The LysR-type transcriptional regulator CysB controls the repression of *hslJ* transcription in *Escherichia coli*

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The LysR-type transcriptional regulator (LTTR) CysB is a transcription factor in *Escherichia coli* cells, where as a homotetramer it binds the target promoter regions and activates the genes involved in sulphur utilization and sulphonate-sulphur metabolism, while negatively autoregulating its own transcription. The *hslJ* gene was found to be negatively regulated by CysB and directly correlated with novobiocin resistance of the bacterium. *cysB* mutants showed upregulation of the *hslJ*::*lacZ* gene fusion and exhibited increased novobiocin resistance. In this study the *hslJ* transcription start point and the corresponding putative σ^{70} promoter were determined. The *hslJ* promoter region was defined by employing different *hslJ*–*lacZ* operon fusions, and transcription of the *hslJ* gene was shown to be subject to both repression imposed by the CysB regulator and direct or indirect autogenous negative control. These two regulations compete to some extent but they are not mutually exclusive. CysB acts as a direct repressor of *hslJ* transcription and binds the *hslJ* promoter region that carries the putative CysB repressor site. This CysB binding, apparently responsible for repression, is enhanced in the presence of the ligand *N*-acetylserine (NAS), hitherto considered to be a positive cofactor in CysB-mediated gene regulations. Interallelic complementation of characterized CysB mutants I33N and S277Ter partially restored the repression of *hslJ* transcription and the consequent novobiocin sensitivity, but did not complement the cysteine auxotrophy.

INTRODUCTION

The LysR-type transcriptional regulator (LTTR) CysB controls the biosynthesis of cysteine and sulphur utilization in *Escherichia coli*. CysB is an activator of the *cys* regulon, *tau* operon, *cbl* gene and *spt* gene, and in association with the Cbl (CysB-like) protein it downregulates the expression of the *ssu* operon (Kredich, 1996; van der Ploeg *et al.*, 2001; Parry & Clark, 2002). Like many other members of the LTTR family, CysB is a repressor of its own transcription (Kredich, 1996). The small metabolite *N*-acetylserine (NAS) is an inducer that facilitates binding of the CysB tetramers to the regulatory regions of target genes, while preventing binding to the *cysB* control region and the consequent negative autogenous control (Kredich, 1996). In the absence of cysteine, NAS is produced by nonenzymic conversion of *O*-acetylserine. This conversion and the synthesis of NAS are blocked in the presence of cysteine (Kredich, 1996).

The CysB protein consists of several functional domains (Lochowska *et al.*, 2001). The N-terminal region comprises the helix–turn–helix (HTH) DNA-binding motif flanked by other amino acids responsible for specificity of binding to DNA sequences. The central region is important for ligand (e.g. NAS) recognition and binding inside the cavity formed by the homodimer (Tyrell *et al.*, 1997; Verschueren *et al.*, 2001). The C-terminal domain is proposed to be involved in homo-oligomerization of this protein as well as in DNA binding of the HTH motif (Lochowska *et al.*, 2001). Transcription factors that contain the HTH motif bind DNA as dimers with the binding motifs positioned in parallel (Pabo & Sauer, 1992; Perez-Rueda & Collado-Vides, 2000). The CysB crystal structure predicts HTH motifs being positioned perpendicularly in the dimer, suggesting an unusual mechanism of binding to DNA (Tyrell *et al.*, 1997). Although there is no strict binding consensus sequence on DNA according to data collected to date, the CysB tetramer recognizes AT-rich sequences in the context of the features of the 'LysR motif' with the characteristic sequence T-N_{11}-A as the core of an inverted repeat (IR) found to be the binding

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Abbreviations: HTH, helix–turn–helix; IR, inverted repeat; LTTR, LysR-type transcriptional regulator; NAS, *N*-acetylserine; OMP, outer-membrane protein; TSP, transcription start point; WT, wild-type.

site for most of the LTTR proteins (Goethals *et al.*, 1992; Lochowska *et al.*, 2001). The specific structures of the CysB binding sites have been recognized as wide DNA regions covered with several divergently or convergently oriented half-sites separated either by a few nucleotides (activator sites) or by one or two turns of DNA helix (repressor sites) (Hryniewicz & Kredich, 1994, 1995; Kredich, 1996). Finally, DNA-bending sensitive sites were defined in some regulatory regions characteristic for the *cys* regulon (Kredich, 1996).

Besides being implicated in sulphur utilization and the synthesis of cysteine, CysB activates the expression of the *adi* and *lysU* genes (Shi & Bennett, 1994; Rowbury, 1997). Recently, we found a novel CysB-regulated gene, *hslJ*, involved in displaying a novobiocin resistance (Nov^R) phenotype in *E. coli* shown to be independent of previously characterized genes (e.g. *gyrB*, *cls*, *nov*) implicated in this phenomenon (Lilic *et al.*, 2003). The *hslJ* gene encodes a putative outer-membrane protein (OMP) but the role of HslJ in the mechanism that enables cells to display novobiocin resistance is unknown. Originally, it was shown that in *E. coli* C600 *cysB* (SY380), AB1157 *cysB*(Ts) (SY381) and MC4100 *cysB* (SY602) mutants the novobiocin resistance was increased 6–15-fold in comparison to *cysB* wild-type (WT) isogenic strains (Rakonjac *et al.*, 1991; Lilic *et al.*, 2003). Using the random insertion of the *lacZ* reporter gene we discovered the *hslJ* gene, whose expression is negatively regulated by CysB (Lilic *et al.*, 2003). Expression of the *hslJ* : : *lacZ* gene fusion is elevated in *cysB* mutants and the five- to sixfold overproduction of HslJ in either $cysB^+$ or *cysB* mutant strains increases the novobiocin resistance. A *cysB hslJ* double mutant does not exhibit the resistance to novobiocin. The *hslJ* gene expression is found to be negatively autogenously controlled *in trans*. However, according to analysis of the deduced amino acid sequence of HslJ this protein resides in the outer membrane. This prediction is strengthened by the fact that the *hslJ* : : ΩKan mutation induces the *psp* operon (Lilic *et al.*, 2003). The *psp* operon is specifically induced by overexpression of WT and mutant OMPs (e.g. pIV, and a number of other secretins) as well as by expression of mutant envelope proteins (Model *et al.*, 1997). Hence, it is very likely that HslJ is localized in the outer membrane, implying the existence of an additional factor involved in negative regulation of *hslJ* expression.

The results presented by Lilic *et al.* (2003) did not answer the question whether CysB plays the role of a repressor acting directly and regulating the transcription of *hslJ*. In this study, we defined the *hslJ* promoter region and determined the mechanism of negative regulation imposed by CysB. Also, we addressed the question whether transcription of the *hslJ* is subject to double negative control by both CysB repression and the HslJ feedback autoregulation. We characterized the MC4100 *cysB* mutated allele in strain SY602 and tested it and other *cysB* alleles in the context of either *hslJ* regulation or control of the *cys* regulon.

