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Identification of the CysB-regulated gene, *hslJ*, related to the *Escherichia coli* novobiocin resistance phenotype

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Abstract

The *cysB* gene product is a LysR-type regulatory protein required for expression of the *cys* regulon. *cysB* mutants of *Escherichia coli* and *Salmonella*, along with being auxotrophs for the cysteine, exhibit increased resistance to the antibiotics novobiocin (Nov) and mecillinam. In this work, by using λp*lac*Mu9 insertions creating random *lacZ* fusions, we identify a gene, *hslJ*, whose expression appeared to be increased in *cysB* mutants and needed for Nov resistance. Measurements of the *hslJ*::l*acZ* gene fusion expression demonstrated that the *hslJ* gene is negatively regulated by CysB. In addition we observe the negative autogenous control of HslJ. When the control imposed by CysB is lifted in the *cysB* mutant, the elevation of Nov resistance can be achieved only in the presence of wild-type *hslJ* allele. A double *cysB hslJ* mutant restores the sensitivity to Nov. Overexpression of the wild-type HslJ protein either in a $cysB^+$ or a $cysB^-$ background increases the level of Nov resistance indicating that *hslJ* product is indeed involved in accomplishing this phenotype. The *hslJ*::ΩKan allele encodes the C-terminaly truncated mutant protein HslJ Q121Ter which is not functional in achieving the Nov resistance but when overexpressed induces the *psp* operon. Finally, we found that inactivation of *hslJ* does not affect the increased resistance to mecillinam in *cysB* mutants.

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Keywords: *cysB*; *hslJ*; Negative regulation; Resistance to novobiocin

1. Introduction

The *cys* regulon of *Escherichia coli* consists of more than 20 genes that function in the transport and synthesis of L-cysteine from inorganic sulfate [1]. Full expression of the *cys* genes requires an active, *cysB*-encoded CysB protein, inducer *N*-acetyl-serine (NAS), and sulfur limitation [1]. CysB multidomain protein belongs to the LysR family of transcriptional regulators with a helix-turn-helix, DNA-binding motif at the amino-terminus [1,2]. A CysB tetramer binds DNA upstream of the −35 box of promoters of the target genes and activates their transcription in the presence of NAS [1]. CysB, like most members of the LysR family, acts as a repressor of its expression by binding to its own promoter region and the presence of NAS inducer decreases CysB binding affinity [1].

Besides being a positive regulator of the *cys* regulon and controlling the biosynthesis of cysteine, CysB in conjunction with CysB-like transcription factor, Cbl [3], is involved in sulfur metabolism by upregulating the sulfate starvation-inducible taurine operon, *tauABCD* [4] and downregulating the *ssuEADCB* operon necessary for utilization of the sulfur from aliphatic sulfonates [5]. Recently, a CysB-regulated gene involved in the transport of glutathione has been identified [6].

It has been shown in our laboratory that CysB, a global regulator of the *E. coli*, may take part in cellular activities not directly related to the biosynthesis of cysteine and the utilization of sulfur. We have shown that mutations in *cysB* (and *cysE*) increase resistance of *E. coli* to the gyrase inhibitor novobiocin (Nov), and this resistance is not related to either the topoisomerase–gyrase system or outer

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membrane permeability [7]. Subsequent studies also demonstrated various nonconventional roles of CysB in cellular metabolism, such as resistance to the β-lactam antibiotic mecillinam [8], and induction of acid-inducible genes [9]. Nov inhibits DNA replication by blocking the ATPase activity of the β subunit (GyrB) of DNA gyrase [10], and conventional Nov^r mutants map within the *gyrB* gene [11]. Some other mutations, such as *ileS*, *alaS*, *argS*, and *rpoN*, also increase resistance to Nov [12], while mutations in the genes *cls* (*nov*) [13,14] and *prc* [15] decrease *E. coli* resistance to Nov. The precise role of these genes in the resistance mechanisms is not known. Recent studies showed that the BaeR response regulator from the BaeSR two-component signal transduction system that responds to envelope stress controls expression of the multidrug transporter ABC, and confers resistance to Nov [16].

This work is a continuation of our original observation on the alternative cellular function of the CysB regulator [7] with the aim of identifying the postulated CysB-regulated gene involved in Nov resistance. In this study we identify a new gene, *hslJ*, regulated by the CysB protein, and correlated to the *E. coli* resistance to the antibiotic Nov.

