAMP-regulated genes in *S. typhimurium fur* cells

The synthesis of flagella in *S. typhimurium* requires more than 50 genes, which are distributed in 17 identified operons (Chilcott & Hughes, 2000). Expression of these genes follows a hierarchic cascade, known as the flagellar regulon, in which three classes of promoters have been identified. Class 1 consists of the promoter of the transcriptional unit *flhDC*, also known as the master operon (Chilcott & Hughes, 2000). The master operon is under cAMP control (Yokota & Gots, 1970; Silverman & Simon, 1974). FlhD and FlhC are the activators of Class 2 promoters (Kutsukake *et al.*, 1990; Liu & Matsumura 1994). They regulate, among other genes involved in flagellum biosynthesis, the expression of *fliA*, which is an alternative sigma factor (σ^{28}) specifically required for the transcription of Class 3 promoters (Ohnishi *et al.*, 1990). Promoters of the *fliC* and *fljB* genes encoding, respectively, each of the two different flagellins which *S. typhimurium* cells can display, belong to Class 3 (Chilcott & Hughes, 2000).

To determine the effect of the *fur* mutation in the flagellar regulon, genes belonging to each one of the three promoter classes were selected. Expression of *flhD* (Class 1), *fliA* and *flgA* (Class 2), and *fliC* (Class 3) promoters was analysed through *lacZ* fusions. Results obtained indicated that all three genes display a significantly lower transcription in the *fur* mutant than in the wild-type strain (Fig. 1). From these data it can be inferred that the inhibition of *fliC* gene expression should be attributed to the hierarchic organization of the flagellar regulon. Thus, the decrease in *flhCD* operon transcription would lead to a lower concentration of sigma factor σ^{28}, which, consequently, would give rise to a lower expression of Class 3 promoters.

It must be noted that the addition of DPD did not modify either *fliC* expression in wild-type or *fur* cells, nor the difference existing between these strains (Fig. 1).

