ANEXO II

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Intracellular cyclic AMP concentration is decreased in *Salmonella typhimurium fur* mutants

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It is known that the Fur protein negatively regulates iron-uptake systems in different bacterial species, including *Salmonella typhimurium*. In this study it has been shown that the intracellular concentration of cyclic AMP (cAMP) is lower in a knockout *S. typhimurium fur* mutant than in the wild-type strain. According to this, the expression of two cAMP-regulated genes, such as *pepE* (encoding an *x*-aspartyl dipeptidase) and the *Escherichia coli lac* operon, is decreased in *S. typhimurium fur* cells in comparison with wild-type cells. Introduction of an additional mutation in *cpdA*, encoding a cyclic 3′,5′-cAMP phosphodiesterase, recovers wild-type intracellular cAMP concentration in the *S. typhimurium fur* mutant. Likewise, expression of *pepE* and the *E. coli lac* operon was the same in the *S. typhimurium fur cpdA* double mutant and the wild-type strain. Moreover, these results also demonstrate that the *S. typhimurium* Fur protein positively regulates the expression of the *flhD* master operon governing the flagellar regulon. This positive control must be mediated by binding of the *S. typhimurium* Fur protein to the *flhD* promoter as indicated by the fact that this promoter tests positive in a Fur titration assay.

Keywords: gene regulation, iron-uptake system, *cpdA*

INTRODUCTION

In *Escherichia coli* the iron-uptake system is under the control of the *fur* gene product (Hantke, 1984), a 17 kDa protein presenting Fe^{2+}-dependent DNA-binding activity (Bagg & Neilands, 1987). Genes under *fur* control require the presence in their promoters of at least three contiguous NAT(A/T)AT-like hexamers, in either direct or inverse orientation, to which the Fur protein binds (Escolar *et al.*, 1999). This sequence, known as the Fur box, seems to be widespread in bacteria since its presence and functionality have been described in the promoter of iron-regulated genes of several bacterial species belonging to groups as different as *Enterobacteriaceae*, *Pseudomonadaceae*, *Neisseriaceae* and Gram-positive bacteria (Escolar *et al.*, 1999; Ratledge & Dover, 2000). It has also been reported that the *Escherichia coli* Fur protein is a positive regulator of *sodB* gene expression, although the precise mechanism of this stimulatory effect has not been established since a putative Fur box seems not to be present in the promoter of this gene (Dubrac & Touati, 2000). However, the *Helicobacter pylori* Fur protein can activate *frpB* gene transcription by directly binding its promoter (Delany *et al.*, 2001). The *Salmonella typhimurium fur* gene and several genes which are under its control have been identified (Ernst *et al.*, 1978; Foster & Hall, 1992; Tsolis *et al.*, 1995). The Fur protein is also involved in the acid tolerance response of *S. typhimurium* (Wilmes-Riesenberg *et al.*, 1996), although its role in iron uptake and acid resistance is physiologically and genetically separable (Hall & Foster, 1996).

The product of the *crp* gene is another global regulator which, by binding to cyclic AMP (cAMP), controls cellular catabolism (including aerobic and anaerobic respiration), at least in the *Enterobacteriaceae* (Kolb *et al.*, 1993). Intracellular cAMP concentration is negatively modulated by the presence of glucose. As the glucose level decreases, the intracellular level of cAMP rises and an active cAMP-CRP complex is formed which transcriptionally regulates the expression of numerous genes (Ishizuka *et al.*, 1993).

Abbreviations: DPD, 2,2-dipyridyl; FURTA, Fur titration assay.

The GenBank accession number for the sequence reported in this paper is AF268282.

It has been suggested that the Fur protein could also act as an internal iron chelator, avoiding a dangerously high increase in reactive ferrous iron concentrations within bacterial cells (Abdul-Tehrani *et al.*, 1999). In this respect, it is known that double *recA fur* mutants of *E. coli* are not viable when growing in the presence of oxygen (Touati *et al.*, 1995). This fact is attributed to the interaction of reactive oxygen species (such as the superoxide radical O_2^- generated during aerobic respiration) with a higher availability of free Fe(II) in the cytoplasm of such double mutants (Touati *et al.*, 1995; Henle & Linn, 1997; Abdul-Tehrani *et al.*, 1999).