2. Materials and methods

2.1. Bacterial strains and media

The bacterial strains, phages, and plasmids used in this study are listed in Table 1. Complex liquid medium was Luria–Bertani broth (LB), solidified when necessary with 1.5% (w/v) agar (LA). Minimal medium was minimal A medium supplemented when necessary with amino acids (aa), as described elsewhere [27]. When appropriate, antibiotics were used at the following concentrations: 10–500 μg Nov ml^{-1}, 0.01–10 μg mecillinam ml^{-1}, 80 μg ampicillin ml^{-1}, 15 μg tetracycline (tet) ml^{-1}, 50 μg spectinomycin (spc) ml^{-1}, and 25 μg kanamycin (kan) ml^{-1}. To detect LacZ$^+$ cells and phages, 0.1 ml of a 10 mg ml^{-1} solution of X-Gal in *N*,*N*-dimetylformamide was added to LB agar plates or LB soft agar, respectively.

2.2. Construction of random translational lacZ fusions

A stock of λp*lac*Mu9 phages was prepared on strain MC4100, whereas λp*lac*Mu507 was propagated on strain MBM7014. For the construction of *lacZ* fusion strains, a fully grown culture of strain SY600 was coinfected with the λp*lac*Mu9 (multiplicity of infection (MOI) approx. 0.1) and λp*lac*Mu507 (MOI approx. 1). The cultures were incubated at room temperature for 30 min without shaking and washed three times with LB medium containing 20 mM Na-citrate to remove excess phages. The cells were then resuspended in 1 ml LB medium and 0.1-ml aliquots of serial dilutions were plated on LB plates containing kan and Na-citrate. After 16 h incubation at 42°C, blue colonies were isolated and tested for β-galactosidase (β-Gal) activity [27] at 30°C. Otherwise, the β-Gal assay was performed with cells grown at 37°C except for the cells tested on a heat shock, grown at 30°C and transiently induced at 37°C, 42°C and 50°C. Finally, the β-Gal assay was performed as well under growth conditions proposed by Anton [28] and we did not see any difference in the activity of the *hslJ*::*lacZ* gene fusion dependent on the late exponential phase, hence the protocol proposed by Miller [27] was used.

2.3. Phage manipulations and transductions

The isolated fusion was transferred by P1 transduction [27] from strain SY601 into strain SY602, previously isolated as a spontaneous Nov^r *cysB* mutant of strain MC4100. Excision of the λp*lac*Mu9 prophage by UV light from the fusion strain was done as follows. Strain SY604 was grown to an OD_{600} of 0.4–0.8, washed with 10 mM $MgSO_4$, and resuspended in 0.5 volume of 10 mM $MgSO_4$. Aliquots were irradiated with UV light (254 nm) for 1–5 min in an open Petri dish and transferred into 10 volumes of LB medium. After 4–6 h incubation in the dark at 37°C, the lysate was centrifuged to remove cell debris. Dilutions of the supernatant were plated on a lawn of strain SY602 on LB plates with X-Gal and incubated overnight at 37°C. The phage from a single blue plaque was further propagated on strain SY602. Phage titers, obtained by spot tests [29], were approximately 10^{10} pfu (plaque-forming units) ml^{-1}. For fusion mapping by transduction, P1 lysates were prepared on strains constructed by Singer et al. [21] CAG12081 (carrying a Tn*10* at minute 29.50) and CAG12026 (carrying a Tn*10* at minute 31.00). The phage lysates obtained were used to transduce the kan^r strain SY604. The resulting tet^r transductants were then tested for kan sensitivity.

2.4. Construction of strains with the hslJ::ΩKan mutation

Strains with the *kan* cassette (ΩKan) inserted into the *hslJ* gene (strains SY605–608) (Fig. 1) were obtained in the following manner. The plasmid pHV3004 (Table 1) was transformed into strain C600 and transformants were selected on LA plates containing kan. In the next step, P1 transducing phage was prepared on the culture of one of the obtained kan^r transformants. The resulting lysate was used to transduce the *kan* gene into recipient strains MC4100, SY602, C600 and SY380, obtaining in this way final constructs SY605, SY606, SY607 and SY608, respectively (Table 1). Transductants with *hslJ*::ΩKan inserts obtained by double-crossover event via the *hslJ* sequences were discriminated from those obtained through single-crossover event or those carrying extrachromosomal plasmid (pHV3004), by nonselective analysis, i.e. isolation of kan^r spc^s clones among kan^r transductants.