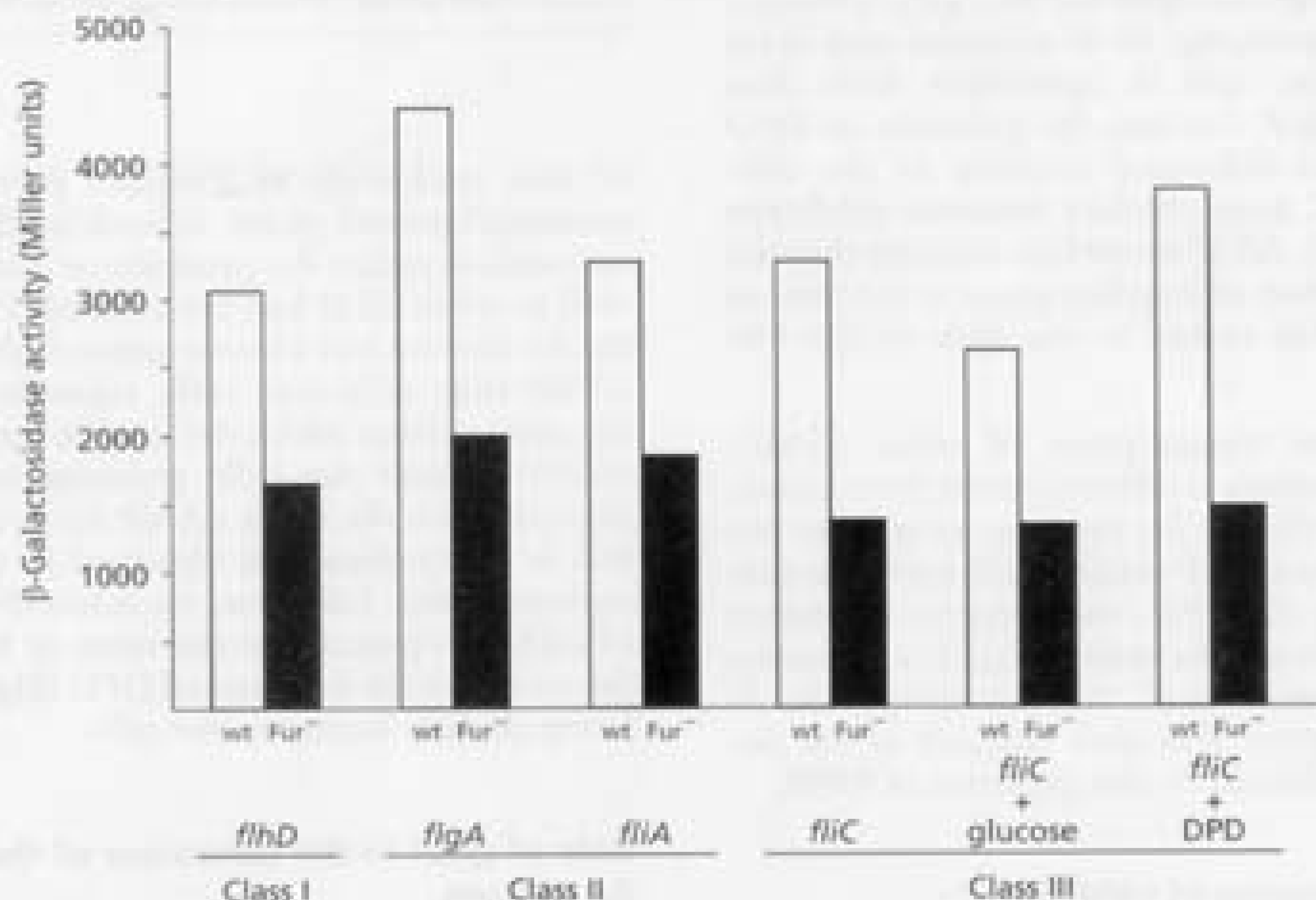

Fig. 1. Basal expression of several promoters belonging to the *S. typhimurium* flagellar regulon. β-Galactosidase synthesis was measured from a fusion of each promoter with *lacZ* in both wild-type (wt) and *fur* (Fur⁻) strains growing in LB medium in the absence or presence of either glucose or DPD. β-Galactosidase activities were measured from samples taken from mid-exponential-phase cultures (OD_{550} about 0·4).

