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Regulation of Sulfur Assimilation Pathways in *Burkholderia cenocepacia*: Identification of Transcription Factors CysB and SsuR and Their Role in Control of Target Genes▽

Roksana Iwanicka-Nowicka,[1] Agata Zielak,[1] Anne M. Cook,[2] Mark S. Thomas,[2] and Monika M. Hryniewicz[1]*

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland,[1] and Division of Genomic Medicine, School of Medicine and Biomedical Sciences, University of Sheffield, Sheffield, United Kingdom[2]

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Two genes encoding transcriptional regulators involved in sulfur assimilation pathways in *Burkholderia cenocepacia* strain 715j have been identified and characterized functionally. Knockout mutations in each of the *B. cenocepacia* genes were constructed and introduced into the genome of 715j by allelic replacement. Studies on the utilization of various sulfur sources by 715j and the obtained mutants demonstrated that one of the *B. cenocepacia* regulators, designated CysB, is preferentially involved in the control of sulfate transport and reduction, while the other, designated SsuR, is required for aliphatic sulfonate utilization. Using transcriptional promoter–*lacZ* fusions and DNA-binding experiments, we identified several target promoters for positive control by CysB and/or SsuR—*sbpp* (preceding the *sbp cysT cysW cysA ssuR* cluster), *cysIp* (preceding the *cysI cysD1 cysN cysH cysG* cluster), *cysD2p* (preceding a separate cluster, *cysD2 cysNC*), and *ssuDp* (located upstream of the *ssuDCB* operon)—and we demonstrated overlapping functions of CysB and SsuR at particular promoters. We also demonstrated that the *cysB* gene is negatively controlled by both CysB and SsuR but the *ssuR* gene itself is not significantly regulated as a separate transcription unit. The function of *B. cenocepacia* CysB (in vivo and in vitro) appeared to be independent of the presence of acetylserine, the indispensable coinducer of the CysB regulators of *Escherichia coli* and *Salmonella*. The phylogenetic relationships among members of the "CysB family" in the γ and β subphyla are presented.

The gram-negative genus *Burkholderia* is a member of the β-proteobacterial subphylum and comprises nutritionally versatile, nonsporulating bacilli that inhabit diverse environments, including freshwater, soil, and plant rhizospheres. Although, *Burkholderia* isolates were initially identified as plant pathogens causing soft onion rot (6), potentially beneficial properties of members of the genus attracted interest as well, e.g., the ability to utilize groundwater pollutants and chlorinated aromatic compounds as nutrient sources and their antagonistic effects on the growth of soilborne plant pathogens (32, 41). Perhaps the major interest in *Burkholderia* spp. emerged from their pathogenic traits in humans. Many studies have documented the occurrence of *Burkholderia cepacia* complex (BCC) infections of the respiratory tracts of immunocompromised individuals, in particular those with cystic fibrosis (CF) and chronic granulomatous disease (see references 35, 36, and 52 for reviews). BCC isolates were formerly classified into nine closely related "genomovars" that have been recently assigned species designations (4, 54). Although essentially all BCC genomovars have been isolated from infected CF patients, *Burkholderia multivorans* (formerly genomovar II) and *Burkholderia cenocepacia* (genomovar III) predominate among the isolates, and the latter has been also associated with both increased virulence and epidemic transmission. It is possible that BCC species responsible for infection in humans are genetically indistinguishable from soil isolates (33). The high degree of adaptability of *Burkholderia* species to different lifestyles correlates with the large sizes of their multireplicon genomes (6 to 9 Mb), as well as the presence of a variety of insertion sequences that promote genomic plasticity (32). Complete genome sequences for *Burkholderia pseudomallei* and *Burkholderia mallei* have been published (19, 39), and those of several members of the BCC, including *B. cenocepacia* strain J2315 (a CF isolate, originally documented as strain CF5610) are publicly available (http://www.sanger.ac.uk/Projects/B_cenocepacia/) but await formal annotation. In the past few years, a number of studies have been devoted to assessing the significance of potential virulence factors of BCC species (35, 36). Some features connected with virulence, e.g., intrinsic resistance to multiple antibiotics, production of catalase and superoxide dismutase, unusual lipopolysaccharide structure, presence of LuxR-type quorum-sensing systems, formation of flagella, ability to form biofilms, and production of siderophores, are hallmarks of all BCC species. Genomewide analyses of BCC bacteria provide a further opportunity to recognize regulatory connections between the production of virulence factors and general anabolic pathways. Recent studies on the codependence of siderophore biosynthesis and sulfate transport by *B. cenocepacia* (15) prompted us to assess experimentally the regulatory circuits involved in sulfur assimilation by *B. cenocepacia* as a model for ubiquitous BCC species.

The assimilation of sulfur from inorganic sulfate through the cysteine biosynthetic pathway has been best studied in *Salmo-*

* Corresponding author. Mailing address: Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawinskiego 5A, 02-106 Warsaw, Poland. Phone: 48 22 592 13 06. Fax: 48 39 121 623. E-mail: monikah@ibb.waw.pl.

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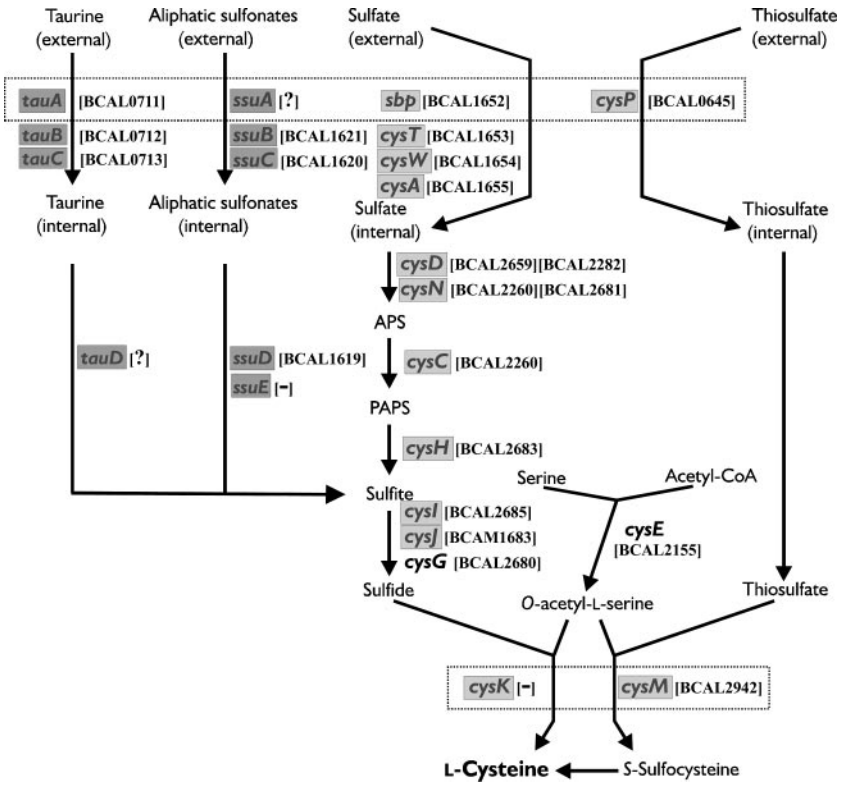


FIG. 1. Genes involved in the L-cysteine biosynthetic pathway in *E. coli* and their putative counterparts identified in *B. cenocepacia.* Genes of *E. coli* and *S. enterica* serovar Typhimurium involved in the sulfate/thiosulfate assimilatory pathway are shown according to the scheme of Kredich (30); those activated by CysB (as transcription units: *sbp*, *cysPTWA*, *cysDNC*, *cysJIH*, *cysK*, and *cysM*) are highlighted in light gray. Shaded in dark gray are the genes of *E. coli* participating in sulfonate-sulfur utilization (transcription units *tauABCD* and *ssuEADCB*) and requiring Cbl (as a direct activator) and also CysB (as an activator of the *cbl* gene) according to a scheme adapted from van der Ploeg et al. (57). The *E. coli* genes whose products display overlapping functions (Sbp/CysP and CysK/CysM) or partially overlapping functions (TauA/SsuA) are boxed. The putative counterparts of *E. coli* genes identified by us in the *B. cenocepacia* genome are indicated in brackets; "–" indicates no counterpart found, and "?" indicates that several ORFs of limited homology to the *E. coli* counterparts were identified by TBLASTN search. For the deduced functions of *B. cenocepacia* ORFs, see Discussion.

nella enterica serovar Typhimurium and *Escherichia coli* (30). Over 20 genes participating in this process form a cysteine (*cys*) regulon, and most *cys* genes are coordinately controlled by the LysR-type transcriptional activator CysB. CysB is highly conserved among gram-negative bacteria examined so far, but *E. coli* and several other species (although not all) also possess another LysR-type regulator, designated Cbl (*CysB-l*ike) for its high (60%) similarity to CysB (22). Studies on *E. coli* Cbl ascribed its regulatory function to the *tauABCD* and *ssuEADCB* operons, which encode proteins involved in transport and desulfonation of the organic sulfur sources taurine and aliphatic sulfonates, respectively (58, 59). In *E. coli*, CysB and Cbl are encoded by loci unlinked to their target genes, and expression of *cbl* itself is positively controlled by CysB (22).

Our inspection of the genomic sequence of the *B. cenocepacia* J2315 strain allowed us to identify preliminarily several potential counterparts of *E. coli* genes participating in sulfur flux from substrates to cysteine, as shown in Fig. 1. In addition, we noticed that two open reading frames (ORFs) (BCAL1656 and BCAL2686) present in chromosome 1 are predicted to encode polypeptides exhibiting homology to either of the *E. coli* regulators CysB and Cbl, as well as high mutual similarity on the amino acid level.