The presence of a putative sequence to which the cAMP-CRP complex binds in the *E. coli fur* promoter has been suggested on the basis of computational analysis (Zheng *et al.*, 1999; Gelfand *et al.*, 2000). In agreement with this possibility, it has been recently demonstrated that the *fur* gene of *Pasteurella multocida*, which belongs to the *γ-Proteobacteria*, as does *E. coli*, is positively regulated by the cAMP-CRP complex (Bosch *et al.*, 2001). On the basis of these data, a close relationship between the metabolism of both cAMP and iron in bacterial cells could be hypothesized. To test this putative relationship, the intracellular levels of cAMP and the expression of several genes regulated by this nucleotide have been studied in an *S. typhimurium fur* knockout mutant.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* and *S. typhimurium* strains were grown in LB broth (Miller, 1991). CAS plates (Schwyn & Neilands, 1987) were used to confirm the constitutive synthesis of siderophores characteristic of *fur* mutants. Antibiotics were added to the culture medium at the concentrations reported by Jordan *et al.* (1996). When necessary, chelating agent 2,2-dipyridyl (DPD) was used at 50 μg ml^{-1}. Induction of the *lac* operon in *S. typhimurium* cells carrying the F′128 (Pro$^+$ Lac$^+$ *zzf*::Tn*10 dtet*) plasmid was analysed by the addition of IPTG to the desired culture at a final concentration of 10 mM. To measure expression of *lacZ* fusions, samples for the β-galactosidase assays were taken, in all cases, from cultures in mid-exponential-growth phase (OD_{550} about 0·4) and enzymic activity was determined as reported by Miller (1991). In the qualitative Fur titration assay (FURTA), 1 mM $FeSO_4$-supplemented Lac EMBO agar plates (Stojiljkovic *et al.*, 1994) were used. For quantitative analysis of FURTA experiments, cells grown on these plates were collected, resuspended in LB medium and their β-galactosidase activities measured.

Genetic techniques and DNA manipulations. Biparental and triparental matings using pRK2013 as the mobilizing plasmid were performed as described by Jordan *et al.* (1996). *S. typhimurrium* chromosome exchange markers, P22 HT-mediated transductions and plasmid electroporation were performed as described by Jordan *et al.* (1996). In all cases, the absence of the P22 HT prophage in the transductants obtained was determined by streaking them on green plates (Davis *et al.*, 1980).

Standard DNA techniques, including restriction enzyme digests, ligation, transformation and plasmid purification, have been described elsewhere (Jordan *et al.*, 1996). *cpdA* and promoters of *pepE*, as well as of all flagellar genes used in this work, were isolated from *S. typhimurium* ATCC 14028 chromosomal DNA by PCR amplification using the appropriate oligonucleotide primers. These primers (Table 2) were designed based on data obtained through early release of the *S. typhimurium* genome sequence (http://www.genome.wustl.edu/gsc) by the Genome Sequencing Center of Washington University, USA. Oligonucleotide primers were supplied by Roche Diagnostics. To facilitate subcloning of PCR DNA fragments and construction of the *lacZ* fusions, specific restriction sites were incorporated at their 5′ ends (Table 2).

Isolation of a *S. typhimurium fur* knockout mutant. To isolate the *fur* gene, a pRK404 plasmid-based genomic library of *S. typhimurium* was introduced by triparental mating into the H1780 *E. coli fur* reporter strain, which is a *fur*-deficient mutant containing a fusion between the *fur*-controlled promoter of the *fiu* gene and *lacZ* in its chromosome (Hantke, 1987). After plating in LB medium supplemented with X-Gal, ferric sulfate (100 μM) and kanamycin (50 μg ml^{-1}), five white clones were detected whose plasmids were retransformed into H1780, again giving white colonies. Since restriction analysis indicated that all five clones contained the same 1 kb size fragment, only one of these plasmids (pUA931) was selected for subsequent work.

Further subcloning and sequencing of several internal fragments enabled us to obtain the sequence of the *S. typhimurium fur* gene present in plasmid pUA931 (GenBank accession no. AF268282).

To obtain an *S. typhimurium fur* knockout mutant, a 3·5 kb chloramphenicol resistance cassette was inserted into the internal *Asp700* site of the cloned *fur* gene. A *KpnI–SacII* 4·5 kb fragment containing the *fur*::Cm construction was then cloned in the pGP704 suicide vector and introduced into a Rif^R derivative of the *S. typhimurium* ATCC 14028 wild-type strain by triparental mating. Chloramphenicol-resistant transconjugants were screened for loss of vector-mediated ampicillin resistance to detect putative mutants which had exchanged their wild-type gene for the inactivated *fur* gene as a consequence of a double cross-over event. For one of these strains, UA1784, this was unequivocally confirmed by PCR amplification of chromosomal DNA using Furup and Furdw primers, Southern dot blotting and constitutive synthesis of siderophores on CAS plates (data not shown).

It has been suggested that most rifampicin-resistant mutants of *S. typhimurium* are affected in their gene expression pattern (Björkman *et al.*, 1998). To prevent any putative interference of the Rif^R mutation in the behaviour of our *fur* mutant, the *fur*::Cm region from strain UA1784 was transferred by P22-mediated transduction to wild-type (Rif^S) cells of *S. typhimurium* ATCC 14028. The PCR profile of the chromosome of 10 Cm^R transductants, when amplified with Furup and Furdw primers, and inoculation in CAS plates revealed that all of them contained the desired *fur*::Cm mutation. One of these transductants, UA1779, was kept for further work.