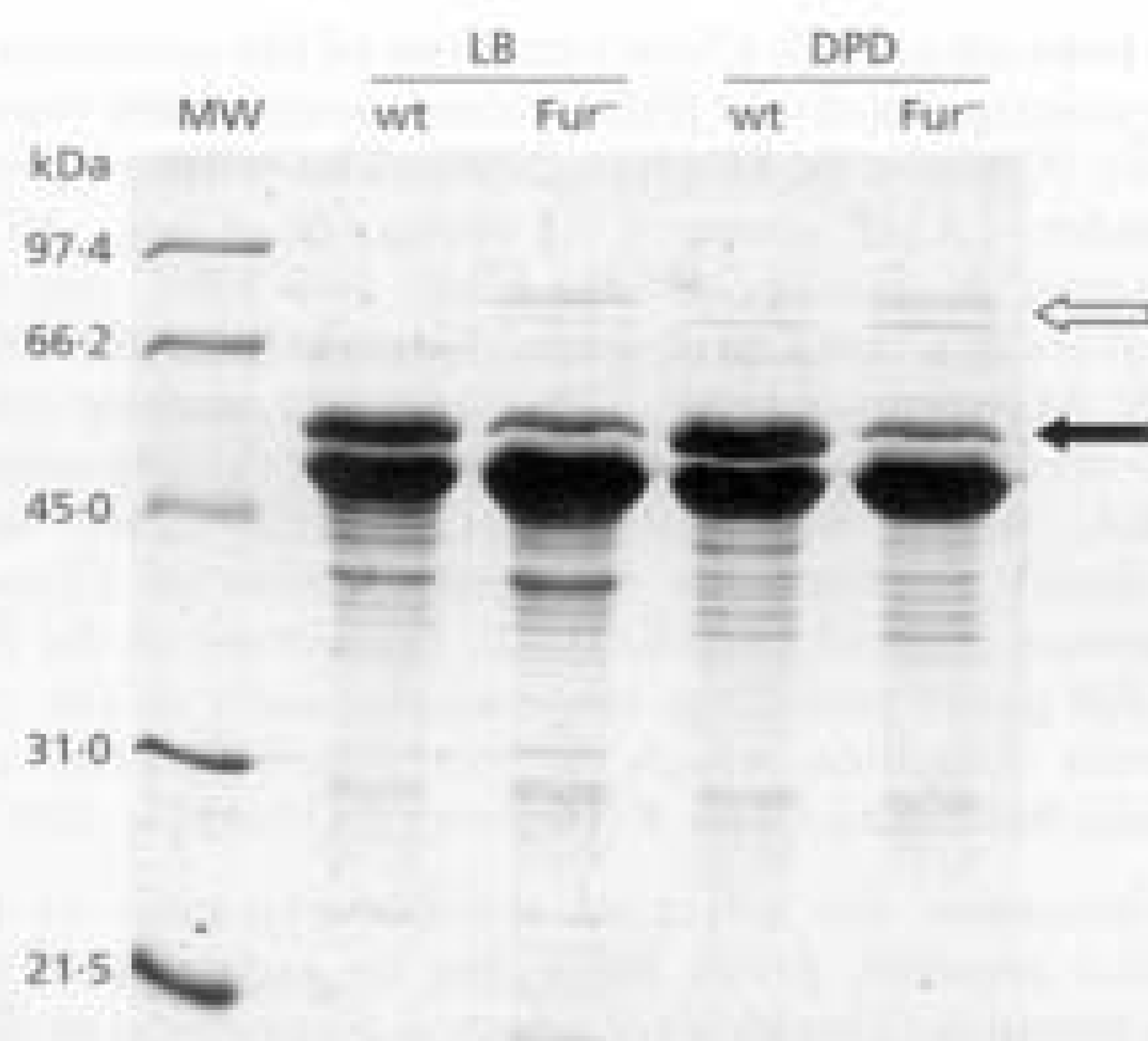

Fig. 2. SDS-PAGE profiles of outer-membrane proteins from wild-type and *fur* cells growing in LB medium in the presence or absence of DPD. White and black arrows indicate the *fur*-dependent high-molecular-mass proteins and the 52 kDa protein corresponding to the product of *fliC*, respectively.

Furthermore, the presence of glucose produces a decrease in *fliC* expression in the wild-type strain of a similar magnitude as that reported for *E. coli* (Bertin *et al.*, 1994), but does not produce any effect in the *fur* mutant (Fig. 1). The same results were obtained when SDS-PAGE profiles of outer-membrane proteins of wild-type and *fur* cells were analysed. Hence, the *fur* mutant showed a significant decrease in the amount of a 52 kDa protein (Fig. 2) corresponding to the *fliC* gene product, as determined by sequencing its N-terminal end (data not shown). Likewise, and in agreement with data obtained from *fliC-lacZ* fusions, the presence of DPD does not abolish the difference existing in the concentration of the *fliC* gene product between wild-type and *fur* strains (Fig. 2). All of these data indicate that the decrease in transcription of flagellar genes is not related to iron starvation, but rather to the lack of the Fur protein.

To ascertain whether transcription of other cAMP-CRP-dependent promoters is affected in the *fur* mutant, the induction of the *E. coli lac* operon, as well as the basal expression of the cAMP-regulated *S. typhimurium pepE* gene (Conlin *et al.*, 1994), encoding an α-aspartyl dipeptidase, was analysed. As seen in Fig. 3, expression of both transcriptional units (*E. coli lac* operon and *S. typhimurium pepE* gene) was also reduced in the *fur* strain, but was not affected by the presence of DPD.

Intracellular concentration of cAMP in *S. typhimurium fur* cells

The data shown above confirm that a connection between the behaviour of *fur* cells and cAMP may exist. For this reason, we decided to analyse the concentration