In this study, we focused on the cloning, expression in *E. coli*, and functional characterization of the two "*cysB*/*cbl*-like" genes of *B. cenocepacia* 715j. We also identify some target promoters for both gene products, and in consequence, we propose the designations SsuR and CysB for the *B. cenocepacia* regulators. The specific or overlapping functions of SsuR and CysB at target promoters are discussed.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The strains of *E. coli* and *B. cenocepacia* used in this study are listed in Table 1. All *E. coli* strains were grown at 37°C with aeration either in Luria-Bertani (LB) medium (44) or in modified M9 medium supplemented with 0.2% glucose, thiamine (0.2 mM), and tryptophan (0.2 mM). In the modified M9 medium (55), $MgSO_4$ was replaced by an equimolar concentration of $MgCl_2$, and sulfur sources were provided as sulfate, taurine, ethanesulfonate (sodium salts; 0.25 mM each), L-cysteine (0.1 mM), or L-djenkolic acid (1 mM). L-Djenkolic acid (S,S′-methylene-bis-cysteine) was used routinely as a "derepressing" sulfur source for *E. coli* and *B. cenocepacia* that satisfied the cellular requirement for cysteine but obviated the usual repressing effect of L-cysteine itself on the expression of sulfur metabolism-related genes. To monitor the ability of *B. cenocepacia* to utilize different sulfur sources, overnight cultures grown in brain-heart infusion broth (Difco) were washed once in modified M9 medium containing 0.5% glucose, and either no sulfur source or magnesium sulfate (1 mM), sodium sulfite (1 mM), sodium ethanesulfonate (0.5 mM), or cysteine (0.5 mM) was added. For performing β-galactosidase assays on

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description (genotype or relevant features)	Reference or source
E. coli K-12 strains		
DH5α	*supE44* Δ*lacU169* (ϕ*80 lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Laboratory collection
CC118(λ*pir*)	*araD139* Δ(*ara*, *leu*)*7697* Δ*lacX74 phoA20 galE galK thi rpsE rpoB argEam recAl* λ*pir*	18
S17-1(λ*pir*)	*thi proA hsdR recA* RP4-2-*tet*::Mu-1 *kan*::Tn*7* integrant (Tp^r Sm^r) λ*pir*	47
MC4100	*araD139* Δ(*lac*)*U169 strA thi*	8
EC1250	MC4100 *trp-1*	Laboratory collection
EC2541	EC1250 Δ*cbl*::*kan*	59
EC2549	EC1250 Δ*cysB*::*kan*	34
EC2625	SE70 Δ*cbl*::*cam*	Laboratory collection
EC2626	EC1250 $sbpp_{Bc}$-*lacZ*	This study
EC2627[a]	EC2626 *cysB*	This study
EC2628	EC1250 $cysBp_{Bc}$-*lacZ*	This study
EC2629[a]	EC2628 *cysB*	This study
EC2630	EC1250 $cysIp_{Bc}$-*lacZ*	This study
EC2631[a]	EC2630 *cysB*	This study
EC2635	EC1250 $ssuDp_{Bc}$-*lacZ*	This study
EC2636[a]	EC2635 Δ*cysB*::*kan*	This study
EC2637	EC1250 $cysD2p_{Bc}$-*lacZ*	This study
EC2638[a]	EC2637 *cysB*	This study
EC2655	EC1250 $ssuRp_{Bc}$-*lacZ*	This study
EC2656	EC2655 *cysB*	This study
EC2672[a]	EC2541 Δ*cysB*::*cam*	This study
EC2673[b]	EC2626 *cysE*	This study
EC2674[b]	EC2630 *cysE*	This study
EC2676[a]	EC2626 Δ*cysB*::*cam*	This study
EC2677[a]	EC2673 Δ*cysB*::*cam*	This study
EC2678[a]	EC2630 Δ*cysB*::*cam*	This study
EC2679[a]	EC2674 Δ*cysB*::*cam*	This study
SE40	EC1250 *ssuE-lacZ* Δ*ssuADCB*	58
SE45	SE40 Δ*cbl*::*kan*	58
SE47	SE40 *trpB*::Tn*10 cysB*	58
SE70	EC1250 *tauA-lacZ* Δ*tauBCD*	Laboratory collection
SP53	$lacI^q$ *rrnB* Δ*lacZ hsdR514* Δ*araBAD* Δ*rhaBAD* Δ*cysB*::*cam*	40
B. cenocepacia strains		
715j	CF isolate; prototroph	10, 15, 37
715j-*ssuR*::Tp	715j with *dfrB2* gene inserted in *ssuR*	This study
715j-*cysB*::Tp	715j with *dfrB2* gene inserted in *cysB*	This study
Plasmids		
p34E-Tp	Source of trimethoprim resistance cassette *dfrB2*; Tp^r	12
pACYC184	Medium-copy-number cloning vector; Cm^r Tc^r	9
pBBR1MCS	Broad-host-range cloning vector; Cm^r	29
pGEM T Easy	Vector for direct cloning of PCR fragments; Ap^r	Promega
pKAGd4	Broad-host-range *lacZ* transcription fusion vector; Ap^r Cm^r	2
pRS551	$lacZYA^+$; used for construction of chromosomal *lacZ* fusions in *E. coli*; Ap^r Kan^r	48
pSHAFT	Mobilizable *pir*-dependent suicide vector; Ap^r Cm^r	2
pTrc99A	Expression vector; *trc* promoter; pBR322 ori; Ap^r	Pharmacia
pMH176	cbl_{Ec} ORF under *trc* promoter control in pTrc99A	59
pMH199	$cysB_{Ec}$ ORF under *trc* promoter control in pTrc99A	34
pMH260[c]	$ssuR_{Bc}$ ORF under *trc* promoter control in pTrc99A; encodes SsuR with substitution F87S	This study
pMH262	$ssuR_{Bc}$ ORF (wt) under *trc* promoter control in pTrc99A	This study
pMH266	Derivative of pMH262 with *dfrB2* cassette inserted into BclI site internal to *ssuR*; Ap^r Tp^r	This study
pMH267	$ssuR_{Bc}$ ORF under *trc* promoter control cloned in pACYC184 EcoRV-BamHI sites; Cm^r	This study
pMH269	Derivative of pSHAFT containing $ssuR_{Bc}$::*dfrB2* inserted into NotI site; Ap^r Cm^r Tp^r	This study
pMH284	$cysB_{Bc}$ ORF (wt) under *trc* promoter control in pTrc99A	This study
pMH289	Derivative of pMH284 with *dfrB2* cassette inserted into NruI site internal to $cysB_{Bc}$; Ap^r Tp^r	This study
pMH291	Derivative of pSHAFT containing $cysB_{Bc}$::*dfrB2* inserted into NotI site; Ap^r Cm^r Tp^r	This study
pMH292	$sbpp_{Bc}$-*lacZ* fusion in pRS551; Ap^r Kan^r	This study
pMH293	$cysBp_{Bc}$-*lacZ* fusion in pRS551; Ap^r Kan^r	This study
pMH294	$cysIp_{Bc}$-*lacZ* fusion in pRS551; Ap^r Kan^r	This study
pMH295	$ssuRp_{Bc}$-*lacZ* fusion in pRS551; Ap^r Kan^r	This study
pMH297	$ssuDp_{Bc}$-*lacZ* fusion in pRS551; Ap^r Kan^r	This study
pMH299	$cysD2p_{Bc}$-*lacZ* fusion in pRS551; Ap^r Kan^r	This study
pMH609	$cysIp_{Bc}$-*lacZ* fusion in pKAGd4; Ap^r Cm^r	This study
pMH610	$sbpp_{Bc}$-*lacZ* fusion in pKAGd4; Ap^r Cm^r	This study
pMH611	$ssuDp_{Bc}$-*lacZ* fusion in pKAGd4; Ap^r Cm^r	This study
pMH612	$cysD2p_{Bc}$-*lacZ* in p KAGd4; Ap^r Cm^r	This study
pMH637	$ssuR_{Bc}$-$cysB_{Bc}$ (tandem) under *trc* promoter control in pTrc99A; Ap^r	This study
pMH648	$ssuRp_{Bc}$-*lacZ* fusion in pKAGd4; Ap^r Cm^r	This study
pMH651	$cysB_{Bc}$ amplified with primers CB2 and CB3 and cloned in pBBR1MCS BamHI site	This study
pMH653	$ssuR_{Bc}$ amplified with primers MX3 and MX12 and cloned in pBBR1MCS BamHI site	This study

[a] *cysB* (point mutation; CysB-null phenotype), Δ$cysB_{Ec}$::*kan*, or Δ$cysB_{Ec}$::*cam* allele was P1-transduced to relevant recipient strains from SE47, EC2549, or SP53, respectively.

[b] *cysE*-null mutation was P1-transduced to relevant recipient strains from EC1250 *cysE* Tn*10 zia*-207 (laboratory collection).

[c] $ssuR_{Bc}$ with asingle nucleotide change resulting in substitution F87S in the gene product, obtained fortuitously during PCR.

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence[a]	Region[b]
CB1	5′-GGTTAT*TC*ATGAACCTGCACC-3′	*cysB*
CB2	5′-CATAC*GGATCC*GTCAAAGCTC-3′	*cysB*
CB3	5′-TGTA*GGATCC*GTTCTGCAGA-3′	*cysB*
CB4	5′-GTAG*GGATCC*TTGGCGGC-3′	*cysB*
CB5	5′-TCGA*GGATCC*TCTGGTCG-3′	*cysI*
CB6	5′-GGCGATCGCGGCCGGCAG-3′	*cysB*
CD1	5′-CA*GAATTC*GGCCCAGGTCTG-3′	*cysD2*
CD2	5′-AGC*GGATCC*AGGTGGGTCAA-3′	*cysD2*
SD1	5′-G*GAATTC*GGATAGGTGCGC-3′	*ssuD*
SD2	5′-AA*GGATCC*AGAACACATTC-3′	*ssuD*
SB6	5′-GGTCAT*GGATCC*ACTGTATGG-3′	*sbp*
SB7	5′-AGTC*GGATCC*GCCTGGGC-3′	*sbp*
SR1	5′-TAC*TCATGA*ATTTTCAGC-3′	*ssuR*
SR2	5′-GCAC*GTCGAC*AACGGCTTCG-3′	*ssuR*
SR3	5′-CG*GGATCC*GGTCAAACGGCTTC-3′	*ssuR*
SR4	5′-GCCGTGCAC*CAGCTG*ATCG-3′	*ssuR*
SR5	5′-GTCG*TCGCGA*CGACGAGG-3′	*ssuR*
MX3	5′-CG*GGATCC*GGTCAAACGGCTTC-3′	*ssuR*
MX10	5′-CACA*GCGGCCGC*AGACCATGAATT TTCAGC-3′	pTrc99A
MX11	5′-CCTG*GCGGCCGC*CTCTAGAGGATC CGGTCAAA-3′	pTrc99A
MX12	5′-AGGC*GGATCC*CGACCGCGAC-3′	*ssuR*
MX13	5′-GTCA*GGATCC*TGTTCTGGCGC-5′	*ssuR*
TRCBam	5′-*GGGATCC*AGGAAACAGACCATG-3′	pTrc99A
cysBfor	5′-CGACAGCCTAAGAGGCAT-3′	*ssuR*
cysBrev	5′-GTGCTTCAGCCGTGATGG	*ssuR*
cysBfor2	5′-GCTGGATTGCTAATGACG-3′	*cysB*
cysBrev2	5′-ATGCGGGAATCTCCATCT-3′	*cysB*

[a] Nucleotides indicated in italics were changed to create restriction sites (underlined).

[b] Regions are indicated according to the current gene predictions in *B. cenocepacia* (Fig. 3).

B. cenocepacia strains harboring promoter-*lacZ* fusions, overnight cultures were washed twice in modified M9 medium containing glucose (0.5%) plus "18 amino acids" (10 μg/ml each amino acid, except cysteine and methionine), djenkolic acid, and chloramphenicol. Strains containing plasmids were grown with appropriate antibiotics: ampicillin (100 μg/ml), tetracycline (15 μg/ml), chloramphenicol (20 μg/ml for *E. coli*; 50 μg/ml for *B. cenocepacia*), kanamycin (25 μg/ml), or trimethoprim (50 μg/ml).