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2.5. Sensitivity tests

Minimal inhibitory concentration (MIC) was determined by plating 0.01-ml samples of diluted overnight cultures (approximately 10^3 cells) on LB plates containing serial dilutions of the antibiotic to be tested. Appropriate dilutions spread on LB plates without antibiotic served to assess the number of cells plated. Growth was scored after 2 days of incubation at 37°C. Survival values less than 10% were recorded as inhibition.

2.6. DNA manipulations

Plasmid isolation, restriction, ligation, electroelution, transformation and other DNA recombinant techniques were performed by standard procedures [30]. DNA from transducing phages was isolated as published [19]. The chromosomal location of the CysB-regulated *lacZ* gene fusion was determined by hybridizing DNA probe with Kohara's lambda clones [24]. The DNA probe was labeled with biotin and signals were detected by the Photo Gene System (Bethesda Research Laboratories, Bethesda, MD, USA). All sequencing reactions were performed by the dideoxy termination method [31].

3. Results and discussion

3.1. Isolation and characterization of the CysB-regulated lacZ fusion

To identify CysB-regulated genes involved in bacterial resistance to Nov, random translational fusions were made with λp*lac*Mu9 phage [32] in strain SY381 carrying a temperature sensitive *cysB*(Ts) mutation. As shown in our original report [7], this *cysB* Ts mutant (SY381) at the permissive temperature (30°C) exhibits wild-type resistance to Nov (100 μg ml^{-1}) and a $CysB^+$ phenotype. A temperature shift to 42°C causes an increase in Nov resistance (400 μg ml^{-1}) and a growth requirement for cysteine [13].

Numerous λp*lac*Mu9 insertion mutants in strain SY600 (Lac^- derivative of strain SY381) were obtained on

Table 1
E. coli K-12 strains, phages and plasmids used in this study

	Relevant genotype	Source
Strain		
AB1157	*thi-1 thr-1 leuB6* Δ(*gpt-proA*)62 *argE3 hisG4 lacY1 galK2 ara-14 xyl-5 mtl-1 tsx 33 rpsL31 supE44 rfbD1 mgl-51 kdgK51 rac*$^-$	[17]
C600	*thi-1 thr-1 leuB6 lacY1 tonA21 supE44 rfbD1*	[18]
MC4100	*araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 deoC1 ptsF25 rbsR flbB5301*	[19]
MBM7014	*araC*(am) *araD* Δ(*argF-lac*)*U169 trp*(am) *malB*(am) *rpsL relA1 thi supF*	[20]
CAG12081	*zcj-3061*::Tn*10 fnr-501*	[21]
CAG12026	*trg-2*::Tn*10*	[21]
SY380	C600 *cysB*	[7]
SY381	AB1157 *cysB*(Ts)	[7]
SY600	SY381 Δ(*argF-lac*)*U169*	Laboratory collection
SY601	SY600 ϕ(*hslJ*::*lacZ*) λp*lac*Mu9 kan^r	This work
SY602	MC4100 *cysB*; isolated as Nov^r mutant	This work
SY603	MC4100 ϕ(*hslJ*::*lacZ*) λp*lac*Mu9 kan^r	This work
SY604	SY602 ϕ(*hslJ*::*lacZ*) λp*lac*Mu9 kan^r	This work
SY605	MC4100 *hslJ*::ΩKan	This work
SY606	SY602 *hslJ*::ΩKan	This work
SY607	C600 *hslJ*::ΩKan	This work
SY608	SY380 *hslJ*::ΩKan	This work
MC4100λpsp3	MC4100 λϕ(*pspA-lacZ*)	[22]
Phage		
λp*lac*Mu9	*imm* λ′*lacZlacY*$^+$*lacA*″*ara*′*Xho*::*kan* Mu[*cI*(Ts)*62 ner*$^+$ *A*$^+$ *S*]	[23]
λp*lac*Mu507	*cI857 Sam7* Mu(*cI*(Ts)*62 ner*$^+$ *A*$^+$ *B*$^+$)	[23]
λ265	*hslJ*$^+$	[24]
Plasmid		
pMS421	Recombinant cloning vehicle, spc^r	[25]
pUC4K	Recombinant cloning vehicle containing the *kan* gene cassette (ΩKan)	[26]
pHV2810	pBR322::*cysB*$^+$	[7]
pHV2814	pBR322 *cls*::Tn*5lacZ* fusion	Laboratory collection
pHV3001	6.6-kb *Sal*I fragment containing *hslJ*::*lacZ* fusion joint cloned into the *Sal*I site of pMS421	This work
pHV3002	3.7-kb *Bam*HI–*Pst*I fragment from λ265 containing the *hslJ*$^+$ gene cloned into the *Bam*HI–*Pst*I digested pMS421	This work
pHV3003	5-kb *Eco*RI fragment containing *hslJ*::*lacZ* fusion cloned in *Eco*RI site of pHV2814	This work
pHV3004	1.28-kb fragment from pUC4K containing the kan gene inserted into the *Hpa*I site of pHV3002	This work