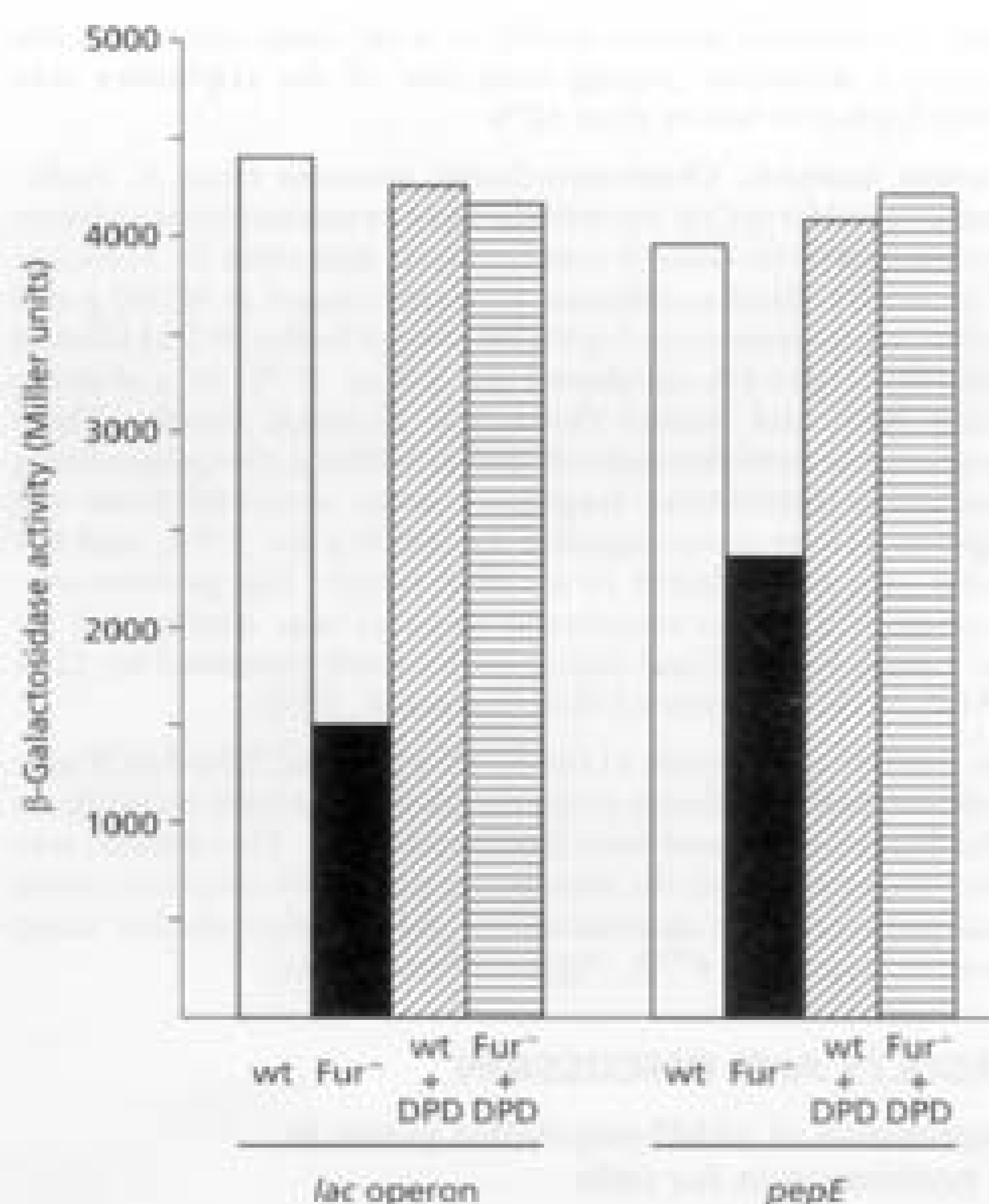

Fig. 3. Basal expression of the *S. typhimurium pepE* promoter and IPTG-mediated induction of the *E. coli lac* operon. Expression of the *lac* operon was measured 90 min after IPTG addition. The *lac* operon was present on the F′128 plasmid in both wild-type and *fur* strains of *S. typhimurium* growing in LB medium in the presence or absence of DPD. β-Galactosidase activities were measured from samples taken from mid-exponential-phase cultures (OD_{550} about 0·4).

of this nucleotide at different points during the exponential growth phase, in both wild-type and *fur* strain cultures, in either the presence or absence of glucose, as well as when DPD had been added. Fig. 4(a) shows how the *fur* mutant has a lower intracellular concentration of cAMP than wild-type cells, regardless of the OD_{550} of the culture from which the sample was taken. Moreover, wild-type and *fur* cells growing in the presence of glucose have the same cAMP level (Fig. 4b), although this is lower than that observed in the absence of this carbon source. Likewise, the intracellular concentration of cAMP is practically the same in both wild-type and *fur* strains in the presence of DPD (Fig. 4c), with the level being slightly higher in *fur* cells.

Role of *cpdA* in the behaviour of the *S. typhimurium fur* mutant

It has been shown that the enzymic activity of the cyclic 3′,5′-cAMP phosphodiesterase of *E. coli* encoded by *cpdA* is strongly stimulated *in vitro* by iron (II) (Nielsen *et al.*, 1973; Imamura *et al.*, 1996). Likewise, it is known