DNA manipulations and reagents. Standard procedures (3) were used for restriction enzyme digestions, ligation, 5′-end labeling of DNA fragments, and transformation of *E. coli.* Plasmid DNA was isolated by using a Plasmid Midi Kit (QIAGEN). Restriction endonucleases, DNA-modifying enzymes, T4 polynucleotide kinase, and T4 DNA ligase were obtained from MBI Fermentas or Invitrogen. *Taq* polymerase was from MBI Fermentas, and High Fidelity PCR Master from Roche. [γ-^{32}P]ATP used for 5′-end labeling was from Amersham Pharmacia Biotech, and all other chemicals (of the highest purity grade available) were from Sigma-Aldrich, Fluka, Promega, or Merck. Oligonucleotide synthesis and DNA sequencing (using the dideoxy chain termination method and an ABI Prism 3730 DNA sequencer [Applied Biosystems]) were performed at the Institute of Biochemistry and Biophysics, Warsaw, Poland.

Plasmid constructions. The plasmids used in this study are listed in Table 1. All the *B. cenocepacia* 715j sequences used for plasmid constructions were amplified by PCR using SacII-digested total genomic DNA as a template and appropriate oligonucleotide primers listed in Table 2. Routinely, two PCR amplifications of each sequence were performed, the obtained fragments were ligated with pGEM-T-easy vector, and independent isolates of each construct were sequenced to ensure that no undesired mutations had been introduced during PCR. Inserts were recovered from pGEM-T-easy derivatives by restriction enzyme digestions, and they were subsequently cloned into appropriate vectors. The sequences of *ssuR* and *cysB* (the ORF plus the upstream intergenic region) were amplified with primers MX3/MX12 or CB2/CB3, respectively, and inserted into the BamHI site of pBBR1MCS. For expression of *ssuR* and *cysB* in *E. coli*, the respective ORFs were amplified with primers SR1/SR3 or CB1/CB2, and the PCR products (containing flanking BspHI and BamHI sites) were inserted into the NcoI/BamHI sites of pTrc99A. In the resultant plasmids, pMH262 and pMH284, wild-type (wt) *ssuR* and *cysB* were placed under the control of the *trc* promoter and an appropriately positioned Shine-Dalgarno (SD) sequence of *E. coli*. Plasmid pMH637, simultaneously expressing *ssuR* and *cysB*, was obtained by PCR amplification of a fragment containing the *cysB* ORF and SD from a pMH284 template (with primers TRCBam and CB2), digestion of the obtained product with BamHI, and ligation with the BamHI-cleaved pMH262. To obtain derivatives with interruptions of *ssuR* and *cysB*, the trimethoprim resistance (Tp) cassette (~600-bp, containing the *dfrB2* gene) was excised from plasmid p34E-Tp with either BamHI or SmaI and inserted into the BclI site of pMH262 (internal to the *ssuR* ORF) or the NruI site of pMH284 (internal to the *cysB* ORF), respectively, to give plasmids pMH266 and pMH289. The *dfr*-interrupted genes were amplified by PCR with primers MX10 and MX11 (for *ssuR*) or primers CB1 and CB2 (for *cysB*), and the PCR products were ligated with pGEM-T-easy. The respective sequences were recovered as NotI fragments and subsequently cloned into the NotI site of the suicide plasmid pSHAFT, giving rise to pSHAFT-*ssuR*::Tp and pSHAFT-*cysB*::Tp. The resultant plasmids, pMH269 and pMH291, were selected from trimethoprim-resistant transformants of *E. coli* strain CC118(λ*pir*), and they were exploited further to obtain the respective gene knockouts in the genome of *B. cenocepacia* 715j by an allele replacement procedure (see below).

Plasmids carrying promoter-reporter (*lacZ*) fusions were constructed with the aid of *E. coli* plasmid pRS551 and with pKAGd4, which replicates in both *E. coli* and *B. cenocepacia*. The putative regulatory regions of selected *B. cenocepacia* genes were chosen as sequences encompassing small portions of downstream open reading frames (including the ATG start codon) and extending upstream at least 200 bp (see Fig. 4B). These sequences were amplified from 715j genomic DNA by PCR as follows: *sbpp* fragment with primers SB6 and SB7, the intergenic fragment between *cysB* and *cysI* with primers CB4 and CB5, the *ssuDp* fragment with primers SD1 and SD2, the *cysD2p* fragment with primers CD1 and CD2, and the *ssuRp* fragment with primers MX12 and MX13. Each of the fragments obtained was sequenced, cleaved with the corresponding restriction enzymes, and inserted into the BamHI or EcoRI/BamHI sites of pRS551, in front of the promoterless *lacZ* gene; the intergenic fragment between *cysB* and *cysI* was inserted in both orientations. The pRS551 derivatives, carrying transcriptional fusions *sbpp-lacZ* (pMH292), *cysIp-lacZ* (pMH293), *cysBp-lacZ* (pMH294), *ssuDp-lacZ* (pMH297), *cysD2p-lacZ* (pMH299), and *ssuRp-lacZ* (pMH295), were subsequently exploited to transfer the respective fusions into the chromosome of *E. coli.* In parallel, each of the promoter fragments described above was inserted into pKAGd4 MCS upstream of the *lacZ* gene. The pKAGd4 derivatives carrying the analogous fusions *sbpp-lacZ* (pMH610), *cysIp-lacZ* (pMH609), *ssuDp-lacZ* (pMH611), *cysD2p-lacZ* (pMH612), and *ssuRp-lacZ* (pMH648) were used as multicopy promoter-reporter systems in *B. cenocepacia*.

Construction of single-copy *B. cenocepacia* promoter-*lacZ* fusions in *E. coli*. The transcriptional fusions of *B. cenocepacia* promoter regions with *lacZ*, encoded by derivatives of pRS551, were transferred to the chromosome of *E. coli* strain EC1250 (Δ*lac recA*$^+$) by the method of Simons et al. (48) utilizing the transducing phage λRS45. The transductants, containing single-copy chromosomal fusions, were selected on LB agar containing kanamycin and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), streak purified, screened for loss of ampicillin resistance, PCR verified, and then used for β-galactosidase assays.

β-Galactosidase assays. β-Galactosidase activities were assayed by the method of Miller (38), with ONPG (*o*-nitrophenyl-β-galactopyranoside) as a substrate, in cells taken from mid-log-phase cultures of either *E. coli* or *B. cenocepacia.* Some assays with *E. coli* were performed with 4-methylumbelliferyl-β-D-galactopyranoside as a substrate, and methylumbelliferon released from the substrate was assayed fluorometrically as previously described (55). Routinely, triplicate cultures were grown for each assay, and the assays were repeated at least twice.

Construction of *B. cenocepacia ssuR* and *cysB* mutants by allelic replacement. Plasmids pMH269 (pSHAFT-*ssuR*::Tp) and pMH291 (pSHAFT-*cysB*::Tp) were mobilized from *E. coli* S17-1(λpir) into *B. cenocepacia* 715j as previously described (11, 18), and recombinants were selected on M9-glucose agar containing Casamino Acids (0.5%), cysteine (40 μg/ml), trimethoprim (50 μg/ml), and kanamycin (50 μg/ml). Candidate double-crossover recombinants, in which vector sequences and the wild-type copy of *ssuR* or *cysB* were lost, were identified by virtue of their sensitivity to chloramphenicol (50 μg/ml). The presence of the desired genomic insertion mutation in the candidate null mutants was confirmed by performing PCRs on boiled lysates using primers cysBfor and cysBrev (for candidate *ssuR*::Tp knockouts) and cysBfor2 and cysBrev2 (for candidate *cysB*::Tp knockouts).

Protein preparations. A procedure described earlier for partial purification of the *E. coli* CysB and Cbl regulators (34, 58) was followed to obtain preparations of *B. cenocepacia* SsuR and CysB. Briefly, *E. coli* strain EC2672 (Δ*cysB* Δ*cbl*) was transformed with pMH262 or pMH284 (expressing the *ssuR* and *cysB* genes from the IPTG [isopropyl-thiogalactopyranoside]-inducible *trc* promoter), the transformed cells were grown in LB-ampicillin to early exponential phase (A_{600} =

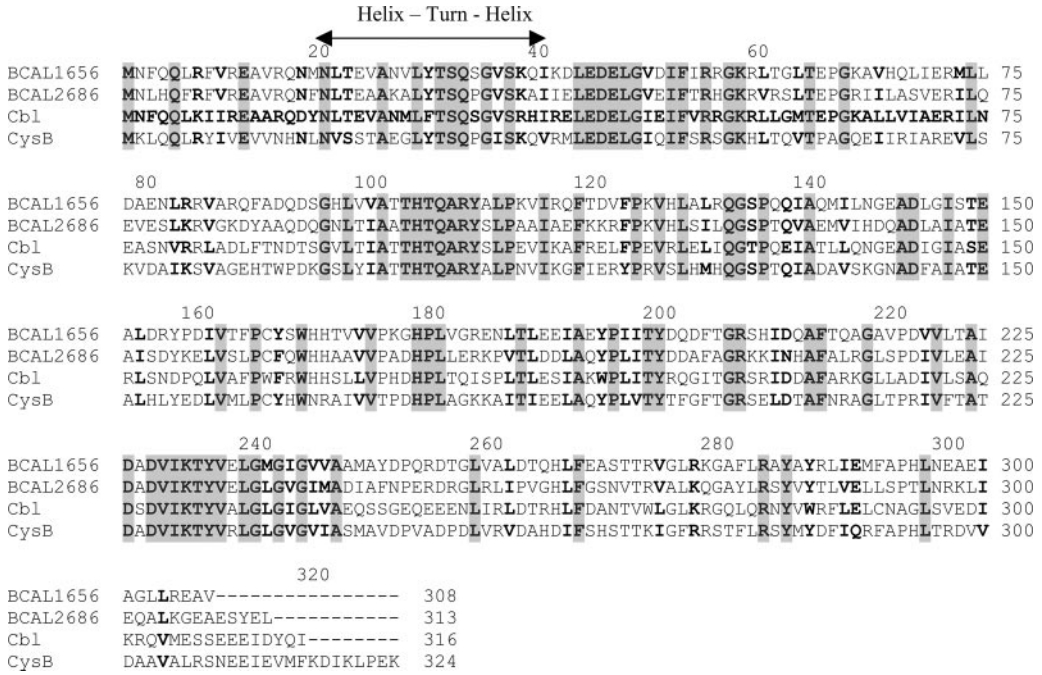


FIG. 2. Amino acid sequence alignment of the *E. coli* CysB and Cbl proteins and deduced products of *B. cenocepacia* BCAL1656 and BCAL2686, designated *ssuR* and *cysB*, respectively. Identical amino acid residues at the same relative position in all four proteins are shaded, and similar residues are shown in boldface. The helix-turn-helix motif typical of LysR family members (45) is shown by arrows.