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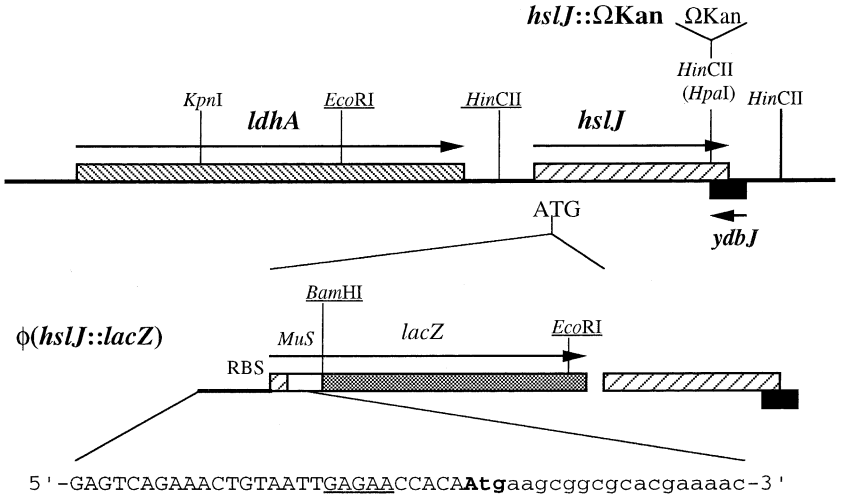

Fig. 1. Schematic representation of the *hslJ* locus, *hslJ*::*lacZ* gene fusion, and *hslJ*::ΩKan mutation. The upper part of the figure represents the *hslJ* locus (*ldhA* and *hslJ* genes and ORF *ydbJ*) positioned on 30.9 min on the *E. coli* chromosome and the partial restriction map. The position of *kan* cassette (ΩKan) insertion in the 3′ region of the *hslJ* gene is presented above. The lower part of the figure is the illustration of λp*lac*Mu9 insertion in the *hslJ* gene start codon ATG, just after the A nucleotide creating the gene fusion *hslJ*::*lacZ*. Restriction sites used for subcloning the *hslJ*::*lacZ* gene fusion joint (*Eco*RI) and constructing the probe for hybridization (*Hin*CII–*Bam*HI) are indicated as underlined sites. The stretch of nucleotides below depicts DNA around the splicing point between the *hslJ* gene and λp*lac*Mu9 phage DNA. Capital letters denote the nucleotides upstream of the *hslJ* gene and the first nucleotide of the initiation codon; small letters denote the λp*lac*Mu9 DNA. Hatched box on the left, *ldhA* gene; hatched box on the right, *hslJ* gene; black box, ORF *ydbJ*; shaded box, *lacZ* gene; open box, *MuS* sequence; RBS, ribosome-binding site (underlined in the sequence depicted).

X-Gal-kan plates. One kan^r mutant exhibited different expression of the fused *lacZ* gene at 30°C (white colonies) and at 42°C (blue colonies). Hence, the strain carrying this gene fusion obtained in this quest exhibited on X-Gal plates blue colonies at 42°C (*cysB* mutant) and white colonies at 30°C (*cysB*$^+$). Since this mutant (SY601) did not require cysteine for its growth at 30°C we assumed that the observed phenotype was not due to the insertion of the phage into genes of the *cys* regulon. Finally, the strain demonstrated five-fold decreased resistance to Nov at 42°C in comparison to the parental strain SY600 (Table 2).

Table 2
Nov resistance of *cysB* and double *cysB hslJ* mutants

Strain	Relevant genotype	MIC[a] (μg ml^{-1})
AB1157	*cysB*$^+$	100
SY381[b]	*cysB*(Ts)	100
SY381[c]	*cysB*(Ts)	400
SY600[b]	*cysB*(Ts)	100
SY600[c]	*cysB*(Ts)	400
SY601[c]	*cysB*(Ts) *hslJ*::*lacZ*	< 100 (80) [blue][d]
MC4100	*cysB*$^+$	< 25 (20)
SY602	*cysB*$^-$	< 400 (300)
SY603	*cysB*$^+$ *hslJ*::*lacZ*	< 25 (20) [white][d]
SY604	*cysB*$^-$ *hslJ*::*lacZ*	> 50 (60) [blue][d]

[a]MIC, minimal inhibitory concentration of Nov. When needed two-fold dilution was changed in order to determine the more precise MIC (round brackets). The cells were grown at 37°C on LB plates supplemented with 0.1 mM cysteine and antibiotic.
[b]Incubation at 30°C.
[c]Incubation at 42°C.
[d]White and blue in the square brackets describe the appearance of the strains with a *hslJ*::*lacZ* fusion on X-Gal LB plates.