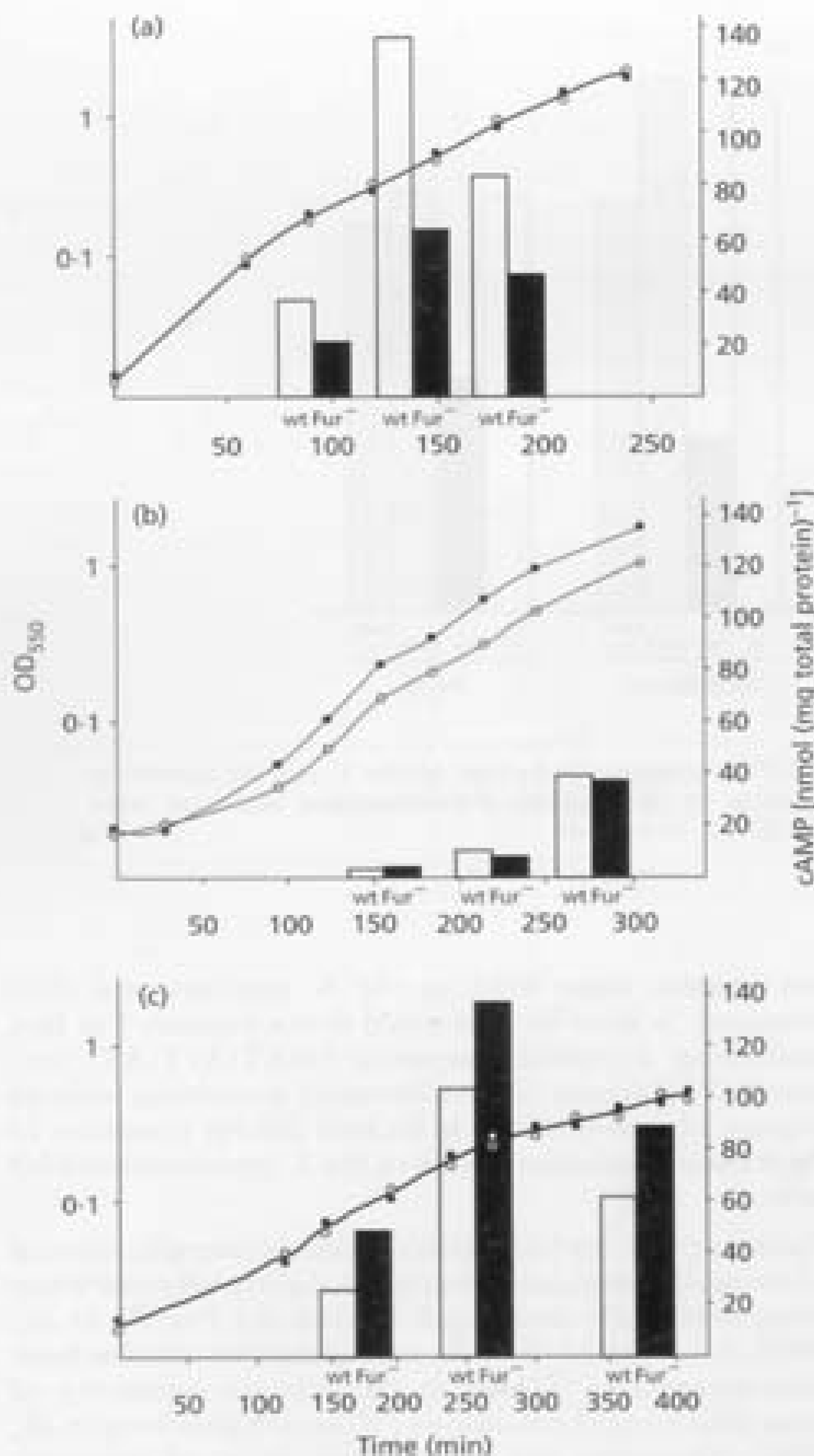

Fig. 4. Intracellular cAMP concentration of wild-type and *fur* strains of *S. typhimurium* at different points during exponential growth phase. Growth occurred in LB medium (a) in the presence of either glucose (b) or DPD (c). Growth of wild-type and *fur* cells is represented by closed and open circles, respectively. Samples were taken at OD_{550} values of 0·1, 0·2 and 0·4, or at OD_{550} values of 0·2, 0·4 and 0·8, depending on whether DPD was added or not, respectively. In all cases, values are presented in nmol cAMP (mg total protein of culture samples)$^{-1}$.