0.15), IPTG was added to a final concentration of 0.1 mM, and growth was continued for a further 2 to 3 h. Cells were collected by centrifugation, resuspended in buffer A (50 mM Tris-Cl, pH 7.5, 1 mM Na_2EDTA, 1 mM phenylmethylsulfonyl fluoride), and disrupted by sonication. The cellular debris was removed by centrifugation, and clear extracts were fractionated by ammonium sulfate precipitation. The protein fractions precipitated with 229 mg/ml of ammonium sulfate were collected, suspended in buffer A at a total protein concentration of 1 to 2 mg/ml, and stored in aliquots at −70°C. The amounts of both *B. cenocepacia* proteins in the preparations obtained were estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie blue staining to be not less than 50% of the total protein. Protein extracts prepared in an analogous way from the same host cells but transformed with an empty vector (pTrc99A) served as a control in DNA-binding studies utilizing partially purified *B. cenocepacia* proteins.

DNA-binding assays. The abilities of *B. cenocepacia* proteins to bind DNA at putative target promoter regions were tested by the electrophoretic mobility shift assay (EMSA) (16). DNA fragments containing promoter regions of interest were the same as those exploited to construct the respective promoter-reporter fusions. PCR-amplified promoter fragments were labeled at the 5′ ends with [γ-^{32}P]ATP and polynucleotide kinase. Reaction mixtures (20 μl) contained approximately 10 ng of labeled DNA fragment and 2 μg of sonicated calf thymus DNA per ml (as a nonspecific competitor) in a buffer consisting of 40 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 100 mM KCl, 1 mM dithiothreitol, and 100 μg bovine serum albumin per ml. The protein preparations were added at various final concentrations (typically 1 to 20 μg/ml), and the reaction mixtures were incubated at 37°C for 5 min. Some samples also contained 10 mM *O*-acetyl-L-serine (OAS), which was tested as a potential cofactor of the *B. cenocepacia* regulatory proteins. After incubation, the reaction mixtures were separated in a 5% acrylamide-bisacrylamide (82:1) nondenaturing gel in 0.05 M Tris-borate-EDTA buffer (pH 8.3) for 1.5 h at 10 V/cm. Radiolabeled bands were visualized by autoradiography.

Sequence analysis. For phylogenetic analysis, the amino acid sequences of ORFs similar to CysB and Cbl were retrieved from the GenBank Sequence Database. The sequences were aligned with the Clustal W program (http://www.ebi.ac.uk/clustalw/index.html) (53) using the default parameters. The alignments were edited with the BioEdit software package (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). A phylogenetic tree was constructed by the neighbor-joining and minimal evolution methods implemented in MEGA 3.1 (http://www.megasoftware.net) (31), using distance matrix calculations for all pairs from the sequence alignments and the *p* distance and Poisson correction substitution models. The bootstrap values for confidence limits of branch points were estimated from 10,000 replicates.

RESULTS

Two genes in chromosome 1 of the *B. cenocepacia* genome encode proteins similar to the *E. coli* CysB and Cbl regulators. Examination of the genomic sequence of *B. cenocepacia* J2315 (http://www.sanger.ac.uk/Projects/B_cenocepacia/) (not formally annotated) revealed that chromosome 1 contains two open reading frames, BCAL1656 and BCAL2686, whose deduced products share homology with both *E. coli* CysB and Cbl transcriptional regulators. It has been previously reported that one of "*cysB*/*cbl*-like" sequences in *B. cenocepacia* strain 715j is adjacent to the locus encoding a putative sulfate transporter (homologs of *E. coli* Sbp, CysT, CysW, and CysA), and it was tentatively designated "*cysB*" (15). The second "*cysB*-like" gene, BCAL2686, present in the J2315 genome, is oriented divergently to a cluster of genes encoding putative enzymes of sulfate activation and reduction (homologs of *E. coli* CysI, CysH, CysD, CysN, and CysG, as inferred from TBLASTN analysis) (Fig. 1) (see Discussion). After inspection of available genomic sequences of other members of the genus *Burkholderia*, *B. pseudomallei* and *B. mallei*, we noticed the presence of potential orthologs of BCAL1656 and BCAL2686 in genomic contexts analogous to those present in *B. cenocepacia* J2315.

Cloning of BCAL1656 and BCAL2686 from *B. cenocepacia* and complementation studies in *E. coli*. The two "*cysB*-like" ORFs of *B. cenocepacia* were amplified from genomic DNA of strain 715j and sequenced, which revealed 100% conservation of deduced products between strains 715j and J2315. As can be seen in Fig. 2,

the mutual similarity of these products is higher than that of *E. coli* CysB and Cbl, and their functional analogy to *E. coli* regulators cannot be simply deduced. The BCAL1656 and BCAL2686 sequences were cloned in the vector pTrc99A under the control of the *trc* promoter and an *E. coli* Shine-Dalgarno sequence to give plasmids pMH262 and pMH284, respectively. Both gene products could be effectively overproduced from these plasmids in *E. coli* strain DH5α, as well as in a Δ*cysB* Δ*cbl* derivative of strain MC4100 (EC2672). The latter strain was exploited to obtain cellular extracts enriched with each of the *B. cenocepacia* proteins, which were subsequently used in DNA-binding experiments.

To investigate whether any of these proteins replace the functions of the *E. coli* CysB and Cbl regulators in vivo, we tested the abilities of plasmids pMH262 and pMH284 to complement the growth of *E. coli cysB* and *cbl* mutants in the presence of selected sulfur sources. The *E. coli cysB*-null mutant requires cysteine for growth and is unable to utilize sulfate as a sulfur source (due to a lack of expression of nearly all the *cys* genes involved in the sulfate assimilatory pathway); this mutant is also unable to utilize taurine and aliphatic sulfonates, since sulfite released from these substrates must be further metabolized by the *cys* pathway, which is dependent on genes controlled by CysB (Fig. 1). In contrast, the *E. coli cbl*-null mutant grows well on sulfate but does not utilize taurine and aliphatic sulfonates, since expression from the *tau* and *ssu* promoters is strictly dependent on Cbl. Since expression of *E. coli cbl* itself is dependent on CysB (22), the *cysB*-null mutant is practically devoid of both CysB and Cbl functions. We found that neither of the *B. cenocepacia* genes could complement the cysteine deficiency of the *E. coli cysB*-null mutant. In contrast, utilization of ethanesulfonate (but not taurine) by the *E. coli cbl*-null strain SE45 was supported in the presence of pMH262 containing BCAL1656. To examine whether the partial cross-activity of the BCAL1656 product in *E. coli* correlates specifically with expression of the *ssuEADCB* operon, we assayed the β-galactosidase levels in strains with chromosomal *ssuE-lacZ* and *tauA-lacZ* fusions in the presence of plasmid pMH262. The results (Table 3) confirmed that the BCAL1656 product is able to activate expression from the *E. coli ssuE* promoter (although to a lesser extent than the cognate Cbl regulator), but it is inactive in upregulation of the *E. coli tauA* promoter (consistent with the inability of strain Δ*cbl*/pMH262 to utilize taurine as a sole sulfur source).

The above-mentioned results provided a first indication that the BCAL1656 product may be a functional analogue of the *E. coli* Cbl regulator rather than the CysB regulator. Since the designation "*cbl*" in *Burkholderia* species has already been exploited for genes involved in the formation of cell surface structures termed "cable pili" (43), we propose to designate BCAL1656 "*ssuR*." For BCAL2686, we propose the designation "*cysB*" on the basis of further characterization of its function in *B. cenocepacia* (see below). These designations are used throughout the following text and figures.

Construction and phenotypes of *cysB* and *ssuR* mutants of *B. cenocepacia*. The *B. cenocepacia* mutants devoid of CysB or SsuR function were constructed by an allele replacement technique using plasmids carrying corresponding ORFs interrupted with a Tp cassette. The growth of both mutant strains 715j-*ssuR*::Tp and 715j-*cysB*::Tp on various sulfur sources was compared with that of the parental strain 715j. As shown in Fig. 3, the *cysB* mutant was not able to utilize any of the tested potential substrates (sulfate, sulfite, and ethanesulfonate) for cysteine biosynthesis, and hence, its phenotype is analogous to that of an *E. coli cysB* mutant. In contrast, the *ssuR* mutant was able grow well with sulfate but was unable to utilize ethanesulfonate as a sulfur source, a hallmark phenotype of an *E. coli cbl* mutant. The growth of the *cysB* mutant on sulfate and ethanesulfonate was restored to the wild-type level in the presence of plasmid pMH651 carrying wt *B. cenocepacia cysB* ($cysB_{Bc}$), and growth of the *ssuR* mutant on ethanesulfonate was restored by plasmid pMH653, carrying wt $ssuR_{Bc}$ (not shown). It has also been noted that L-cysteine as a sole sulfur source does not support full growth of strain 715j even over a 24-h period. As shown in Fig. 3, supplementation with 18 amino acids significantly increased growth with L-cysteine. This effect is consistent with earlier observations in *E. coli* of transient amino acid starvation (especially for threonine and branched-chain amino acids) caused by cysteine excess (17, 50, 51).

TABLE 3. Effects of BCAL1656 product on the activities of the *E. coli ssu* and *tau* promoters

Strain[a]	Plasmid[b]	β-Galactosidase activity (Miller units)[c]
SE40 (*ssuE-lacZ*)	None	888 ± 84
SE45 (*ssuE-lacZ* Δ*cbl*)	None	28 ± 2
SE45	pMH176 (cbl_{Ec})	2,676 ± 252
SE45	pMH260 (BCAL1656*)	139 ± 13
SE45	pMH262 (BCAL1656)	1,139 ± 18
SE70 (*tauA-lacZ*)	None	1,037 ± 108
EC2625 (*tauA-lacZ* Δ*cbl*)	None	34 ± 4
EC2625	pMH176	958 ± 78
EC2625	pMH260	70 ± 5
EC2625	pMH262	55 ± 7

[a] *E.* coli strains harboring single-copy chromosomal *ssuE-lacZ* or *tauA-lacZ* fusions.

[b] Plasmids were derivatives of pTrc99A; BCAL1656* contained on pMH260 encodes a mutant protein with a single amino acid substitution, F87S.

[c] β-Galactosidase was assayed in cells from mid-log-phase cultures grown in minimal medium with L-djenkolic acid (1 mM) as a sulfur source. Triplicate assays were performed for each culture of at least three independent transformants.

Identification of *B. cenocepacia* genes controlled by SsuR and CysB. Following the TBLASTN analysis of the genomic sequence of *B. cenocepacia* J2315 for the presence and organization of ORFs encoding putative enzymes of the sulfate and sulfonate assimilatory pathways (Fig. 1) (see Discussion), we chose four regions, shown in Fig. 4, for further analysis. We noted that the organizations of these regions are similar (but not strictly identical) to that found in genomic sequences of other *Burkholderia* spp. published to date. In all of these genomic sequences, ORFs encoding the same pair of potential regulators, here referred to as *ssuR* and *cysB*, are clustered with the "*cysA* locus" and the "*cysI* locus," respectively.