To verify the nature of the obtained construct, we transferred the fusion by P1 transduction into another strain, SY602, harboring a *cysB* mutation. This MC4100 derivative carries a *cysB* gene with an IS*1* insertion in its 3′ region (unpublished data) and exhibits the auxotrophy to cysteine and resistance to Nov. Colonies of the resulting strain (SY604) were blue on X-Gal plates and the strain showed five-fold decreased resistance to Nov relative to the Nov^r parental strain SY602 (Table 2). Hence, the inactivation of an unknown gene in the *cysB* mutant decreases the resistance to Nov. The control strain SY603 (*cysB*$^+$), formed white colonies on X-Gal plates and demonstrated low resistance to Nov (Table 2). These results showed that the insertion mutant containing the *lacZ* reporter gene fused to an unknown gene is related to bacterial resistance to Nov and that this gene might be negatively controlled by CysB. They also eliminated the possibility that the observed behavior of the fusion is temperature dependent.

3.2. *Mapping and sequence determination of the* lacZ *insertion site*

The chromosomal location of the *lacZ* (λp*lac*Mu9) insertion in strain SY604 was determined by P1-mediated transductions as described in Section 2.3. The approximate position of the insert was determined between minutes 29.50 and 31.00 of the *E. coli* chromosomal map by using CAG strains (data not shown). For more precise mapping, the λp*lac*Mu9 phage was induced by UV irradiation from strain SY604. The lysate obtained was used for backcross to infect a *cysB*$^-$ host (SY602), and the phage/bacteria

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mixture was plated on X-Gal LB plates. Lac$^+$ transducing phages were picked from blue plaques and further propagated on strain SY602 as a source of phage DNA. By using the restriction mapping, the insertion site of the *lacZ* and the fusion joint was localized on the 5-kb*Eco*RI fragment of the phage/chromosome DNA (Fig. 1). This fragment was subcloned in the vector pHV2814 and the 1-kb *Bam*HI–*Hin*CII fragment obtained from that clone was used as a probe for hybridization analysis with Kohara's clones [24] encompassing that region of the chromosome (Fig. 1). The probe hybridized with λ265 (data not shown), which among other genes carries the *hslJ* gene. Finally, sequencing established that the insertion is in the *hslJ* gene. The nucleotide sequence analysis of the DNA segment from plasmid pHV3003, containing the junction between *lacZ* and the unknown gene, and subsequent search of the deposited *E. coli* genome sequence [33], revealed that *lacZ* was fused to the very beginning (after the A in ATG start codon) of the *hslJ* gene (Fig. 1), previously described as a heat shock-responsive gene [34]. The 423-nucleotide-long *hslJ* gene is not in an operon and there is no open reading frame (ORF) downstream. The small convergently oriented *ydbJ* ORF positioned in the *hslJ* 3′ region does not account for the phenotype observed for the *hslJ*::*lacZ* gene fusion since this *hslJ* null mutation falls into the very beginning of the gene leaving the small ORF intact (Fig. 1). The *ldhA* gene is positioned 110 nucleotides upstream of *hslJ* and possesses a strong stem-loop structure behind the gene (5′-GCTCCCC-TGcattcCAGGGGAGC-3′).

The *hslJ* gene encodes the 140-residue-long HslJ protein with a molecular mass of 15 kDa. The analysis of the deduced aa sequence showed the following: (1) the first 20–25 residues of the N-terminus comprise the hydrophobic sequence with a high probability for α-helix secondary structure and resembles either the signal sequence or the transmembrane region; (2) the central region (15–116 aa) contains a DUF 306 (*d*omain of *u*nknown *f*unction) found in proteins of unknown function, some of them secreted and involved in motility of bacteria and some related to the virulence of bacterial pathogens [35]; (3) the C-terminus (97–124 aa) is predicted to have the coils structure with a long α-helix superimposed; this structure is predisposed for protein–protein interactions that form the more complex coiled coils structure; (4) very strict criteria predicted that the HslJ protein was located either in the outer membrane (probability 0.79) or in the inner membrane (probability 0.70) of the bacterium. Hydropathy and topology of the protein were predicted using programs 'DNA star 3.87 – Protean', 'TopPred 2 – Stockholm University' and 'SignalP V1.1'. The secondary structure of the protein was analyzed by using 'Profile network prediction Heidelberg' and 'NCBI – MMDB Structure Summary'.