that the Fe(II)/Fe(III) ratio is higher in Fur mutants than in wild-type *E. coli* cells and that part of this Fe(II) is not bound to iron-storage proteins as firmly in this mutant as in the wild-type strain (Abdul-Tehrani *et al.*, 1999). Results reported above indicate that the lower concentration of cAMP in *S. typhimurium fur* mutants is related to intracellular iron availability. So, iron-depleted cells (those being cultured in the presence of DPD) of both wild-type and *fur* strains showed the same cAMP levels (Fig. 4c). In contrast, the *fur* mutant, which shows a constitutive expression of iron-uptake mechanisms and lacks the predicted protective effect of the Fur protein in iron-overloading conditions (Touati *et al.*, 1995), presents a lower level of cAMP (Fig. 4a) when growing in an iron-rich medium (in the absence of DPD). For all of these reasons, we decided to analyse whether the product of *cpdA* is responsible for the low cAMP level of *S. typhimurium fur* cells. To carry this out, the *S. typhimurium cdpA* gene was isolated by using oligonucleotide primers shown in Table 2 and mutagenized by insertion of an ΩKm resistance cassette into its internal *Hin*dIII site. This *cpdA*::ΩKm construction was introduced by marker exchange into both *S. typhimurium* Rif^R wild-type and Fur^- strains and its presence confirmed by both PCR and Southern analysis (data not shown). Fig. 5 indicates that the intracellular concentration of cAMP is restored in double *cpdA fur* mutants, this value being slightly higher than that shown by wild-type cells.