To investigate whether the intergenic regions shown in Fig. 4A contain target sequences for CysB or/and SsuR, appropriate "promoter fragments" (the sequences are shown in Fig. 4B) were amplified from genomic DNA of *B. cenocepacia* 715j and used to construct transcriptional fusions with the promoterless *lacZ* gene contained on plasmid pKAGd4. Plasmids carrying promoter-reporter fusions were introduced into the *B.*

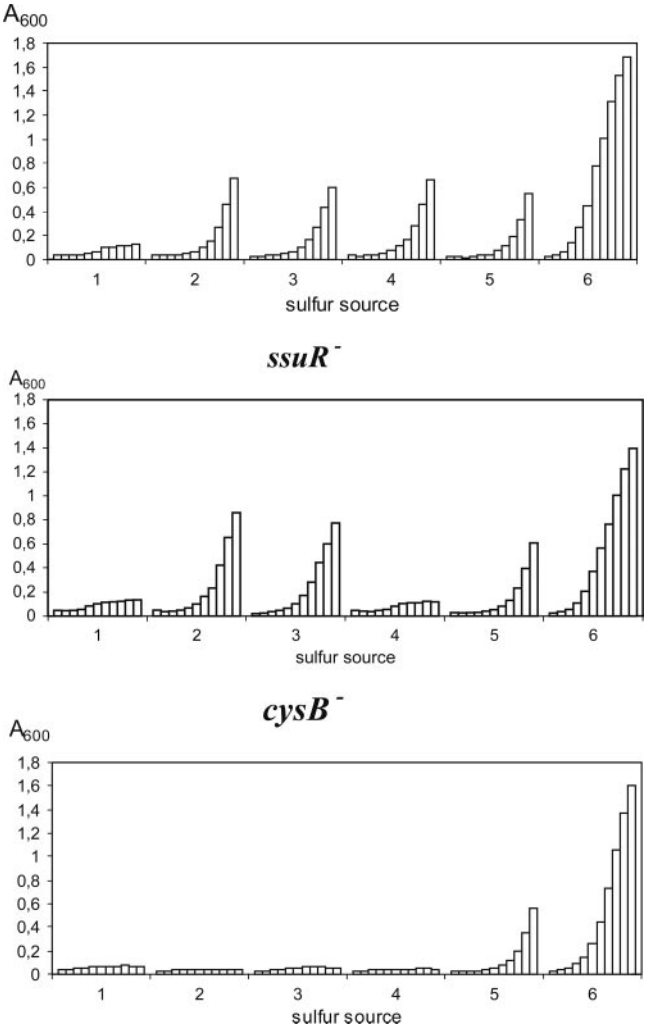
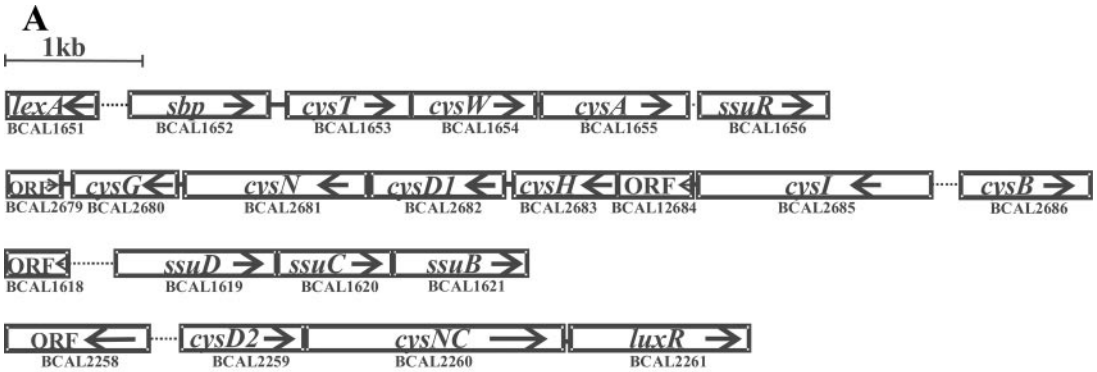


FIG. 3. Growth of *B. cenocepacia* 715j and isogenic *ssuR* and *cysB* mutants in the presence of various sulfur sources. Growth was monitored by A_{600} measurements over 11 h after inoculation of cells taken from LB cultures and washed in modified (i.e., sulfate-free) M9 minimal medium supplemented with (1) no sulfur source, (2) sulfate (1 mM), (3) sulfite (1 mM), (4) ethanesulfonate (0.5 mM), (5) L-cysteine (0.5 mM), or (vi) L-cysteine (0.5 mM) plus 18 amino acids.

cenocepacia wt strain 715j and its derivative *cysB*-null or *ssuR*-null. Table 4 shows the results of β-galactosidase assays, reflecting transcription initiating from within the five intergenic regions (cloned in pKAGd4) in the three *B. cenocepacia* genetic backgrounds. High promoter activity above the background level (afforded by pKAGd4) was detected in *sbpp*-, *cysIp*-, and *ssuDp-lacZ* fusions in wt 715j. In the absence of functional *cysB*, expression from *cysIp* was moderately decreased (2.3-fold), while the absence of functional *ssuR* strongly affected expression from *ssuDp* (170-fold) and also that from *sbpp*, albeit less severely (2.4-fold). It was noted that β-galactosidase activity measured with a plasmid containing *ssuDp-lacZ* in an *ssuR*-null strain was lower than background levels afforded by pKAGd4, which might suggest the presence of a strong transcription termination signal residing in a cloned *ssuDp* fragment upstream of the SsuR-dependent promoter. The expression levels of *lacZ* from *cysD2p* and *ssuRp* were lower by an order of magnitude than those of other fusions, indicating lower promoter activities, but in both cases, some negative effect of either a *cysB* or *ssuR* knockout was detectable.

Suspecting that the roles of *B. cenocepacia* CysB and SsuR at particular targets may partially overlap, we decided to test the individual contribution of each regulator to expression from promoter regions of interest in a heterologous system, using *E. coli* as a host. Transcriptional fusions of the respective *B. cenocepacia* promoter regions (the same as those present in the pKAGd4 derivatives) with promoterless *lacZ* were constructed in the vector pRS551, and they were subsequently recombined into the *E. coli* chromosome. To avoid possible cross talk between *B. cenocepacia* promoters and resident *E. coli* regulators ($CysB_{Ec}$ and Cbl_{Ec}), all of the strains obtained with chromosomal *B. cenocepacia* promoter-*lacZ* fusions were made $cysB_{Ec}$ null. As the expression of cbl_{Ec} is under the control of $CysB_{Ec}$, this background indicated the absence of both CysB and Cbl activities. β-Galactosidase activity was assayed in the resultant strains in the absence and in the presence of *B. cenocepacia* CysB and SsuR expressed from plasmids (Table 5). Consistent with the results obtained with the *B. cenocepacia* host strains (compare Table 4), the *ssuDp-lacZ* fusion was highly and specifically upregulated by SsuR in *E. coli*. Expression of two other fusions, *sbpp-lacZ* and *cysIp-lacZ*, was upregulated by SsuR and CysB; however, *cysIp* was much more sensitive to the stimulatory activity of CysB, whereas *sbpp* appeared more sensitive to that of SsuR. The activity of β-galactosidase in *E. coli* harboring the *cysD2p-lacZ* fusion was barely detectable in the presence of SsuR or CysB in a standard Miller assay, but a more sensitive method revealed elevation of *cysD2p* activity when *B. cenocepacia* SsuR and CysB were delivered jointly (expressed from two plasmids present in the same cell). Using the same system, we observed that expression of *lacZ* from $cysBp_{Bc}$ was negatively affected by the presence of either SsuR (3.8-fold) or $CysB_{Bc}$ (2.8-fold), while expression of *lacZ* from "*ssuRp*" (taken as a *cysA-ssuR* intergenic region) was barely detectable.

Binding of SsuR and CysB proteins to DNA at target promoter regions. The promoter regions of *B. cenocepacia* identified as targets for CysB- and SsuR-mediated regulation in vivo were subsequently tested by EMSA for the ability to bind each regulator in vitro. The DNA fragments used as promoter probes (*ssuDp*, *cysIp*/*cysBp*, *sbpp*, and *cysD2p*) (Fig. 3B) encompassed the same DNA regions as those used to construct the respective promoter-*lacZ* fusions. As a source of *B. cenocepacia* regulators, we used protein extracts of a Δ*cysB* Δ*cbl E. coli* strain enriched with either $CysB_{Bc}$ or $SsuR_{Bc}$ (following their overproduction from the corresponding plasmids, pMH262 and pMH284). As shown in Fig. 5A, the *ssuDp* promoter fragment was able to bind SsuR only, giving a single shifted band. Further increase of the SsuR concentration (data not shown) did not result in the appearance of additional bands, suggesting that a single binding site for this regulator may exist in the *ssuDp* region. The intergenic region separating *cysI* and *cysB* (*cysIp*/*cysBp*) (Fig. 5B) gave rise to shifted bands with both SsuR and CysB. It is also evident that CysB was able to produce more than one distinct complex with the *cysIp*/*cysBp* probe, suggesting the presence of at least two target sequences



B

sbpp

*GGATCC*ACTGTATGGATAGACAGTGAGCTGTATTTTTATACAGTACTTGGTGAATTTCAAGTGTTACT
TGAGGTTCGACGCGATCGCGCCGGTTCCGGCGCGATTTCGCGACGCCCGGACGCCGGTTTATCCGCCG
CAACCGTCCATCCCGTCTGCGTTAGCACTTTTGAGTATTAAAAAATGAGGAACGATTATTTTTAATCA
TGTAACCCGTTCGATAGACTGGCCCGACATCGCAATACCGACAACACTGGAGAACCAAGG**ATG**GCGAA
GCGCAACACGGGGCTGGTGGGCGGAGTGGGCCGCCTGATCGCAACACTCGCGCTGGGCGCGGCGGCGG
CGCTGGGCGTCGTGACGCATGCCCAGGC*GGATCC*

cysBp/cysIp

*GGATCC*TTGGCGGCCTCGGTGAGGTTGAAATTCTGCCGGACGGCCTCGCGCACGAAGCGAAATTGGTG
CAGGTT**CAT**TTATAACCCTTCCGCATATCAACAGAATTTTTTAGTCGTTTGAAATATAAGGCGAGTTT
ATTACGATTCACCGGAGTTTTTCAAATATGGATATCTGTTTTCGTCATTAGCAATCCAGCCGGCAGCG
GATGCGGCGGTGCGGCGGAACGGAGATTCGGGCGCGACGTGGCTAGGACGTCGCGCGGTCACCACGAA
AACCCTGGGGGTCCCCGA**ATG**TATCAGTACGACCAATACGACCAGA*GGATCC*