METHODS

Bacterial strains, media and growth conditions. The strains of *E. coli* and plasmids used in this study are listed in Table 1. Strains SY511, SY512, SY513 and SY514 were constructed by transducing strains MC4100, SY602, EC2275 and EC2549, respectively, with $P1_{vir}$ phages grown on RH90 and selecting for Tet^R transductants. Strain SY515 was constructed by transducing strain SY602 with $P1_{vir}$ phage grown on SY605 and selecting for Kan^R transductants. Media used for bacterial growth were rich medium (LB) or minimal medium (A) solidified when necessary with 1·5 % (w/v) agar (LA) as described by Miller (1992). All strains were grown at 37 °C as described previously (Lilic *et al.*, 2003). When required, the antibiotics were added to the media at the following concentrations: 100 μg ampicillin (Amp) ml^{-1}; 25 μg kanamycin (Kan) ml^{-1}; 50 μg spectinomycin (Spc) ml^{-1}; 15 μg tetracycline (Tet) ml^{-1}; 10–400 μg novobiocin (Nov) ml^{-1}. To detect $LacZ^+$ cells, 0·1 ml of a 10 mg ml^{-1} solution of X-Gal in *N*,*N*-dimethylformamide was added to LB agar plates or LB soft agar. Transformation of bacteria and transduction by $P1_{vir}$ were performed as described by Miller (1992).

DNA manipulations. Plasmids pVGM3, pVGM4 and pVGM5, containing the 666 nt (region A), 150 nt (region B) and 93 nt (region C) upstream of the *hslJ* start codon, respectively, fused to promoterless *lacZYA* genes on plasmid pRS415 were constructed and used for determination of the *hslJ* promoter region. We used PCR to amplify different DNA fragments, regions A, B and C. As a template we used the MC4100 chromosomal DNA isolated as described by Hopwood *et al.* (1985). The PCR was performed using the forward primers (Operon Tech): region A, HSLJ-rr1 (5′-GGAATTCGGTACCGGTAAAATC-3′); region B, HSLJ-fw1 (5′-GGAATTCTGGAAAAAGGCG-3′); and region C, HSLJ-fw2 (5′-GGAATTCCAGGGGAGCTGATTC-3′) (the *Eco*RI site is underlined). The reverse primer used for all fragments was HSLJ-rev1 (5′-GCGGATCCTTGTGGTTCTCAAT-3′) (the *Bam*HI site is underlined). The purified PCR products were digested with *Eco*RI and *Bam*HI and ligated into the high-copy-number vector pRS415 digested with *Eco*RI and *Bam*HI, creating plasmids pVGM3, pVGM4 and pVGM5. To transfer regions A, B and C onto a low-copy-number (5–6) vector pMS421 we subcloned the constructed *hslJ*–*lacZ* operon fusions from pVGM3, pVGM4 and pVGM5 by digesting these plasmids with *Eco*RI and *Sal*I and ligating the corresponding DNA fragments (Table 1) with pMS421 digested with the same enzymes, creating the plasmids pVGM6, pVGM7 and pVGM8, respectively. All constructs were checked by DNA sequencing performed by the dideoxy chain-termination method (Sanger *et al.*, 1977).

To characterize the *cysB* mutation in strain SY602 (MC4100 *cysB*) we isolated the chromosomal DNA of SY602 and performed the PCR using the primers (IMGGE, Belgrade) CYSB-fw1 (5′-TTTAGCATGC-AATTACAACAAC-3′), carrying the *Sph*I restriction site (underlined), and CYSB-rev1 (5′-GAAGATCTTTTTTCCGGCAGTTT-3′), carrying the *Bgl*II restriction site (underlined). A 1 kb DNA fragment from PCR carrying the mutated *cysB* allele was then digested by *Sph*I and *Bgl*II and cloned into vector pQE70, creating plasmid pVGM9. The entire *cysB* allele was sequenced.

RNA manipulations. Total RNA was isolated according to Aiba *et al.* (1981) and Gerendasy & Ito (1990). Briefly, strains MC4100 and SY602 (MC4100 *cysB*) were cultured in 10 ml minimal medium A supplemented with cysteine overnight at 37 °C. The cells were harvested and resuspended in 10 ml protoplast buffer (15 mM Tris/HCl pH 8, 0·45 M sucrose, 8 mM EDTA) and then 80 μl lysozyme (1 mg ml^{-1}) was added. Protoplasts were centrifuged and the pellet was resuspended in 0·5 ml lysis buffer (20 mM Tris/HCl pH 8, 10 mM NaCl, 1 mM NaCl, 1 mM sodium citrate, 1·5 % SDS), then 15 μl diethyl pyrocarbonate (DEPC) was added and the suspension was incubated for 5 min at 37 °C and then put on ice. After treatment

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant characteristic(s)	Source/reference
Strains		
XL-1 Blue	*endA1 hsdR17* (r_k^-, m_k^+) *supE44 thi-1 recA1 gyrA96*	Bullock *et al.* (1987)
MC4100	F^- *araD139* Δ(*argF–lac*)*U169 deoC1 flbB5301 rbsR rpsL150 relA1 ptsF25*	Casadaban (1976)
SY602	MC4100 *cysB** Nov^R	Lilic *et al.* (2003)
SY605	MC4100 *hslJ*::ΩKan Kan^R	Lilic *et al.* (2003)
SY515	SY602 *hslJ*::ΩKan Kan^R	This work
EC2275	MC4100 *cysB*† Nov^R	Lochowska *et al.* (2001)
EC2549	MC4100 Δ*cysB* Nov^R	Lochowska *et al.* (2001)
RH90	MC4100 *rpoS359*::Tn*10* Tet^R	Lange & Hengge-Aronis (1991)
SY511	MC4100 *rpoS359*::Tn*10* Tet^R	This work
SY512	SY602 *rpoS359*::Tn*10* Tet^R	This work
SY513	EC2275 *rpoS359*::Tn*10* Tet^R	This work
SY514	EC2549 *rpoS359*::Tn*10* Tet^R	This work
SY380	C600 *cysB* Nov^R	Rakonjac *et al.* (1991)
Plasmids		
pRS415	*lacZYA*$^+$ promoterless vector used for construction of *lacZ* operon fusions, Amp^R	Simons *et al.* (1987)
pMS421	Low-copy-number cloning vector, Spc^R	Grana *et al.* (1988)
pQE70	Expression vector, Amp^R	Qiagen
pREP4	Low-copy plasmid, carries *lacI* gene, Kan^R	Qiagen
pJOH1	pBR322 carrying WT *cysB*, Amp^R	Ostrowski *et al.* (1987)
pHV3002	pMS421 carrying WT *hslJ* on 3·7 kb DNA fragment from λ265	Lilic *et al.* (2003)
pVGM1	pQE70 carrying WT *cysB*	This work
pVGM3	665 bp DNA fragment upstream of *hslJ* cloned into pRS415, $\phi hslJ_A$–*lacZ*	This work
pVGM4	150 bp DNA fragment upstream of *hslJ* cloned into pRS415, $\phi hslJ_B$–*lacZ*	This work
pVGM5	93 bp DNA fragment upstream of *hslJ* cloned into pRS415, $\phi hslJ_C$–*lacZ*	This work
pVGM6	6·95 kb *Eco*RI–*Sal*I DNA fragment from pVGM3 cloned into pMS421, $\phi hslJ_A$–*lacZ*	This work
pVGM7	6·45 kb *Eco*RI–*Sal*I DNA fragment from pVGM4 cloned into pMS421, $\phi hslJ_B$–*lacZ*	This work
pVGM8	6·38 kb *Eco*RI–*Sal*I DNA fragment from pVGM5 cloned into pMS421, $\phi hslJ_C$–*lacZ*	This work
pVGM9	0·97 kb *Sph*I–*Bgl*II DNA fragment carrying the *cysB831*::IS*1* allele cloned into pQE70	This work