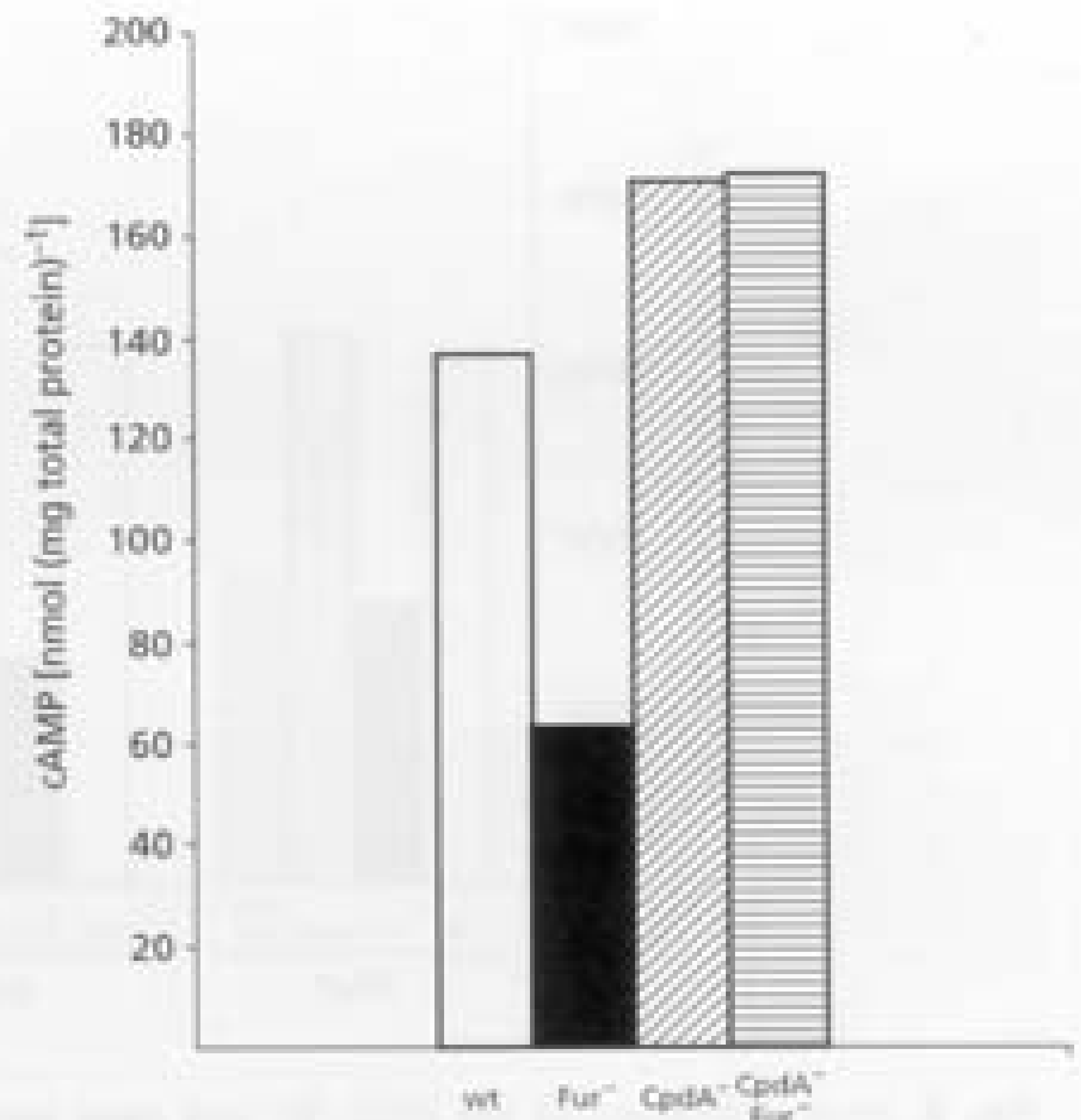

Fig. 5. Intracellular cAMP concentration in wild-type, *fur*, *cpdA* and *fur cpdA* cells of *S. typhimurium* in mid-exponential-phase growth (OD_{550} about 0·4). In all cases, values are presented in nmol cAMP (mg total protein of culture samples)$^{-1}$.

According to these results, expression of both *pepE* and the *E. coli lac* operon in the *S. typhimurium cpdA fur* double mutant is practically the same as in wild-type cells (Fig. 6). In agreement with previous studies (Nielsen *et al.*, 1973; Imamura *et al.*, 1996), the higher level of Fe(II) in *S. typhimurium* Fur cells could stimulate the activity of the cyclic phosphodiesterase encoded by *cpdA* which would then produce a decrease in the