ssuDp

*GAATTC*GGATAGGTGCGCCATTATAGGGCCCGGCCGCGGCGATATTCGCTCCAGCGTCGTGCTAATCA
CGAAAGCGGAAAACCTAAGCTCAAAAAAATCGTTCCAAAGCCCTGTACCGATCCCTAGACTGATTCCT
CAGAAACAGCCGTGCGATCGCGTCCTCGCCCGCTGCACGGCCTGACCGGCGAGCCATCCCGATTCGAA
TGCACACCATGCATGCGTCGCTCCTCCGCGACGCGAGCAGGGCGACGTTTTTTTCAAATTTCGGCAGT
GACAGTAAAGCAGGAGCAGCAG**ATG**AATGTGTTCT*GGATCC*

cysD2p

*GAATTC*GGCCCAGGTCTGGCCCGAAAGCAATTGCGTGGCGCGTGTGTCATCCGTCATGTGTCGTCTCC
GCCGATGGTCGAGCGCTCACCTTAAGCGGCGCCGCCGCGCGCCACTTCGTCCGGGTGGACTAAGGAAA
GATCCGCTCCACGGCCGCGTGCCGGTTTAGTCCACCCGGACGAAGAGCGGCATGCCGCCCGCTGGATA
CCGTTGGCGCCATGCCCGCAACGCCGCACATGTCGCGCGGCGCCGGCATGAAACAAGGAGACGAACG**A**
TGTTGACCCACCT*GGATCC*

ssuRp

*GGATCC*CGACCGCGACGCATGGCGGGCGCTGTCGCTGCAGGTCGGCGACGGTGCGACGGCCGTGCCGC
GCGCCGTGCGGGTATTTCCCGCGCGT**TGA**CGAGACAATGCAGGGCAGAGGGCGGGCATCGCTCCGATT
CGACAATCAAACAACGACAGCCTAAGAGGCATACCC**ATG**AATTTTCAGCAATTGCGGTTCGTGCGCGA
AGCGGTGCGCCAGAACA*GGATCC*

FIG. 4. Organization of chromosomal regions encoding putative enzymes of sulfate and sulfonate assimilatory pathways in *B. cenocepacia* J2315. (A) The ORF numbers are shown according to current gene predictions in *B. cenocepacia* J2315 (http://www.sanger.ac.uk./projects/B_cenocepacia); the gene designations are proposed on the basis of homology of the predicted products to their counterparts in *E. coli* (or other bacteria) (see Discussion); "ORF" denotes a gene of unknown function; transcription directions are indicated by arrows. Intergenic regions indicated by dots were analyzed in this study. (B) Sequences of promoter fragments (isolated from the strain 715j) with indicated ATG start codons (boldface) and predicted SD elements (underlined); the restriction sites (introduced by primers) used for cloning in *lacZ* vectors are shown in italics.

(binding sites) for CysB in this region. SsuR produced only one shifted band with *cysIp*/*cysBp*, but the electrophoretic mobility of the DNA-SsuR complex decreased with the protein concentration; this effect might also suggest some changes in the DNA-binding stoichiometry. The *sbpp* probe was up-shifted by both SsuR and CysB (Fig. 5C); however, apparently weaker and diffuse shifted bands observed with CysB might reflect lower affinity of CysB for the target site(s) in the *sbpp r*egion than that of SsuR.

Using the *cysD2* promoter region as a probe, a shifted band

TABLE 4. Effects of mutant *cysB* and *ssuR* alleles on the activities of promoters of sulfur metabolism genes in *B. cenocepacia*

Plasmid (fusion)	β-Galactosidase activity (Miller units)[a] in *B. cenocepacia* 715j strain:		
	Wild type	*cysB*-null	*ssuR*-null
pKAGd4 (none)	136 ± 5	157 ± 16	126 ± 4
pMH610 (*sbpp-lacZ*)	13,462 ± 1,159	15,906 ± 450	5,731 ± 423
pMH609 (*cysIp-lacZ*)	4,509 ± 351	2,003 ± 198	4,451 ± 196
pMH611 (*ssuDp-lacZ*)	5,726 ± 530	5,390 ± 1,056	31 ± 1
pMH612 (*cysD2p-lacZ*)	568 ± 25	433 ± 18	258 ± 10
pMH648 (*ssuRp-lacZ*)	456 ± 12	336 ± 13	292 ± 11

[a] β-Galactosidase was assayed in cells from mid-log-phase cultures grown M9 with 18 amino acids (minus cysteine and methionine) and L-djenkolic acid (1 mM) as a sulfur source. Triplicate cultures were grown for each transformant, and each culture was assayed in duplicate for enzyme activity.

was detected after incubation with SsuR, but not with a CysB preparation (Fig. 5D). The same result (a single shifted band corresponding to the presumed SsuR-DNA complex) was obtained when both SsuR and CysB (individually overproduced from plasmids pMH262 and pMH284, respectively) were added together to the reaction mixture (data not shown). However, a distinct retarded band (a very slowly migrating complex) was clearly seen after incubation of the *cysD2p* probe with protein extract containing SsuR and CysB overproduced simultaneously in the same cell (from plasmid pMH637; *trcp-ssuR-cysB*). This suggests that *cysD2p* may be regulated by some cooperation of SsuR-CysB. No complex of either CysB or SsuR was detectable by EMSA using the intergenic region *cysA-ssuR* (*ssuRp*) as a probe (not shown).

A hallmark feature of the CysB proteins from *E. coli* and *S. enterica* serovar Typhimurium ($CysB_{St}$) is either qualitative or quantitative modulation of their DNA-binding mode in the presence of a cognate inducer, acetylserine (30, 34). Using the *B. cenocepacia* promoter probes *sbpp* and *cysD2p* (data not shown) and *cysIp*/*cysBp* (Fig. 5E), we found no effect of *O*-acetylserine on either the abundance or the relative mobilities of complexes formed by SsuR and/or $CysB_{Bc}$. The possibility that $CysB_{Bc}$ may function without a coinducer (in contrast to $CysB_{Ec}$ and $CysB_{St}$) was further tested in vivo (see below).

The function of $CysB_{Bc}$ in vivo is independent of acetylserine. In *S. enterica* serovar Typhimurium and *E. coli*, OAS is synthesized in the reaction catalyzed by serine acetyltransferase (the product of the *cysE* gene) and serves as an acceptor of the sulfide moiety in the final reaction of cysteine biosynthesis. In addition, OAS, and also the product of its nonenzymatic conversion, *N*-acetyl-L-serine, serve as coinducers of CysB-activated transcription in both these species (30, 34). Thus, the phenotypes of *cysB* and *cysE* null mutants are very similar in terms of the lack of expression from CysB-dependent promoters. In order to test if $CysB_{Bc}$-mediated activation of *B. cenocepacia* promoters requires OAS as an inducer, expression of chromosomal $sbpp_{Bc}$-*lacZ* and $cysIp_{Bc}$-*lacZ* fusions was measured in *E. coli* strains $cysB_{Ec}$-null and $cysE_{Ec}$-null in the presence of $CysB_{Bc}$ or $CysB_{Ec}$ expressed from corresponding plasmids (Table 6). The $CysB_{Bc}$-activated expression from $sbpp_{Bc}$ and $cysIp_{Bc}$ was maintained at the same level, irrespective of the presence of CysE (an enzyme producing OAS). In addition, expression of the $sbpp_{Bc}$-*lacZ* and $cysIp_{Bc}$-*lacZ* fusions was unchanged by growth of the corresponding strains (EC2676/pMH284 and EC2678/pMH284) with cysteine, which acts as an inhibitor of serine acetyltransferase (CysE) activity (data not shown). It can be noted that a low level of expression from $sbpp_{Bc}$ was observed in the presence of $CysB_{Ec}$, which

TABLE 5. Effect of SsuR and CysB on the activity of *B. cenocepacia* promoters of sulfur metabolism genes measured in a heterologous system (*E. coli*)

E. coli strain	Chromosomal fusion (*B. cenocepacia* promoter-*lacZ*)	Plasmid[b] (relevant *B. cenocepacia* gene)	β-Galactosidase activity[a]	
			Miller units[c]	MUF units[d]
EC2627	*sbpp-lacZ*	pTrc99A	12 ± 2	ND
EC2627	*sbpp-lacZ*	pMH262 (*ssuR*)	7,600 ± 491	ND
EC2627	*sbpp-lacZ*	pMH284 (*cysB*)	4,560 ± 366	ND
EC2631	*cysIp-lacZ*	pTrc99A	42 ± 2	ND
EC2631	*cysIp-lacZ*	pMH262 (*ssuR*)	950 ± 99	ND
EC2631	*cysIp-lacZ*	pMH284 (*cysB*)	4,370 ± 260	ND
EC2638	*cysD2p-lacZ*	pTrc99A	ND	33 ± 5
EC2638	*cysD2p-lacZ*	pMH262 (*ssuR*)	ND	61 ± 7
EC2638	*cysD2p-lacZ*	pMH284 (*cysB*)	ND	68 ± 6
EC2638	*cysD2p-lacZ*	pMH267 (*ssuR*)	ND	61 ± 7
EC2638	*cysD2p-lacZ*	pMH284 + pMH267	ND	257 ± 20
EC2636	*ssuDp-lacZ*	pTrc99A	10 ± 1	ND
EC2636	*ssuDp-lacZ*	pMH262 (*ssuR*)	3,480 ± 360	ND
EC2636	*ssuDp-lacZ*	pMH284 (*cysB*)	40 ± 7	ND
EC2629	*cysBp-lacZ*	pTrc99A	116 ± 7	ND
EC2629	*cysBp-lacZ*	pMH262 (*ssuR*)	31 ± 3	ND
EC2629	*cysBp-lacZ*	pMH284 (*cysB*)	42 ± 2	ND
EC2656	*ssuRp-lacZ*	pTrc99A	ND	37 ± 3
EC2656	*ssuRp-lacZ*	pMH262 (*ssuR*)	ND	65 ± 2
EC2656	*ssuRp-lacZ*	pMH284 (*cysB*)	ND	60 ± 2

[a] In all EC strains, the wild-type $cysB_{Ec}$ was replaced with a *cysB*-null allele from SE47. ND, not determined.
[b] Plasmids were derivatives of pTrc99A (Ap^r), with the exception of pMH267(Cm^r), which contains *ssuR* under the *trc* promoter in pACYC184.
[c] Assays were performed under the same conditions as indicated in Table 4.
[d] MUF (4-methylumbelliferone) units are shown as pmol MUF/ml/min/A_{600} released from 4-methylumbelliferyl-β-D-galactopyranoside. In our hands, 100 MUF units correspond approximately to 1 Miller unit of β-galactosidase activity.