**cysB* mutant gene characterized in this study, *cysB831*::IS*1*, which encodes the CysB S277Ter protein.
†*cysB* mutant gene that encodes the CysB I33N protein.

with 250 µl NaCl (40 %, w/v) and incubation on ice for 10 min, the suspension was centrifuged for 1 h. The RNA was extracted with phenol/chloroform (1::1, v/v) at room temperature and precipitated by adding 1 ml ethanol (at −70 °C). The RNA pellet was washed with 70 % ethanol and dissolved in water. We added 3 vols 4 M sodium acetate to concentrate the RNA solution and then reprecipitated. The RNA quality and concentrations were determined by measurement of A_{260} and A_{280}. RNA with a A_{260}/A_{280} ratio of 1·5–2·0 was used for further experiments.

Primer extension analysis was performed according to Sambrook *et al.* (1989) using primer HSLJ-rev3 (5′-GCCATCAGCAGGCTTAGC-3′) (Genosys), 43–26 nt downstream from the translational start, and 100 µg of total RNA isolated from strain MC4100 or SY602 (MC4100 *cysB*). RNA samples were resuspended in RNase-free water and incubated at 37 °C for 1 h in the presence of RNase-free DNase (Promega). Primer was labelled at its 5′ end by using [γ-^{32}P]dATP and T4 polynucleotide kinase (New England Biolabs). The cDNA was extended at 37 °C for 40 min with MMLv Reverse Transcriptase (Pharmacia). The products were loaded on a 5 % polyacrylamide gel together with a nucleotide sequence (Sanger *et al.*, 1977) generated with the same (unlabelled) primer and plasmid pHV3002 as a template.

β-Galactosidase assay. *E. coli* strains carrying the plasmid *hslJ*–*lacZ* operon fusions were grown overnight at 37 °C in LB broth containing the appropriate antibiotic and diluted 100-fold into the same medium. Following growth to mid-exponential phase (OD_{600} 0·4), cultures were assayed for β-galactosidase activity by the method of Miller (1992).

Overproduction and purification of CysB-His$_6$ protein. Plasmid pVGM1 was used for overexpression and purification of the CysB-His$_6$ fusion protein following the protocol in the Qiagen manual. The *E. coli cysB* gene was amplified by PCR using plasmid pJOH1 as a template and the pair of primers CYSB-fw1 and CYSB-rev1. The PCR product was digested with *Sph*I and *Bgl*II and cloned into the *Sph*I/*Bgl*II sites of vector pQE70 to generate the plasmid pVGM1. This construct was checked by sequencing (Sanger *et al.*, 1977) using primers Type III/IV (Qiagen) and Reverse sequencing (Qiagen). Plasmid pVGM1 was used to transform *E. coli* strains SY602 and SY380 previously transformed with plasmid pREP4. The recombinant strains were grown in LB medium at 37 °C to mid-exponential phase (OD_{600} 0·4). IPTG (1 mM) was then added and incubation continued for 3 h. Purification of the overproduced protein CysB-His$_6$ (strain SY380/pREP4/pVGM1) was carried out at room temperature using

the Ni-NTA agarose matrix of the QIAexpressionizt kit (Qiagen). Briefly, the cells were centrifuged, resuspended in 1/25 of the culture volume of sonication buffer, disrupted by freezing (in dry ice/ethanol, with thawing in cold water), and sonication, and the cell debris was removed by centrifugation. *E. coli* crude extracts were loaded on a 1 ml Ni-NTA agarose column previously equilibrated with sonication buffer and the CysB-His$_6$-tagged protein was eluted with an imidazole gradient (100–500 mM). The eluted fractions were subjected to SDS-PAGE as described by Laemmli (1970). The protein eluate was later dialysed overnight against a storage buffer (50 mM Tris/HCl pH 8·0, 1 mM EDTA and 20 % glycerol). The concentration of CysB-His$_6$ was determined using the Bradford protein assay (Bio-Rad) (Bradford, 1976). The purified protein was stored at −80 °C.

Nondenatured protein molecular mass determination. Native molecular masses of the fusion protein CysB-His$_6$ were determined by the method of Bryan (1977). Purified CysB-His$_6$ (10 μg), and molecular size protein markers (20 μg) (nondenatured protein molecular mass marker kit, Sigma) in 20 mM Tris/HCl (pH 7·4), 1 mM EDTA and 50 mM NaCl buffer were subjected to electrophoresis on a set of native protein gels that contained various concentrations of acrylamide (6, 7, 8·5 and 10 %), and stained with Coomassie blue. Considering the pI (around 7) of CysB-His$_6$, besides using gels of pH 8·8, we used native gels of pH 9·5. Both sets of gels worked equally well. The relative mobilities (R_F), construction of the Ferguson plots and the coefficients of retardations (K_r; negative slope) of each protein species deduced from the slopes were determined. The logarithm of the K_r of the markers was then plotted versus the logarithm of their molecular masses. The plots obtained were used to determine the molecular sizes [molecular mass (Da) × 1000] of CysB-His$_6$ by extrapolation of their respective K_r.

Electrophoretic mobility-shift experiments. DNA mobility-shift experiments were performed essentially as described by Prentki *et al.* (1987). DNA probes carrying different fragments of the *hslJ* promoter region were obtained by PCR amplification using pHV3002 as a template and different sets of primers: probe 301, HSLJ-fw3 (5′-GCAAAACTTAAGCAATCTGGAAAAAGGCG-3′) (Genosys) plus HSLJ-rev2 (5′-GTCACGGGCTTACCG-3′) (Genosys); probe 135, HSLJ-fw4 (5′-TGAAGAAAGTAGCCGCG-3′) (Genosys) plus HSLJ-rev2; and probe C, HSLJ-fw2 plus HSLJ-rev1. DNA probes 301 and 135 were labelled at the 5′ end by using [γ-^{32}P]dATP and T4 polynucleotide kinase (New England Biolabs). Probe C was used as an unlabelled specific competitor in 50-fold molar excess. Briefly, CysB protein was pre-incubated in binding buffer (10 mM Tris/HCl, pH 7·5; 70 mM KCl; 5 mM $MgCl_2$; 1 mM DTT; 1 mM EDTA; 12·5 %, v/v, glycerol; 0·1 % Triton X-100; 200 μg ml^{-1} BSA) for 5 min at 4 °C before the labelled DNA (1–2 ng) and in some cases NAS (1, 3 or 5 mM) and/or unlabelled specific probe were added. All reaction mixtures contained 1000-fold weight excess of poly(dI-dC) (Pharmacia). Binding reaction mixtures (total volume 20 μl) were incubated for 20 min at 37 °C. After pre-electrophoresis (2 h), the samples were loaded onto 5 % native polyacrylamide gels in 0·5 × TBE and run at a voltage of 12 V cm^{-1}. The gels were then dried and exposed to X-ray film.