3.3. *The* hslJ *expression is not induced by heat shock*

As mentioned above, the *hslJ* gene was previously described as a heat shock-responsive gene [34]. In the original report, the authors demonstrated weak heat shock induction at 50°C of the *hslJ* gene in vivo, but data on whether synthesis of HslJ is stimulated by heat shock-specific σ^{32}-RNA polymerase are missing. We examined the activity of the *hslJ*::*lacZ* gene fusion in vivo in *cysB* mutant strain, SY604, at different temperatures. The β-Gal assay showed that the *hslJ* expression did not respond to heat shock, and the highest value (92 Miller units) was achieved at 37°C as compared to 58, 33 and 30 Miller units obtained at 30°C, 42°C and 50°C, respectively. Moreover, the inspection of DNA sequence in the region upstream of the *hslJ* gene shows no resemblance to either σ^{32}- or σ^{E}-dependent, heat shock-responsive promoters; it rather indicates that *hslJ* expression might be driven by a σ^{70} house-keeping type of promoter. Therefore, all further analyses with strains harboring *hslJ*::*lacZ* gene fusion were carried out at 37°C.

3.4. *CysB negatively regulates the expression of* hslJ *gene*

The results presented in Table 3 show that expression of the chromosomal gene fusion *hslJ*::*lacZ* in both rich LB medium and minimal A medium (containing cysteine) is higher in the *cysB* mutant (SY604) than in the *cysB*$^+$ strain (SY603), indicating that CysB negatively regulates the *hslJ* expression. This regulation persists in trans as

Table 3
β-Gal activities of the gene fusion *hslJ*::*lacZ* on the chromosome and plasmid pHV3001

Strain	Relevant genotype	β-Gal activity[a]	
		A+cysteine	LB
SY603	*cysB*$^+$ *hslJ*::*lacZ*	< 1	28 ± 2
SY604	*cysB*$^-$ *hslJ*::*lacZ*	48 ± 6	90 ± 3
SY604/pHV2810	*cysB*$^-$ *hslJ*::*lacZ*/*cysB*$^+$	40 ± 8	77 ± 3
SY605/pHV3001	*cysB*$^+$ *hslJ*::ΩKan/*hslJ*::*lacZ*	6 ± 2	36 ± 4
SY604/pHV3002	*cysB*$^-$ *hslJ*::*lacZ*/*hslJ*$^+$	NG[b]	24 ± 1

[a]β-Gal activity was expressed in Miller units. The Miller units in the table represent the average values of six independent assays with standard deviations. The β-Gal activity in either A or LB medium was assayed after growing cells at 37°C in the presence of 0.5 mM or 0.1 mM cysteine, respectively.
[b]NG, no growth.

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well. When plasmid-borne *cysB*$^+$ under its own promoter (pHV2810) was introduced into the fusion strain SY604, β-Gal activity in both media exhibited reduced values as compared to the strain without the plasmid (Table 3). In trans negative regulation of the *hslJ*::*lacZ* gene fusion imposed by CysB was more pronounced when the *hslJ*::*lacZ* fusion located on plasmid (pHV3001) was introduced into the *hslJ*::ΩKan mutant strain (see Section 2.4.) with intact *cysB* gene on the chromosome. Hence, the chromosomal copy of the *cysB* gene strongly represses the plasmid-borne *hslJ*::*lacZ* fusion in both media (Table 3). It is possible that the intracellular concentration necessary for CysB to act as a repressor may be attained more easily by the chromosomal than the plasmid copy of *cysB*, since the *cysB* gene expressed from plasmids and under control of the homologous promoter is known not to allow a high accumulation of its transcript [3].

A relatively small difference in β-Gal activities (factor 3) in rich medium between *cysB*$^+$ (SY603) and *cysB*$^-$ (SY604) carrying the *hslJ* null mutation (*hslJ*::*lacZ*) (Table 3) is in accordance with the observed difference in Nov resistance (factor 3) (Table 2). Nevertheless, the difference in resistance between the *cysB*$^+$ and the *cysB*$^-$ strains MC4100 vs. SY602 carrying the wild-type *hslJ* gene is 15-fold (Table 2). The intriguing observation is that the small difference in β-Gal activities SY603 vs. SY604 accounts for the strong difference in the phenotype (white or blue) on LB X-Gal plates (Table 2).