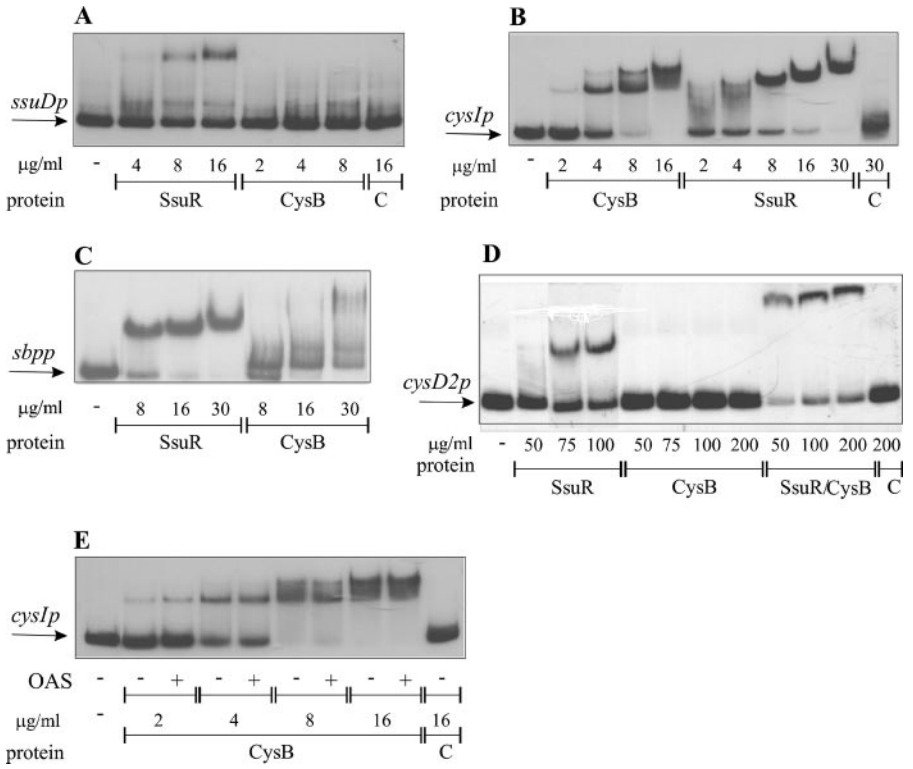


FIG. 5. Binding of SsuR and CysB to DNA at target promoter regions. EMSA was performed with radiolabeled *B. cenocepacia* promoter probes (PCR-amplified fragments of 715j DNA; sequences are shown in Fig. 4B). (A) *ssuDp.* (B and E) *cysBp*/*cysIp.* (C) *sbpp.* (D) *cysD2p*. Protein extracts enriched with SsuR, CysB, or control extract (in panel C, prepared from cells containing vector pTrc99A) were added to the final protein concentrations as indicated (in μg/ml). OAS (10 mM) was included where indicated by + in the reactions shown in panel E. Free probes (unbound DNA) are indicated by arrows.

may reflect a regulatory "cross talk" between the *B. cenocepacia* target and the *E. coli* regulator. Significantly, upregulation of $sbpp_{Bc}$ by $CysB_{Ec}$ was entirely abolished in the absence of functional *cysE*. These pieces of evidence strongly suggest that *B. cenocepacia* CysB may not require acetylserine as a coinducer to act as a transcriptional activator.

DISCUSSION

Relatively little is known about sulfur assimilation pathways and their regulation in ubiquitous bacteria belonging to the genus *Burkholderia*, and the presence of relevant traits can be only tentatively predicted from analysis of available genomic sequences. As a starting point to assess experimentally the selectivity of sulfur source utilization by these bacteria, we focused here on transcriptional regulation of some "signature" genes for sulfur metabolism in *B. cenocepacia* 715j. The TBLASTN search of the *B. cenocepacia* J2315 genome with the *E. coli* proteins involved in sulfur flux from external substrates to L-cysteine (Fig. 1) as queries allowed us to recognize their candidate counterparts in *B. cenocepacia*. Notably, no putative ortholog of the *E. coli* major *O*-acetylserine sulfhydrylase A (CysK) involved in the final reaction of cysteine biosynthesis has been found in *B. cenocepacia.* Perhaps this reaction relies solely on the *O*-acetylserine sulfhydrylase B-like enzyme (CysM), the product of BCAL2942, sharing 60% identity and 73% similarity with *E. coli* CysM.

$SsuR_{Bc}$ is a specific activator of genes involved in aliphatic sulfonate transport and desulfonation. The organization of a gene cluster of *B. cenocepacia* designated by us *ssuD ssuC ssuB* (Fig. 4A) is essentially identical to that found in other *Burkholderia* genomes annotated to date (19, 39). The translated gene products are likely to constitute two components of the transporter for aliphatic sulfonates (SsuB and SsuC) and an $FMNH_2$-dependent monooxygenase-type enzyme (SsuD) involved in desulfonation of substrates. The components of homologous bacterial systems devoted to alkanesulfonate transport and desulfonation (Fig. 1 shows those in *E.* coli) typically include the periplasmic substrate-binding protein (SsuA), in addition to SsuB and SsuC, and the NAD(P)H-dependent flavin mononucleotide reductase (SsuE) acting in complex with SsuD; all of these proteins are usually encoded within a single operon (13, 14, 23, 24, 25, 56, 58). In *B. cenocepacia*, the *ssu* gene cluster does not include an *ssuE*-like ORF, as in *B. subtilis* (56), and the SsuE ortholog is apparently not encoded by any other genomic locus. Possibly, the SsuD monooxygenase in *Burkholderia* may interact with a different type of flavin reductase, as suggested for *B. subtilis* (24). Our search for a gene encoding a potential counterpart of SsuA revealed the presence of several ORFs (e.g., BCAL1552, BCAM1118, and BCAS0769) sharing limited similarities (44 to 59% over the parts of the deduced protein sequences) with SsuA from *E. coli* or *Pseudomonas putida.* The genomic contexts of these ORFs do not encourage speculation on their relevance to sulfonate-

TABLE 6. Expression of *lacZ* from *B. cenocepacia* promoters in *E. coli* in the presence/absence of functional serine transacetylase (CysE)

Strain	Fusion	Background	Plasmid (relevant gene)	β-Galactosidase activity (Miller units)
EC2676	$sbpp_{Bc}$-*lacZ*	Δ*cysB*	pMH289 ($cysB_{Bc}$::Tp)[b]	<5
EC2676	$sbpp_{Bc}$-*lacZ*	Δ*cysB*	pMH284 ($cysB_{Bc}$)	4,769 ± 325
EC2677	$sbpp_{Bc}$-*lacZ*	Δ*cysB* Δ*cysE*	pMH284 ($cysB_{Bc}$)	4,361 ± 134
EC2676	$sbpp_{Bc}$-*lacZ*	Δ*cysB*	pMH199 ($cysB_{Ec}$)[c]	152 ± 3
EC2677	$sbpp_{Bc}$-*lacZ*	Δ*cysB* Δ*cysE*	pMH199 ($cysB_{Ec}$)	<5
EC2678	$cysIp_{Bc}$-*lacZ*	Δ*cysB*	pMH289 ($cysB_{Bc}$::Tp)	<5
EC2678	$cysIp_{Bc}$-*lacZ*	Δ*cysB*	pMH284 ($cysB_{Bc}$)	2,860 ± 260
EC2679	$cysIp_{Bc}$-*lacZ*	Δ*cysB* Δ*cysE*	pMH284 ($cysB_{Bc}$)	2,727 ± 209
EC2678	$cysIp_{Bc}$-*lacZ*	Δ*cysB*	pMH199 ($cysB_{Ec}$)	<5
EC2679	$cysIp_{Bc}$-*lacZ*	Δ*cysB* Δ*cysE*	pMH199 ($cysB_{Ec}$)	<5

[a] Assays were performed under the same conditions as indicated in Table 5.
[b] Plasmid carrying $cysB_{Bc}$ disrupted by a *dfrB2* cassette.
[c] Plasmid encoding wt *E. coli* CysB.

assimilation processes, and the regulation of their expression remains to be investigated. However, we have shown here that expression of contiguous *ssuDCB* genes (presumably an operon), controlled by a promoter preceding *ssuD*, is strictly dependent on the SsuR regulator (the product of BCAL1656) in *B. cenocepacia.* In *E. coli*, the *ssuEADCB* operon is under the positive control of the Cbl regulator (58), and some cross-activation of the $ssuE_{Ec}$ promoter by SsuR was demonstrated in our study. *B. cenocepacia* also possesses the putative orthologs of the *tauABCD* genes (Fig. 1), whose expression in *E. coli* is strictly dependent on Cbl (7, 59). However, we were not able to detect regulation of "$tauAp_{Bc}$" by $SsuR_{Bc}$ (data not shown), and no upregulation of $tauAp_{Ec}$ by $SsuR_{Bc}$ was detected in our assays. Nevertheless, we believe that $SsuR_{Bc}$ is most likely an ortholog of *E. coli* Cbl. It should be stressed, however, that $SsuR_{Bc}$ is not an ortholog of the recently described "SsuR" activator of *ssu* genes in *Corynebacterium japonicum* (27); the latter belongs to the ROK family of proteins, and it was discussed as a functional (but not structural) counterpart of *E. coli* Cbl.

$SsuR_{Bc}$ and $CysB_{Bc}$ control the steps of sulfate transport, activation, and reduction. The region encoding the "sulfate transporter" of *B. cenocepacia* 715j has been identified (15) as a cluster, *sbp cysT cysW cysA*, where Sbp is an ortholog of the periplasmic sulfate-binding protein and CysT, CysW, and CysA represent orthologs of the ABC-type sulfate transporter components of several bacteria (25). The TBLASTN analysis revealed that in *B. cenocepacia*, *B. mallei*, and *B. pseudomallei*, the product of the first ORF in the locus is more similar to Sbp than to CysP of *E. coli* (despite overall similarities between the proteins). In all these species, *cysA* is followed by a gene encoding a transcriptional regulator, designated "*cysB*" in a previous study on *B. cenocepacia* (15) and here renamed "*ssuR*." *E. coli* and *Salmonella* possess the sulfate/thiosulfate transporter locus organized as the *cysPTWA* operon, transcribed from the *cysP* promoter, where CysP is a thiosulfate-binding protein (20) and Sbp is encoded by an unlinked gene. We demonstrated that the *B. cenocepacia* region preceding the *sbp* gene contains a functional promoter whose full activity requires SsuR in vivo. However, since both SsuR and CysB were active in upregulation of *sbpp* in a heterologous system and complexes of *sbpp* probe with both these proteins were detectable in vitro by EMSA, the functions of SsuR and CysB at *sbpp* can apparently replace each other.