RESULTS AND DISCUSSION

Determination of the *hslJ* transcription start point

The *hslJ*::*lacZ* gene fusion activity was increased in a *cysB* mutant (Lilic *et al.*, 2003). Hence, we isolated total RNA from the *cysB* mutant strain, and by using the oligonucleotide HSLJ-rev3 and the primer extension reaction we determined the *hslJ* transcription start point (TSP). The TSP (+1) was found to be an A nucleotide positioned 36 bp upstream of the *hslJ* ATG translation start codon (Fig. 1a, b). We also performed the primer extension experiment with RNA isolated from the *cysB*$^+$ strain MC4100 (data not shown). The analysis of the bands obtained in the primer extension experiments showed about the same moderate signal and a very similar steady-state level of *hslJ* transcription in both the WT and the *cysB* mutant strains. This result implies that an additional negative regulator, other than CysB, controls the transcription of *hslJ*.

Regarding the *hslJ* TSP, we localized a putative σ^{70} *hslJ* promoter with the sequence TAATCC-N$_{17}$-TCTATT at position 71–43 nucleotides upstream of the *hslJ* ORF

(a)

G A T C 1 2

G C
A T
A T
T A
T A
T A +1
T A
A T
T A
C G

(b)

-35
5'-CCAGGGGAGCTGATTCAGATAATCCCCAATG

-10 +1
ACCTTTCATCCTCTATTCTTAAAATAGTCCTGAG

RBS *hslJ*
TCAGAAACTGTAATT**GAGAA**CCACA***ATG****AAG*-3'

Fig. 1. Determination of the *hslJ* TSP. (a) Primer extension analysis. Lane 1 contains primer with no RNA; lane 2 contains the primer extension product from RNA isolated from strain SY602. Sequence ladders generated with the same primer are shown. The TSP (A, +1) is indicated. (b) Sequence of the promoter region of *hslJ*. Locations of the TSP (A, +1), σ^{70} (underlined −35 and −10 sequences) and σ^S (open boxes) putative promoters of *hslJ*, the 'LysR motifs' (two head-to-head arrows; arrows indicate the inverted repeat), ribosome-binding site (RBS, bold nucleotides), and the *hslJ* (italic) start codon (bold and underlined) are shown on the nucleotide sequence depicted from the *hslJ* regulatory region (region C).

(Fig. 1b). However, the major parts of the consensus sequence characteristic for the σ^S promoter were also found to reside in this DNA region (Fig. 1b). In order to discriminate between these two putative *hslJ* promoters, we analysed *hslJ* transcription in SY511 (*rpoS*$^-$ *cysB*$^+$) and in SY512, SY513 and SY514 *cysB* mutants carrying the inactive σ^S factor and the constructs pVGM5 or pVGM8 with an *hslJ–lacZ* operon fusion (see below) respectively, under different growth conditions. We showed that *hslJ* transcription does not depend on either the σ^S factor or the stationary growth phase (data not shown).

We searched for the sequence proposed to be the consensus for binding of LTTRs such as the CysB protein. Nucleotide sequence analysis for direct and inverted repeats was done using the PCgene program. Inspection of the *hslJ* regulatory region revealed the IR sequence CTATTcttaaAATAG positioned 32 nucleotides upstream of the *hslJ* ORF (Fig. 1b). This AT-rich sequence overlaps the −10 hexamer of the putative σ^{70} *hslJ* promoter as well the *hslJ* TSP and resembles the conserved base pairs of a generic T-N_{11}-A 'LysR motif' present in the binding sites for CysB and most LTTRs (Goethals *et al.*, 1992; Lochowska *et al.*, 2001). In addition, a second putative CysB binding site aTaATccccaATgAc was found 10 bp upstream of the first one (Fig. 1b). This site is not AT-rich but it contains the core of the 'LysR motif' and the imperfect IR, overlaps the −35 hexamer of the putative *hslJ* promoter, and in concert with the first site resembles the organization of the CysB repressor sites CBS-B and CBS-K2 found in the *cys* regulon promoters (Hryniewicz & Kredich, 1995).

Definition of the *hslJ* promoter region

Different *hslJ–lacZ* operon fusions were constructed to dissect the regulatory region and to determine a DNA fragment that carries an active *hslJ* promoter. The constructs carried either the largest portion of the *hslJ* regulatory region A (pVGM3) or 5′ deletion derivatives resulting in regions B (pVGM4) and C (pVGM5) (Fig. 2). All constructs contained the putative σ^{70} *hslJ* promoter. Since *hslJ* was proposed to be a CysB-regulated gene, the activities of the *hslJ–lacZ* operon fusions were measured in the WT MC4100 and in a *cysB* mutant strains. As shown in Fig. 2, the deletion analysis showed that all constructs, including one with region C carrying the putative σ^{70} *hslJ* promoter with the rest of 5′ region deleted, were active. The β-galactosidase activities were at the same level in all constructs and slightly higher in the *cysB* mutant strain. This result defined region C to be the *hslJ* promoter region containing both the determined putative σ^{70} *hslJ* promoter and the potential CysB repressor site (two 'LysR motifs').

Negative regulation of *hslJ* transcription

Considering the work of Lilic *et al.* (2003) and the results presented above, the moderate increase of the activities of the *hslJ*: : *lacZ* gene fusion and the *hslJ–lacZ* operon fusions, respectively, in the *cysB* mutant, might be due to action of the additional negative regulator that works in concert with CysB. This idea is substantiated by the finding that the WT *hslJ* presented on a plasmid, *in trans*, strongly negatively regulates the expression of the chromosomal *hslJ* gene while increasing the resistance to novobiocin (Lilic *et al.*, 2003). In this previous work the *hslJ*: : *lacZ* gene fusion activities were always measured in conditions where the *hslJ* gene was inactivated either by the *lacZ* gene or by the Kan cassette (Lilic *et al.*, 2003) and did not reflect the regulation of *hslJ* transcription. Therefore, in order to solve this question, the constructs with different *hslJ–lacZ* operon fusions, pVGM6,