Construction of *lacZ* fusions and β-galactosidase assays. A PCR-fragment of about 300 bp containing the promoter and a fragment of its coding region was cloned for each gene in the pGEM-T vector (Promega) to construct the desired *lacZ* fusion. Upper primers used for the construction of *lacZ* fusions contained an *Eco*RI restriction site at their 5′ ends, whereas lower primers presented a *Bam*HI site at their 5′ ends (Table 2). For each fusion, *Eco*RI–*Bam*HI restriction fragments were recovered from the appropriate pGEM-T derivative and subcloned into pUJ8 upstream of the promoterless *trp′-′lacZ* region. Afterwards, the *Not*I fragment harbouring the created fusion was recovered from agarose gels, filled-in

Table 1. Bacterial strains and plasmids used in this study

Organism	Relevant features	Source or reference*
Escherichia coli		
DH5α	*supE4 ΔlacU169 (Φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Clontech
HB101	*supE4 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	Clontech
MC1061 (*λpir*)	*hsdR mcrB araD139Δ(araABC-leu)7679 ΔlacX74 galI galK rpsL thi*; lysogenized with *λpir* bacteriophage	This laboratory
S17 (*λpir*)	*recA1 thi pro hsdR* RP4; 2-Tc::Mu::Km Tn7; Tp^R Sm^R; lysogenized with *λpir* bacteriophage	Herrero *et al.* (1990)
Salmonella typhimurium		
ATCC 14028	Wild-type	ATCC
TT10423	*pro*AB47/F' Pro^+ Lac^+ *zzf*::Tn*10 dtet*	SGSC
TT7557	As ATCC 14028, but Crp^-	SGSC
UA1770	As ATCC 14028, but Rif^R	This study
UA1784	As UA1770, but *fur*::ΩCm	This study
UA1779	As ATCC 14028, but *fur*::ΩCm	This study
UA1794	As UA1770, but *cpdA*::ΩKm	This study
UA1795	As UA1779, but *cpdA*::ΩKm	This study
UA1805	As TT7557, but *fur*::ΩCm	This study
Plasmids		
pRK2013	Tra^+, ColE1 replicon, Km^R	Ditta *et al.* (1985)
pUJ8	Promoterless vector for making *lacZ* fusions; Ap^R	de Lorenzo *et al.* (1990)
pLV106	Low-copy-number, broad-host-range plasmid; Mob^+ Tc^R	Lee & Kaplan (1992)
pGem-T	Cloning vector; Ap^R	Promega
pHP45ΩCm	Source of $ΩCm^R$ cassette	Prentki & Krisch (1984)
pHP45ΩKm	Source of $ΩKm^R$ cassette	Prentki & Krisch (1984)
pRK404	Broad-host-range cloning vector; Mob^+ Tc^R	Ditta *et al.* (1985)
pBluescript SK(+/−)	Cloning vector; Ap^R	Stratagene
F'128	Pro^+ Lac^+ *zzf*::Tn*10 dtet*; Tc^R	SGSC
pUA949	A pBluescript SK(+/−) derivative containing a $ΩKm^R$ cassette cloned in a *Hin*dIII restriction site; Ap^R Km^R	This laboratory
pGP704	Suicide vector; Mob^+ Ap^R	de Lorenzo *et al.* (1990)
pUA931	pRK404 derivative carrying a 1 kb fragment containing the *S. typhimurium fur* gene	This study
pUA932	As pBluescript, but carrying a 1 kb fragment containing the *S. typhimurium fur* gene	This study
pUA933	As pUA932, but carrying a *fur*::ΩCm construction	
pUA934	As pGP704, but carrying a *fur*::ΩCm construction	This study
pUA935	As pGem-T, but carrying a 280 bp PCR fragment containing the *S. typhimurium flbD* promoter	This study
pUA936	As pGem-T, but carrying a 366 bp PCR fragment containing the *S. typhimurium flgA* promoter	This study
pUA937	As pGem-T, but carrying a 403 bp PCR fragment containing the *S. typhimurium fliA* promoter	This study
pUA938	As pGem-T, but carrying a 327 bp PCR fragment containing the *S. typhimurium fliC* promoter	This study
pUA947	As pGem-T, but carrying a 248 bp PCR fragment containing the *S. typhimurium pepE* promoter	This study
pUA939	pLV106 carrying a *flbD*::*lacZ* fusion; Km^R Gm^R	This study
pUA940	pLV106 carrying a *flgA*::*lacZ* fusion; Km^R Gm^R	This study
pUA930	pLV106 carrying a *fliA*::*lacZ* fusion; Km^R Gm^R	This study
pUA929	pLV106 carrying a *fliC*::*lacZ* fusion; Km^R Gm^R	This study