We did not succeed in cloning the *hslJ* gene on a multicopy plasmid, which may indicate cellular sensitivity to a higher concentration of HslJ. We did succeed in cloning that gene on the low copy plasmid pMS421. However, when this construct pHV3002 was introduced into the *cysB* mutant it abolished its ability to grow on minimal medium supplemented with cysteine (Table 3). This phenotype may be of help in future efforts to establish the role of HslJ in the cell. For now, the true function of the *hslJ* gene product remains unclear. In a rich medium the overexpression of the *hslJ* gene in the *cysB*$^-$ background strongly represses the *hslJ*::*lacZ* gene fusion in trans (Table 3), yet significantly increases the resistance to Nov (see Table 4). Considering this autogenous control as well as the negative regulation by CysB and the moderate expression of *hslJ* when these controls are lifted, one may assume that strict control of *hslJ* expression ensures maintenance of the HslJ protein at the physiologically acceptable level. Our failure to clone *hslJ* in a multicopy plasmid could reflect this phenomenon.

The measurements of *hslJ*::*lacZ* gene fusion expression demonstrated that the *hslJ* gene is under negative control imposed by the CysB regulator, but did not reveal whether CysB affects expression of the *hslJ* gene directly as a repressor, or via CysB-controlled intermediary regulator(s). It might be that CysB acts directly to repress the expression of *hslJ* but respecting the genetic studies and the description of the protein proposed to reside in the membrane one cannot rule out an indirect effect for the negative autogenous control of HslJ. Dissection of the *hslJ* regulatory region and experiments that will help us to understand whether CysB acts directly as a repressor or through intermediate(s) are currently underway.

3.5. *The expression of* hslJ *correlates with the resistance to Nov*

It was stated above that *cysB* mutants exhibit resistance to Nov. The results presented in this work show that inactivation of the *hslJ* gene either by insertion of *lacZ* (*hslJ*::*lacZ*) (Table 2) or by the *kan* cassette (*hslJ*::ΩKan) (Table 4) in a *cysB*$^-$ background, SY604 and SY606, respectively, causes a five-fold decrease in resistance to Nov relative to the *cysB* mutant alone (SY602). The same results were obtained with the *cysB*(Ts) mutation (strain SY601 vs. strain SY600) (Table 2). In contrast, the inactivation of *hslJ* either by the *lacZ* or by the *kan* cassette did not affect the resistance to Nov in a *cysB*$^+$ genetic background, strains SY603 and SY605, respectively, in comparison to the wt MC4100 (Tables 2 and 4). Therefore, the phenotype of double *cysB hslJ* mutants implements the role of HslJ in Nov resistance. This statement is strengthened by the fact that overexpression of *hslJ* cloned on low copy plasmid (pHV3002) in either *cysB*$^+$ or *cysB*$^-$ strain increases the resistance to this antibiotic (Table 4). Although the obtained resistance to Nov was the highest in the mutant, the elevation is much higher in the strain that carries a *cysB* wild-type allele than in the *cysB* mutant. This could be due to the contribution of the *hslJ* chromosomal copy overexpressed in *cysB* mutant. The *hslJ*::ΩKan mutation was used as a control and did not change the Nov resistance of recipient strains (Table 4).

The results presented in Tables 2 and 4 show that the inactivation of the *hslJ* gene does not affect cellular resistance to Nov when the *hslJ* is under the negative control of CysB. However, when the negative control of CysB is abolished, the facilitated expression of *hslJ* increases Nov resistance while the inactivation of *hslJ* significantly re-

Table 4
Nov resistance of *cysB* wild-type strain and *cysB* mutant overproducing HslJ

Strain	Relevant genotype	MIC[a] (μg ml^{-1})
MC4100	*cysB*$^+$	< 25 (20)
SY605	*cysB*$^+$ *hslJ*::ΩKan	< 25 (20)
SY602	*cysB*$^-$	< 400 (300)
SY606	*cysB*$^-$ *hslJ*::ΩKan	> 50 (60)
MC4100/pHV3002	*cysB*$^+$/*hslJ*$^+$	100
MC4100/pHV3004	*cysB*$^+$/*hslJ*::ΩKan	< 25 (20)
SY602/pHV3002	*cysB*$^-$/*hslJ*$^+$	400
SY602/pHV3004	*cysB*$^-$/*hslJ*::ΩKan	< 400 (300)

[a]MIC, minimal inhibitory concentration of Nov. When needed two-fold dilution was changed in order to determine the more precise MIC (round brackets). The cells were grown at 37°C on LB plates supplemented with 0.1 mM cysteine and antibiotic.