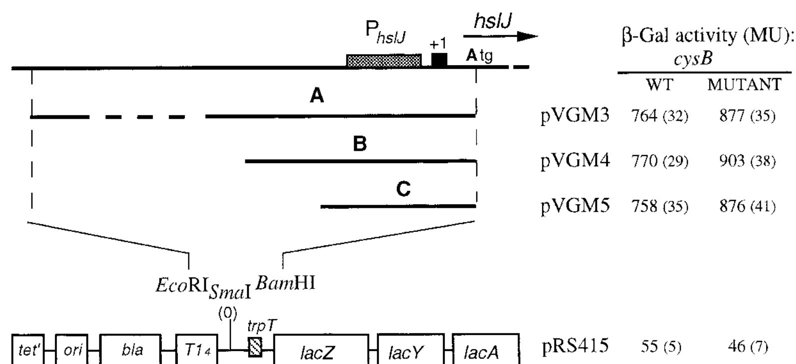

Fig. 2. Construction of different *hslJ–lacZ* operon fusions and determination of the *hslJ* promoter region. Schematic representation of plasmids pVGM3–5, carrying the operon fusions ($hslJ_A$–*lacZ*), ($hslJ_B$–*lacZ*) and ($hslJ_C$–*lacZ*), respectively, constructed by ligating regions A (666 bp), B (150 bp) and C (93 bp) to the *lacZ* gene in *Eco*RI- and *Bam*HI-digested promoterless vector pRS415 (*bla*; Amp^R). Shaded box (P_{hslJ}), σ^{70} putative promoter of the *hslJ* gene; Atg, *hslJ* start codon; black box (+1), *hslJ* TSP. DNA fragments are not to scale. The β-galactosidase activities (Miller units, MU) of the MC4100 *cysB*$^+$ (WT) and SY602 *cysB*$^-$ (Mutant) strains carrying plasmids pVGM3, pVGM4 or pVGM5, or vector pRS415, are shown. The β-galactosidase activities represent the mean values of six independent assays with standard deviations in parentheses. The β-galactosidase activity in LB medium was assayed after growing cells at 37 °C in the presence of 0·1 mM cysteine.

pVGM7 and pVGM8, described above (Fig. 2), were introduced into MC4100 isogenic strains carrying different combinations of the *cysB* and *hslJ* mutated genes (MC4100, *cysB*$^+$ *hslJ*$^+$; SY602, *cysB*$^-$ *hslJ*$^+$; SY605, *cysB*$^+$ *hslJ*$^-$; SY515, *cysB*$^-$ *hslJ*$^-$). In parallel with *β*-galactosidase activities we determined the Nov^R status of the transformants. The results we obtained using different fusions (pVGM6, pVGM7 and pVGM8) and different *cysB* mutated alleles (EC2549 *cysB*^{null}, EC2275 *cysB* and SY602 *cysB*) were essentially the same (data not shown). Hence we present the values obtained with the construct pVGM8 carrying the *hslJ* promoter region C, *hslJ*$_C$–*lacZ*, introduced into the strains with combined mutations, SY602 *cysB* allele and *hslJ*::ΩKan (Table 2). These results revealed that negative regulation of *hslJ* transcription is both CysB- and HslJ-dependent. This control is stronger when one of the proteins is not functional (*cysB*$^+$ *hslJ*$^-$ or *cysB*$^-$ *hslJ*$^+$) in comparison with the WT situation (*cysB*$^+$ *hslJ*$^+$) (Table 2), suggesting that negative regulations imposed by these functionally active proteins are in competition. The similar steady-state level of *hslJ* transcription in either WT or *cysB* mutant strains obtained in the primer extension experiments is in accordance with this result. The strongest repression was obtained by CysB alone while the maximal *hslJ* transcription is achieved in the absence of both functional CysB and HslJ proteins (*cysB*$^-$*hslJ*$^-$) (Table 2). This was the exact genetic background when we found that the *hslJ*::*lacZ* gene fusion expression is elevated in a *cysB* mutant (Lilic *et al.*, 2003). These results imply that *hslJ* transcription is under a double negative control effected by the expression of CysB regulator and the HslJ protein. These regulations are in competition but do not exclude each other.

When the *hslJ*::ΩKan allele encoding the functionally inactive HslJ is present, besides making the strain Nov^S, the HslJ-dependent negative regulation is absent as well (Table 2). The *hslJ* transcription regulated by both LTTR CysB and the expression of HslJ protein is insufficient to produce the critical concentration of the HslJ product and to increase the novobiocin resistance of the WT strain (Table 2). The strong negative regulation of *hslJ* transcription and the parallel increase in novobiocin resistance observed in the strain where the *hslJ* gene is intact and the CysB-imposed negative regulation is abolished (*cysB*$^-$ *hslJ*$^+$) (Table 2) could be due to transient elevation of HslJ production and the immediate downregulation of *hslJ* expression. This negative feedback *hslJ* autoregulation could be important because of the toxicity of HslJ observed upon overexpression of this protein (Lilic *et al.*, 2003). Considering the structure of the HslJ protein and the fact that the *hslJ*::ΩKan allele induces the *psp* operon, HslJ has been predicted to be located in the outer membrane of *E. coli* (Lilic *et al.*, 2003). As mentioned above, among other stimuli, the *psp* operon is induced by expression of mutant OMPs (Model *et al.*, 1997). Here we found the *hslJ*::ΩKan allele to be inactive in autogenous control. Therefore, the HslJ-dependent negative regulation of the *hslJ* transcription is likely to be indirect, involving an additional negative regulator. The action of this regulator might be dependent on the proper HslJ cell localization.

However, a CysB-dependent negative regulation of the *hslJ* transcription should be direct. Since all results were obtained with the construct carrying the *hslJ* promoter region C, and since this region contains a putative CysB repressor site composed of two 'LysR motifs' found to be the consensus sequences for binding of the LTTRs, CysB could bind the *hslJ* regulatory region and act as a direct repressor of the *hslJ* transcription.

Purification of the active CysB regulator

We wanted to analyse the CysB binding properties of the *hslJ* regulatory region *in vitro*. In order to approach this issue, by adding His tag at the C-terminal end of CysB (36·3 kDa), we constructed the fusion protein CysB-His$_6$ (37·3 kDa) encoded from the plasmid pVGM1. The *in vivo* activity of this protein is proved by complementation of the cysteine auxotrophy. The *cysB* cysteine auxotrophs SY602/pREP4 and SY380/pREP4 were transformed with pVGM1. The transformants grew on minimal medium plates in the absence of cysteine (data not shown). Hence, we used strain

Table 2. Activity of the *hslJ*$_C$–*lacZ* operon fusion from the pVGM8 construct in *cysB*, *hslJ* or double *cysB hslJ* mutant backgrounds

Strain	Relevant genotype	*β*-Gal activity (Miller units)*	Nov phenotype†
MC4100/pRS415	WT/promoterless *lacZ*	30±15	S
MC4100/pVGM8	WT/*hslJ*$_C$–*lacZ*	300±35	S
SY602/pVGM8	*cysB*$^-$/*hslJ*$_C$–*lacZ*	273±50	R
SY605/pVGM8	*hslJ*::ΩKan/*hslJ*$_C$–*lacZ*	105±20	S
SY515/pVGM8	*cysB*$^-$ *hslJ*::ΩKan/*hslJ*$_C$–*lacZ*	521±42	S

*The *β*-galactosidase activity in LB medium was assayed after growing cells at 37 °C. Mean values of six independent assays with standard deviations are shown.