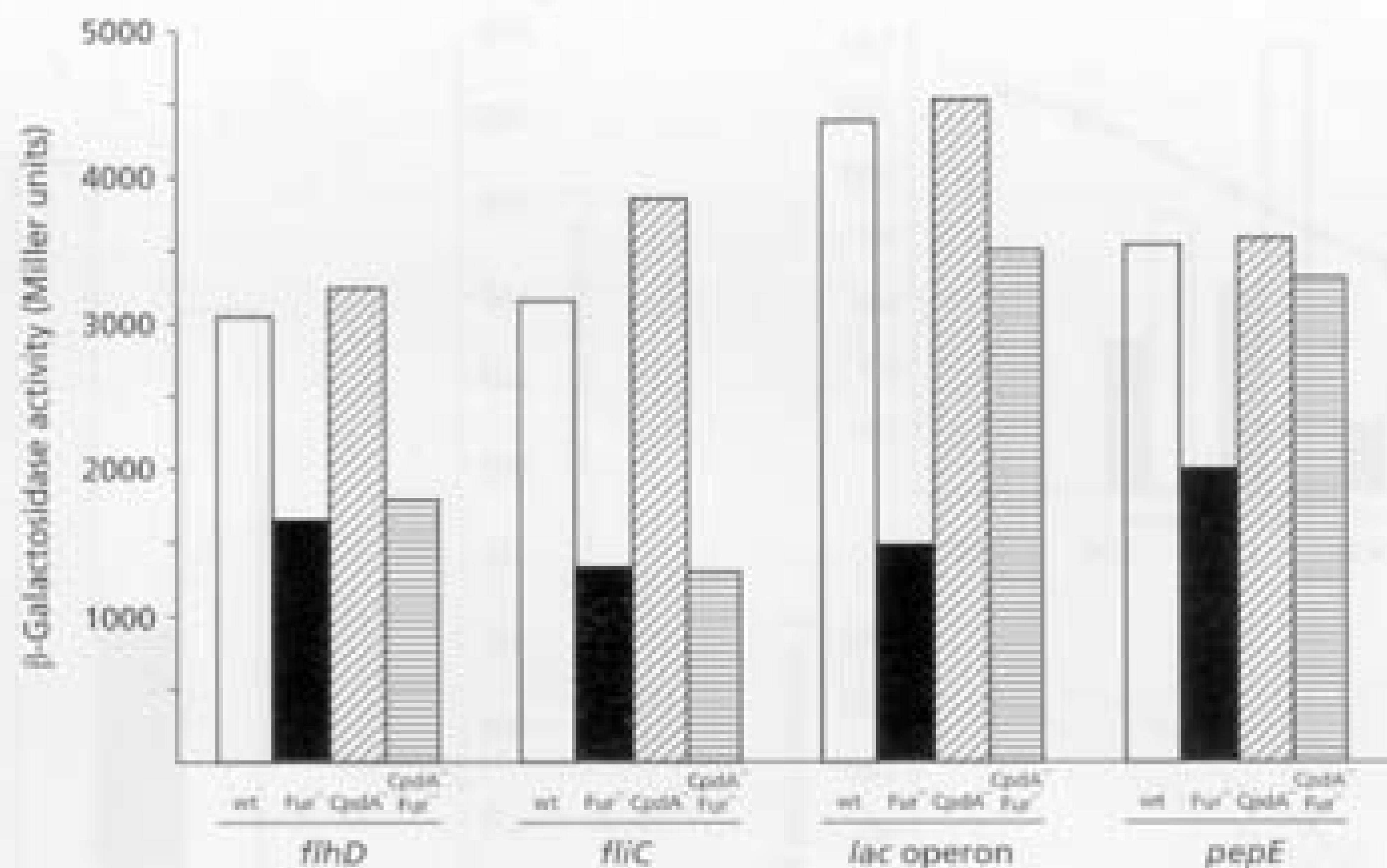

Fig. 6. Basal expression of the *flhD*, *fliC* and *pepE* promoters and IPTG-mediated induction of the *E. coli lac* operon in wild-type, *fur*, *cpdA* and *fur cpdA* cells of *S. typhimurium* growing in LB medium. β-Galactosidase activities were measured from samples taken from mid-exponential-phase cultures (OD_{550} about 0·4).

intracellular cAMP level. In accordance with this hypothesis, the addition of exogenous cAMP to *S. typhimurium fur* cultures did restore the wild-type level expression of either *pepE* or the *E. coli lac* operon (data not shown). Furthermore, and giving support to the role of cAMP in the behaviour of the *S. typhimurium fur* mutant, expression of the *pepE* and *lac* operons was the same in *fur*, *crp* and *fur crp* strains of *S. typhimurium* (data not shown). As anticipated, the level of expression was lower in all these mutants than in wild-type cells (data not shown).

In contrast to the findings described above, *S. typhimurium cpdA fur* cells did not restore wild-type levels of either *flhD* or *fliC* transcription (Fig. 6). Consistent with this finding, the amount of the product of *fliC* in outer-membrane profiles of both *fur* and *fur cpdA* mutants was the same when analysed by SDS-PAGE (data not shown). This fact indicates that, regardless of cAMP, the Fur protein must be involved in the control of the flagellar regulon and that its presence is absolutely required to achieve an optimal expression of the *flhD* promoter, despite there being enough intracellular cAMP concentration. In this respect, it is worth noting that the *S. typhimurium flhD* promoter tested positive, in both qualitative and quantitative assays, in the FURTA test used to determine if the Fur protein binds to a given promoter *in vivo* (Stojilkovic *et al*., 1994). Thus, *E. coli* H1717 (which is the basis of the FURTA test) harbouring a pUA949 plasmid derivative, including the *S. typhimurium flhD* promoter, expressed 80 Miller units of β-galactosidase. This same strain containing the pUA949 plasmid alone showed a β-galactosidase activity of only 2 Miller units. This result demonstrates that the Fur protein must bind to the *S. typhimurium flhD* promoter. It must be also noted that a putative Fur Box containing a perfect Fur-motif [NAT(A/T)AT] surrounded by copies of this hexamer presenting various degrees of conservation is located 107 bp upstream of the translational start codon of the *S. typhimurium flhD* gene.

Virulence of *S. typhimurium fur* cells is strongly reduced when orally inoculated, but only slightly affected when intraperitoneally challenged (Garcia del Portillo *et al*., 1993). Attenuation of orally inoculated *fur* cells has been demonstrated to be due to the extreme sensitivity of these cells to acid conditions (Wilmes-Riesenberg *et al*., 1996). Moreover, the virulence decrease of intraperitoneally inoculated *fur* cells had been attributed to its high sensitivity to superoxide (Touati *et al*., 1995). In contradiction to this last hypothesis, it has been established that *S. typhimurium fur* and wild-type strains present the same viability inside macrophage cells (Garcia del Portillo *et al*., 1993). The lower intracellular concentration of cAMP in *S. typhimurium fur* cells could explain its virulence decrease in comparison to the wild-type strain when such strains are intraperitoneally inoculated into mice, since appropriate levels of cAMP are required for *S. typhimurium* cells to be virulent (Curtiss & Kelly, 1987).

In summary, *S. typhimurium fur* mutants present reduced cAMP levels which results in an indirect reduction of expression of cAMP-regulated genes, such as *pepE* and *lac*. This decrease can be compensated by the introduction of a knockout mutation in *cpdA*, encoding a cyclic 3′,5′-cAMP phosphodiesterase. Moreover, the *S. typhimurium flhD* master operon, con-

trolling flagellar gene expression, is positively regulated by Fur through an iron-independent mechanism that requires further characterization.

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