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duces it. We should emphasize that the inactivation of the *hslJ* gene in *cysB* mutants does not bring cellular resistance back to its native level of 20 μg ml^{-1} in MC4100, suggesting that some other gene(s) controlled by the CysB protein, might also take part in this resistance pathway. However, the overexpression of *hslJ* alone overcomes the negative control of CysB, consequently raising the resistance to Nov. Thus, the conclusion drawn from these results is that an increased intracellular concentration of the HslJ protein causes the increased resistance to Nov in mutants lacking CysB, the negative regulator of *hslJ* expression.

3.6. *The expression of* hslJ*::ΩKan allele induces the* psp *operon*

The mutation *hslJ*::ΩKan truncates the C-terminal region of the HslJ making the functionally inactive HslJ Q121Ter protein in providing the Nov resistance. The ΩKan was inserted in the 3′ region of the *hslJ* gene disrupting the predicted coils region and long putative α-helix secondary structure of the HslJ protein. This mutation somehow abolishes the functionality of HslJ related to Nov resistance suggesting that this region, probably involved in the protein–protein interaction, could be important for the observed phenotype.

The predicted membrane localization of HslJ could have an impact on the function of this protein. The *psp* operon is induced upon exposure to a number of envelope perturbations or by overexpression of a variety of OMPs (e.g. pIV, PulD, OutD, and some other secretins) both mutant and wild-type [36]. The *psp* response is also turned on by expression of mutant envelope proteins (e.g. PhoE, LamB) [36]. Hence, we did the experiment where the wild-type *hslJ* gene or the *hslJ*::ΩKan allele was overexpressed in the host strain MC4100 λpsp3 [22] that carries the chromosomal operon fusion *λϕ(psp-lacZ)*. We found that only expression of the mutated *hslJ* gene induces the *psp* operon five-fold. Namely, the β-Gal activity of MC4100 λpsp3/pHV3004 strain overexpressing the truncated protein HslJ Q121Ter was 423 Miller units in comparison to 86 units found in the control strain MC4100 λpsp3/pMS421. This result suggests that the HslJ could be the cell envelope protein.

3.7. *Inactivation of the* hslJ *gene does not affect the resistance to mecillinam in* cysB *mutant*

The final aspect of this study deals with the resistance of *cysB* mutants of *E. coli* to the β-lactam antibiotic mecillinam. Mecillinam specifically targets penicillin-binding protein 2 and blocks cell wall elongation in *E. coli* [37]. It has been shown that *cysB* mutants of *Salmonella typhimurium* are resistant to mecillinam [8]. Similar to the results obtained with *Salmonella*, *cysB* mutants of *E. coli* also exhibit increased resistance to mecillinam. We showed that the *cysB* mutant of *E. coli*, SY380, exhibited a dramatic increase in mecillinam resistance in rich medium (MIC – 10 μg ml^{-1}) relative to its *cysB*$^{+}$ counterpart, strain C600 (MIC – 0.05 μg ml^{-1}). Following this analogy between the CysB^{-} phenotype on the one hand and the resistance to Nov and mecillinam on the other hand, we investigated whether the lack of HslJ in a *cysB*$^{-}$ genetic background affects the resistance to mecillinam or is only responsible for the decrease in Nov resistance. The results we obtained in rich medium demonstrate that inactivation of the *hslJ* gene by the *kan* cassette in both *cysB*$^{+}$ and *cysB*$^{-}$ strains, SY607 and SY608, respectively, does not cause any change in their resistance to mecillinam as compared to their *hslJ*$^{+}$ parental strains, C600 and SY380, respectively (data not shown). It is worthwhile to note that these experiments were carried out in rich media where the cysteine is either oxidized to cystine or present in oligopeptides. Otherwise, the presence of reduced cysteine in plates inactivates mecillinam and the problem could occur [38].

Therefore, in contrast to the results obtained with induced resistance to Nov in *cysB* mutants, inactivation of *hslJ* does not decrease the resistance to mecillinam, indicating that these two resistance pathways branch off at this point. Isolation and identification of mutants with decreased or abolished resistance to mecillinam in *cysB* mutants would certainly help in unraveling other gene(s) of this resistance pathway.

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