†The cells were grown at 37 °C on LB plates supplemented with antibiotic. Nov^S, sensitive to 60 μg ml^{-1}; Nov^R, resistant to 300 μg ml^{-1}.

SY380/pREP4 harbouring plasmid pVGM1 to overexpress and purify the fusion protein CysB-His_6 placed under the IPTG inducible promoter (see Methods). After 3 h induction in the presence of 1 mM IPTG, we went through the protein purification procedure and eluted >90 % pure CysB-His_6 fusion protein (37·3 kDa) in the fraction obtained by 0·25–0·3 M imidazole (Fig. 3). Only 10 % of the induced protein was soluble (90 % left in the pellet after lysis) (data not shown).

It has been shown that in order to be active, the CysB regulator binds DNA of the target promoters as a homotetramer and positively or negatively regulates transcription (Kredich, 1996). Hence, the activity of the LTTR CysB depends on its potential to oligomerize and form homotetramers. We analysed this property of the purified CysB fusion protein by employing PAGE under nondenaturing conditions and at specific pH, screening the profiles of the CysB mobilities in different gel concentrations, and comparing the profiles with the mobilities of protein standards (see Methods). Knowing the molecular mass of the CysB fusion protein (37·3 kDa) we estimated the molecular masses of the oligomers obtained: CysB-His_6 tetramer, 150 kDa; CysB-His_6 hexamer, 225 kDa. CysB fusion protein oligomerizes up to tetramers and hexamers (data not shown); higher oligomers were also observed but we did not estimate their molecular masses.

CysB binding in the *hslJ* regulatory region

We used the purified CysB fusion protein to analyse binding of this transcription factor in the *hslJ* regulatory region. It has been shown for the *cys* regulon target genes that CysB binds DNA in both promoter and coding regions (−110 to +30) (Kredich, 1996). The protein in different concentrations, probe 301 comprising the 166 nt of the *hslJ* regulatory region (region B, 150 nt, is inside this portion of probe 301) and the 135 nt of the 5′ region of the *hslJ* gene, and probe 135 covering only the 5′ region of the *hslJ* gene were used (Fig. 4a). Gel retardation assay revealed that the CysB fusion protein binds only a DNA fragment containing the *hslJ* regulatory region (166 nt), making distinct DNA–protein complexes Cpx1 and Cpx2 (Fig. 4b, lanes 1–3). The appearance of these complexes was dependent on the protein concentration. Using region C as an unlabelled probe (Fig. 4c, lane 5) diminished the binding of CysB to probe 301. Formation of two complexes, such as Cpx1 and Cpx2, usually indicates two binding sites. As shown above, region C is the *hslJ* promoter region that carries the putative *hslJ* promoter and two 'LysR motifs' (the putative CysB repressor site composed of two binding sites) (Fig. 1b, Fig. 2). Therefore, CysB most likely binds the sequence predicted to be the CysB repressor site in an *hslJ* regulatory region, and upon binding acts directly as a repressor of *hslJ* transcription.

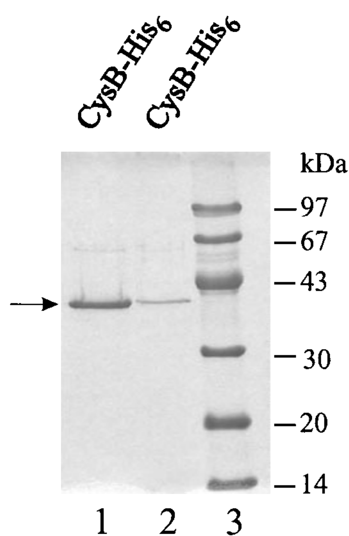

Fig. 3. Purification of the active CysB-His_6 fusion protein. Lanes 1 and 2, purified CysB-His_6, 37·3 kDa (arrow), after elution with 0·25 M (lane 1) and 0·3 M imidazole (lane 2). Lane 3, Protein standards.

The small molecule NAS has been considered to be the CysB ligand (inducer) that binds the cavity formed by two CysB monomers and, depending on the specific binding site in the target promoter, facilitates binding of the perpendicularly positioned HTH motifs of the CysB tetramer(s) to the activator sites while preventing binding to the repressor site in the *cysB* regulatory region (Kredich, 1996). The binding of CysB protein to probe 301 was analysed in the presence of NAS. CysB alone at a concentration of either 0·15 or 0·5 μg ml^{-1} weakly bound probe 301 (Fig. 4c, lanes 3 and 4). However, in the presence of increasing concentrations of NAS (1–5 mM; result shown for 3 mM), CysB used at 0·15 μg ml^{-1} bound probe 301 forming the more pronounced DNA–protein complexes Cpx1 and Cpx2, and the additional Cpx3 complex (Fig. 4c, lane 6). The efficacy of binding depended on NAS concentration as shown for other CysB-NAS-dependent DNA bindings (Kredich, 1996). Using region C as an unlabelled probe (Fig. 4c, lane 7) diminished this binding of CysB in the presence of NAS. Hence, once again, the portion of the *hslJ* regulatory region carrying the putative *hslJ* promoter and the putative CysB repressor site successfully competes for binding of CysB.

In all CysB-dependent regulations described to date, NAS plays the role of an inducer when used in concentrations like those used in this study (Kredich, 1996). We showed that CysB negatively regulates *hslJ* transcription, where it is proposed to bind the 'LysR motifs' that resemble the topology of the CysB repressor site that overlaps the *hslJ* promoter and the *hslJ* TSP. However, in the control of *hslJ* transcription where CysB acts as a repressor, NAS enhances the binding of this regulator to DNA. Therefore, it seems that the term 'inducer' cannot be applied to NAS as regards CysB-mediated regulation of *hslJ* transcription.