It seems that the *ssuR* gene of *B. cenocepacia*, which is clustered with *sbp cysTWA* and separated by 75 bp from the *cysA* ORF, may be expressed at a low level as a separate transcription unit. Our probing of the promoter activity within the *cysA-ssuR* region (as a plasmid-encoded *ssuRp-lacZ* fusion including 172 bp upstream of the *ssuR* start codon, ATG) revealed only a weak decrease in *ssuRp-lacZ* expression in *B. cenocepacia* mutants devoid of CysB or SsuR function. Since we have not detected binding of CysB and SsuR in this "*ssuRp* region," regulation of the *ssuR* gene itself may be not of physiological relevance. This is in contrast to the *E. coli* paradigm, where expression of the *cbl* gene from its own promoter is strongly activated by CysB (22). At the moment, we can speculate that in *Burkholderia* the expression of *ssuR* is regulated in parallel with either the *sbp cysTWA* transcription unit (from *sbpp*) or the *cysTWA* transcription unit, if the latter is preceded by a functional promoter.

Apart from the *sbp cysTWA ssuR* locus, two other gene clusters of *B. cenocepacia* have been recognized by homology searches and designated here as loci devoted to sulfate metabolism: the cluster *cysI cysH orf cysD1 cysN cysG* and the separate cluster *cysD2 cysNC* (Fig. 4). The proteins encoded in the former locus, CysD1 (a putative ATP sulfurylase) and CysN (a putative GTPase coupling GTP hydrolysis to the sulfurylation of ATP) are likely to be associated with sulfate "activation" to the adenosine 5′-phosphosulfate (APS), while CysH, CysI, and CysG are likely to participate in subsequent reduction steps of S^{6+} to S^{2-}. Notably, a gene encoding an ortholog of CysC (an APS kinase producing 3′ phosphoadenosine 5′ phosphosulfate from APS) is absent from this gene cluster. However, there is an ORF encoding a putative ortholog of bacterial "CysH"-type reductases that utilizes APS as a substrate (1, 5, 26, 28). The cluster also encodes a putative sulfite reductase component (CysI), which is similar (43%) to the hemoprotein subunit of *E. coli* sulfite reductase and 78% similar to that of *P. aeruginosa* (21), and a putative ortholog of uroporphyrinogen III methylase (CysG), an enzyme involved in synthesis of the siroheme cofactor of sulfite reductase (60). The arrangement of ORFs in the *cysI-cysG* locus of *B. cenocepacia* is identical to that in two other *Burkholderia* genomes (19, 39), including the presence of

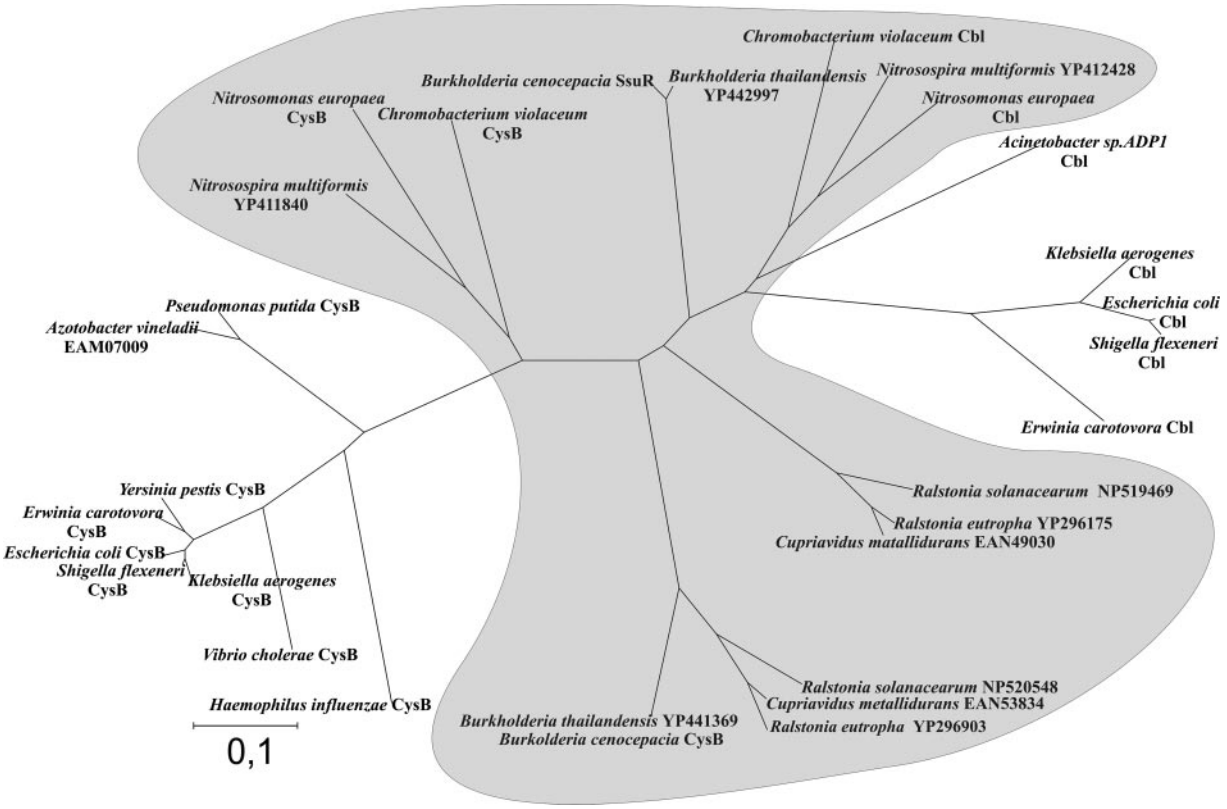


FIG. 6. Dendrogram showing the relationship between "CysB-like" proteins in bacteria. The protein sequences were retrieved from GenBank and analyzed with the programs Clustal and MEGA as described in Materials and Methods. The "CysB" and "Cbl" symbols are assigned to the proteins whose functions were either verified experimentally or annotated as CysB or Cbl in published genomic sequences; the putative members are indicated by accession numbers. The species belonging to the γ and β subphyla are unshaded and shaded, respectively. The scale bar represents the number of amino acid substitutions per site.

an ORF of unknown function located between *cysI* and *cysH* and an ORF encoding a CysG-type protein (annotated as *cobA* in the *B. mallei* and *B. psedomallei* genomes). The deduced functions of proteins encoded in this gene cluster are consistent with a view that in *Burkholderia* they may be sufficient to perform conversion of inorganic sulfate to sulfide.

The gene designated by us *cysB* (BCAL2686) is oriented divergently from *cysI*, and its location is conserved in the *B. mallei* and *B. pseudomallei* genomes. We demonstrated that the intergenic region between *cysI* and *cysB* contains targets for binding of both SsuR and CysB and that expression from *cysIp* is elevated by both CysB and SsuR (albeit to a lesser extent by the latter). This suggests that these regulators may replace each other in positive control of *cysIp*, similarly to *sbpp.* An analogous interpretation of overlapping functions of *B. cenocepacia* regulators may be applied to the observed negative control of the divergently oriented *cysB* gene by either CysB or SsuR.

In addition to *cysD1 cysN* genes contained within the *cysI-cysG* cluster, the *B. cenocepacia* genome contains another "sulfate activation locus," *cysD2 cysNC* (Fig. 4). The translated product, CysD2, shares 44% identity and 66% similarity with CysD1, and it is also similar (~70%) to the NodP-type proteins of *Rhizobiaceae.* The product of *cysNC* is likely to be a fusion protein with ATP sulfurylase and APS kinase activities, as judged by comparisons of the $CysNC_{Bc}$ and CysNC proteins of *P. aeruginosa* and *Mycobacterium tuberculosis* (42) and the NodQ-type proteins of *Rhizobiaceae* (49) and RaxQ of *Xanthomonas oryzae* (46). It seems, therefore, that *Burkholderia* species, like *Rhizobiaceae* and *Mycobacteriaceae* but unlike *Enterobacteriaceae*, can channel the intracellular sulfate either to the reductive pathway (functions encoded in the cluster *cysI cysH orf cysD1 cysN cysG*) or to sulfatation processes that require phosphoadenosine 5′ phosphosulfate as a sulfate donor (the latter being produced by the sulfate-activating complex CysD2/CysNC). In our assays, activity of the *cysD2p* promoter appeared weak (compared with those of other promoters tested), but it showed measurable upregulation by *SsuR* and *CysB* acting in concert in vivo. Also, an EMSA using a *cysD2p* probe detected a high-order complex with protein extract containing SsuR and CysB overproduced jointly. Although elucidation of the nature of this "supercomplex" requires further studies, we think that *cysD2p* is regulated by some cooperation of SsuR-CysB, possibly via formation of mixed hetero-oligomers.

Phylogeny of the "CysB family" of transcriptional regulators. The "*cysB*-like" genes can be identified in silico in many genomes of *Proteobacteria* belonging to the β and γ classes, either as single-copy ORFs (e.g., in *Haemophilus*, *Vibrio*, *Salmonella*, and *Acinetobacter*) or as two ORFs sharing substantial similarity at the amino acid level. It seems that in genomes containing a pair of "*cysB*-like" sequences, they arose from

duplication of a single ancestral gene and subsequent divergence of daughter genes. The functional significance of such divergence has so far been experimentally assessed only in *E. coli*, where the *cysB* and *cbl* (*cysB*-like) gene products activate expression of different target genes and respond to different metabolic signals. The constructed phylogenetic tree of "*cysB*-like" gene products (Fig. 6) illustrates various degrees of their relatedness in representative *Proteobacteria* from the β and γ subphyla. In *Enterobacteriales* (e.g., *Escherichia*, *Erwinia*, *Klebsiella*, and *Shigella*), CysB and Cbl appear to represent a pair of the most divergent paralogs in the "CysB family," while the $CysB_{Bc}$ and $SsuR_{Bc}$ proteins of *B. cenocepacia* and their putative counterparts in other *Burkholderiaceae* (e.g., *Ralstonia* and *Cupriavidus*) appear to be the most closely related paralogous proteins. Nevertheless, it has been demonstrated by this study that the two regulatory proteins of *B. cenocepacia* display distinct preferences for some target genes that justifies a proposed annotation of their genes as $cysB_{Bc}$ (encoding an ortholog of *E. coli* CysB) and $ssuR_{Bc}$ (encoding an ortholog of *E. coli* Cbl). However, our results have highlighted some functional differences between the regulators $CysB_{Bc}$/$CysB_{Ec}$ and $SsuR_{Bc}$/Cbl_{Ec}. In contrast to $CysB_{Ec}$, which activates the target *cys* promoters only in the presence of the inducer acetylserine, the function of $CysB_{Bc}$ (at least at the $cysI_{Bc}$ promoter) seems to be independent of this cofactor. The Cbl regulator of *E. coli* activates the target promoters (*taup* and *ssup*) without any inducing ligand, but its function is negatively affected by APS, the first intermediate of the sulfate assimilatory pathway (7). This explains the strong inhibition of expression from Cbl-dependent promoters in vivo in the presence of inorganic sulfate in the medium (55, 58, 59). In the case of $SsuR_{Bc}$, no decrease in expression of the responsive fusion $ssuD_{Bc}$-*lacZ* was observed in the presence of sulfate in the growth medium (measured in *E. coli*) (data not shown). The intriguing possibility that the function of $SsuR_{Bc}$ is independent of any cofactor is being investigated.

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