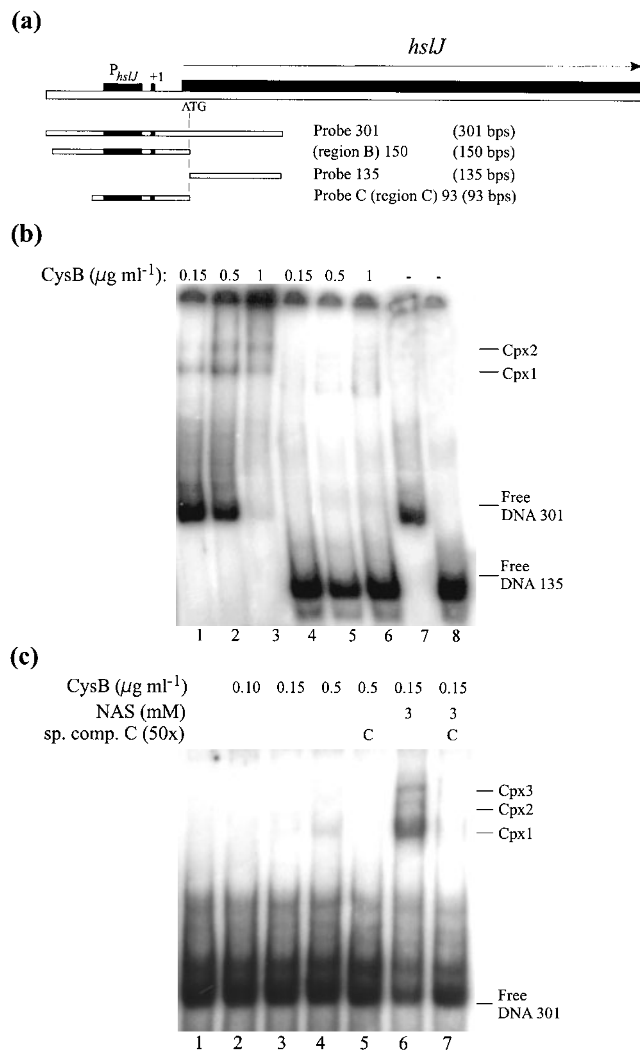

Fig. 4. Binding of CysB-His_6 to the *hslJ* regulatory region. (a) Schematic representation of probes 301, 135 and C in relation to the *hslJ* gene (arrow) and the *hslJ* promoter region [black box (P_{hslJ}), *hslJ* promoter; small black box (+1), *hslJ* TSP; ATG, *hslJ* start codon]. (b) CysB-His_6 binds the *hslJ* regulatory region in a concentration-dependent manner. Lanes 1–3, CysB–DNA 301 complexes, Cpx1 and Cpx2; lanes 4–6, CysB–DNA 135 binding. (c) NAS facilitates binding of the CysB-His_6 to the *hslJ* regulatory region. Lane 1, free DNA 301; lanes 2–4, binding of the CysB fusion protein in increasing concentrations to probe 301 (Cpx1); lane 5, CysB-His_6 preincubated with unlabelled specific competitor (probe C) at 50-fold molar excess; lane 6, binding of CysB-His_6 to probe 301 in the presence of 3 mM NAS (Cpx1–3); lane 7, binding of CysB-His_6 to probe 301 in the presence of 3 mM NAS when the protein was preincubated with unlabelled specific competitor (probe C) at 50-fold molar excess.

cysB mutations

The CysB regulator is a multi-domain protein that contains the functional domains responsible for DNA binding (N-terminus), interaction with ligands (e.g. NAS) (central domain), and oligomerization and DNA binding (C-terminus) (Schell, 1993; Lochowska *et al.*, 2001). The different MC4100 *cysB* mutants used in this work carry *cysB* alleles with specific mutations affecting the function of the entire protein or the specific functional domain. Δ*cysB* is a null mutation with the major portion of the *cysB* gene deleted and hence strain EC2549 entirely lacks the CysB product (Lochowska *et al.*, 2001). However, the CysB I33N mutant protein produced by strain EC2275 carries a missense mutation in the 'recognition' helix of the HTH motif in the CysB DNA binding domain (Lochowska *et al.*, 2001) (Fig. 5b). This mutation has been shown to diminish the binding of CysB to both the activator and the repressor sites in the regulatory regions of the *cys* regulon genes and consequently the active regulation failed (Colyer & Kredich, 1994). The interallelic complementation of this allele with the other specific *cysB* allele encoding CysB T149Ter restored the functionality of the CysB regulator (Colyer & Kredich, 1994). In the previous and in this work we used the spontaneous MC4100 *cysB* mutant SY602 obtained by selection of Nov^R colonies on plates supplemented with 400 μg novobiocin ml^{-1} (Lilic *et al.*, 2003). This mutant, besides exhibiting resistance to novobiocin, was a cysteine auxotroph. We further characterized this mutant and found that the phenotypes obtained were due to mutation in the *cysB* gene (Lilic *et al.*, 2003). The previous work by Rakonjac *et al.* (1991) and Lilic *et al.* (2003) and this study established that the Nov^R phenotype related to the *cysB* mutations is the consequence of elevated HslJ expression upon lack of *hslJ* transcription repression otherwise imposed by CysB. Hence, the repression of *hslJ* transcription, the Nov^S phenotype and the biosynthesis of the cysteine are the outcomes of the WT CysB activities.

In this study we have characterized the MC4100 *cysB* allele in detail by cloning and sequencing the *cysB* gene from the mutant SY602 (see Methods). The sequencing revealed in-frame insertion of the 0·75 kb transposable element IS*1* after nucleotide 831 in the 3′ region of the *cysB* gene (Fig. 5a). We named this *cysB* allele as *cysB831*::IS*1*. This result is in agreement with the restriction pattern and the 1·7 kb length of the PCR fragment obtained using SY602 chromosomal DNA and a pair of primers framing the *cysB* gene assuming the insertion of the 0·75 kb DNA in the 3′ region of the 1 kb long WT *cysB* gene. The IS*1* element with 5′-*cysB831*::IR_L-(IS*1*)-IR_R-3′ orientation is flanked by duplication of the 11 nt from the *cysB* coding region (Fig. 5a). The first three nucleotides GGT of the IR_L (left inverted repeat) are deleted, most likely leaving the IS*1* element fixed and inactive for further transposition (Fig. 5a). All these characteristics of the IS elements (e.g. IS*1*) and their transpositions have been seen previously and have been reviewed by Galas & Chandler (1991).

The insertion of IS*1* element interrupts the region that encodes the CysB domain responsible for the oligomerization and binding of this regulator to DNA (Fig. 5b). In order

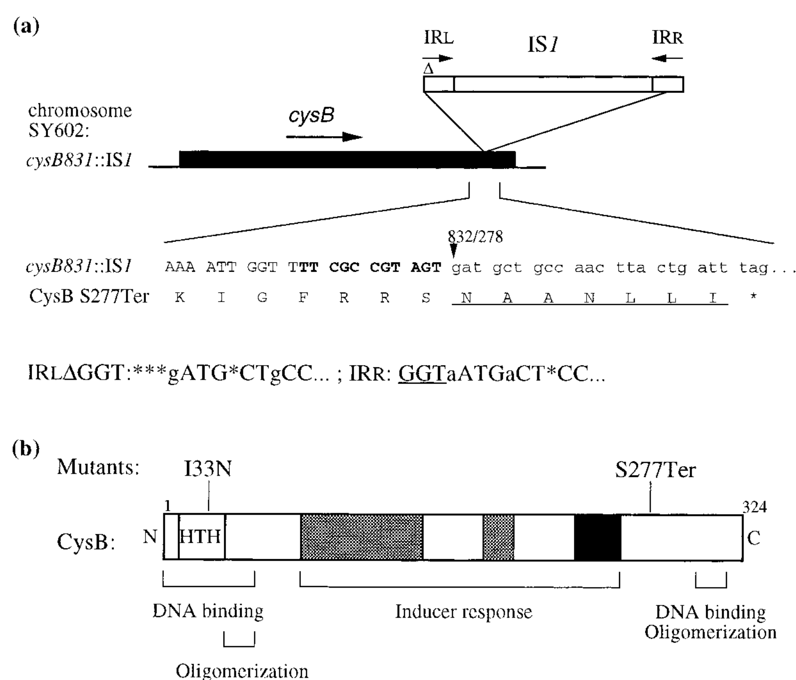

Fig. 5. Characterization of the *cysB* allele from mutant SY602. (a) Schematic representation of the IS*1* element insertion into the 3′ region of the *cysB* allele from strain SY602. IS*1* insertion generated the *cysB831*::IS*1* mutation. IS*1*, insertion sequence 1 with left (IR_L) and right (IR_R) inverted repeats presented; Δ, deletion of the GGT nucleotides at the 5′ end of IR_L presented below as IR_LΔGGT. The position of IS*1* insertion in the 3′ region of the *cysB* gene *cysB831*::IS*1* and the corresponding amino acid sequence (CysB S277Ter) are presented below. Capital letters, *cysB831*::IS*1* sequence; bold nucleotides show the 11 nt duplication upon IS*1* insertion; small letters, IS*1* sequence; 832/278, point of IS*1* insertion. Underlined amino acids are those encoded by IS*1*. (b) Schematic representation of the CysB protein and its mutant forms, CysB I33N and CysB S277Ter. CysB functional domains are presented as described by Lochowska *et al.* (2001). HTH, DNA-binding motif. Positions of the I33N and S277Ter mutations in relation to the CysB domains are shown.

to answer whether the CysB mutant regulator encoded by the *cysB831*::IS*1* allele is produced as a CysB S277Ter protein (Fig. 5a, b) and whether its functionality can be restored by complementation with the other, different, CysB mutant(s), we performed an interalellic complementation experiment. We followed the resistance to novobiocin as a measure of *hslJ* expression and the biosynthesis of cysteine as a functionality of the *cys* regulon control enabled by CysB. The results are presented in Table 3. Plasmid pVGM9 carrying the *cysB831*::IS*1* allele complemented the chromosomal copy of the EC2275 *cysB* allele encoding the CysB I33N protein, decreasing the novobiocin resistance, but it failed to complement the cysteine auxotrophy of strain EC2275. As a control, neither the *cysB831*::IS*1* allele nor the EC2275 *cysB* allele complemented a Δ*cysB* mutation in strain EC2549, while the WT $cysB^+$ efficiently restored the Nov^S and the cysteine prototrophy phenotypes. Hence, we showed that the functional restoration of CysB regarding the repression of the *hslJ* transcription and the consequent Nov^S phenotype can be obtained to a certain extent by complementation of the alleles that encode the CysB I33N and CysB S277Ter proteins. However, the combination of these mutated CysB proteins did not complement the cysteine auxotrophy. This result suggests that the restored

Table 3. Interallelic complementation of *cysB* mutant genes that encode the CysB I33N and CysB S277Ter regulators

Strain	Relevant genotype	Resistance to novobiocin (μg ml^{-1})*	Growth on A medium†
MC4100	$cysB^+$	60	+
EC2275	$cysB^-$‡	300	−
EC2275/pVGM9	$cysB^-$/*cysB831*::IS*1*§	200	−
EC2275/pVGM1	$cysB^-$/$cysB^+$	60	+
EC2549	Δ*cysB*	300	−
EC2549/pVGM9	Δ*cysB*/*cysB831*::IS*1*	280	−
EC2549/pVGM1	Δ*cysB*/$cysB^+$	60	+

*The cells were grown at 37 °C on rich LB plates supplemented with antibiotic.
†The cells were grown at 37 °C on minimal medium A plates with no cysteine supplemented.
‡*cysB* mutant gene that encodes the CysB I33N protein.
§*cysB* mutant gene that encodes the CysB S277Ter protein.

oligomer of the CysB regulator partially represses the *hslJ* transcription while not functioning in regulating the transcription of the *cys* regulon genes. This result is different from that obtained by Colyer & Kredich (1994), where the interallelic complementation of the CysB I33N and the CysB T149Ter mutant regulators restored the functional regulation of the *cys* regulon genes and complemented the cysteine auxotrophy. One may suspect that the DNA-binding property of the LTTR CysB slightly differs in the *hslJ* system in comparison to the *cys* regulon target promoters. It could be that binding is correct but the transcription activation (CysB as an activator) important for the *cys* regulon genes but not for the *hslJ* transcription regulation (CysB as a repressor) failed.

Conclusions

In this work we determined the *hslJ* TSP, the putative σ^{70} promoter and the *hslJ* promoter region. We found that *hslJ* transcription is subject to a complex double negative control by CysB and autogenously upon expression of HslJ. These two regulations compete in controlling *hslJ* transcription but do not exclude each other. CysB is a repressor of *hslJ* transcription that binds a DNA sequence (region C) which carries the putative *hslJ* σ^{70} promoter and the potential CysB repressor site. The putative repressor site is composed of two binding sites, 'LysR motifs', that overlap the -10 and -35 hexamers of the *hslJ* σ^{70} promoter, and the *hslJ* TSP. The CysB ligand, NAS, hitherto considered to be an inducer in CysB-mediated gene regulations, instead of preventing, enhances the CysB-binding in *hslJ* promoter region. The interallelic complementation of the mutated *cysB* genes that encode the CysB I33S and the CysB S277Ter regulators suggests that there may be differences in DNA binding of CysB to the *hslJ* regulatory region in comparison to the regulatory regions of the *cys* regulon genes. The negative autogenous control of *hslJ* transcription is observed but the mechanism of regulation remains elusive. This autoregulation might be indirect and enabled by an assumed factor X dependent on the proper HslJ localization. The toxicity of the overexpressed HslJ, which may be explained by the proposed localization in the outer membrane, could be the reason for the tight negative regulation of the *hslJ* transcription. Only the small concentration of the HslJ produced in WT cells or the limiting higher amount of the HslJ transiently produced in the *cysB* mutants is tolerated by the bacterium. However, the HslJ transiently produced in the higher concentration (e.g. in *cysB* mutants) is sufficient to provide the novobiocin resistance followed by the immediate negative feedback control and the repression of *hslJ* transcription. This model could be analysed by using different approaches. The mechanism of HslJ-expression-dependent autogenous control could be illuminated by using a chase experiment in a *cysB* mutant background, by determining the exact localization of the HslJ protein, and by screening for the HslJ-dependent regulation factor which, when mutated, in the *cysB* mutants leaves the *hslJ* transcription open. Finally, the mechanism of novobiocin resistance, specific or not, the biological significance of the HslJ protein and its role in the physiology of bacteria remain for future studies.

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