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Characterization of the σ^B Regulon in *Staphylococcus aureus*

SILKE GERTZ,[1] SUSANNE ENGELMANN,[1] ROLAND SCHMID,[2] ANNE-KATHRIN ZIEBANDT,[1] KARSTEN TISCHER,[1] CHRISTIAN SCHARF,[1] JÖRG HACKER,[3] AND MICHAEL HECKER[1]*

Institut für Mikrobiologie und Molekularbiologie, D-17487 Greifswald,[1] FB5 AG Mikrobiologie, Universität Osnabrück, D-49069 Osnabrück,[2] and Institut für Molekulare Infektionsbiologie, Universität Würzburg, D-97070 Würzburg,[3] Germany

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The σ^B-dependent stress regulon in gram-positive bacteria might fulfill a physiological role in stress response and virulence similar to that of the σ^S regulon in *Escherichia coli* and other gram-negative bacteria. In order to obtain evidence for the function of the σ^B regulon of *Staphylococcus aureus*, especially in virulence control, σ^B-dependent stress genes were identified. The two-dimensional protein pattern of wild-type cells of *S. aureus* COL was compared with that of an isogenic *sigB* mutant. By this approach, we found that the synthesis of about 27 cytoplasmic proteins seemed to be under the positive control of σ^B. N-terminal sequencing of 18 proteins allowed the identification of their genes on the almost finished genome sequence of *S. aureus* COL and the analysis of the promoter structure. Transcriptional analyses of 11 of these genes confirmed their σ^B dependency, and moreover, about 7 additional σ^B-dependent genes were found which are cotranscribed with the newly detected genes, forming operons. Altogether, we identified 23 σ^B-dependent genes and their corresponding proteins. Among them are proteins probably involved in the generation of NADH or in membrane transport mechanisms. Furthermore, at least one *clpC*-homologous gene was localized on the *S. aureus* sequence solely transcribed by σ^B. In contrast, a second *clpC*-homologous gene in *S. aureus* forming an operon with *ctsR*, *yacH*, and *yacI* was σ^B independently expressed.

Staphylococcus aureus is an important human pathogen. Its pathogenesis is very complex and probably involves the synthesis of cell wall-associated adhesins and the secretion of extracellular toxins with damaging effects on host cells, including those of the immune system (48). Nevertheless, even the ability of *S. aureus* to survive suboptimal growth conditions within the host should be a significant property which contributes to the virulence of this organism and is closely connected with the expression of stress genes (14).

In the gram-positive bacterium *Bacillus subtilis*, the alternative sigma factor σ^B regulates a large number of general stress genes (2, 7, 9, 47, 61; A. Petersohn et al., submitted for publication). Some of these genes are involved in the protection of DNA, membranes, and proteins against oxidative damage, which might represent an important component within the stress response of glucose-starved cells (4, 21, 55). Moreover, σ^B-dependent proteins contribute to survival under extreme environmental conditions such as heat or osmotic stress, repeated freezing and thawing, and acid or alkaline shock of starving *B. subtilis* (23, 63). In summary, the σ^B regulon is expected to provide multiple stress resistance to starving *B. subtilis* cells in anticipation of future stress (26, 60).

A similar physiological role has been postulated for the RpoS (σ^S) regulon in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (27, 39). In this context, it is interesting that orthologues of the σ^B-dependent genes like *katE*, *dps*, *opuE*, and *osmC* in *B. subtilis* are regulated by RpoS in *E. coli* (4, 20, 39, 57, 62). Since *rpoS* mutants of gram-negative pathogens show significantly reduced virulence (45, 51, 66), it has been suggested that in pathogenic gram-positive bacteria, the σ^B regulon also has a function in the ability of bacteria to interact with host defense mechanisms and persist during infection.

Over the last few years, σ^B was identified in the gram-positive pathogens *S. aureus* (34, 67), *Mycobacterium tuberculosis* (16), and *Listeria monocytogenes* (6, 65). As expected, *S. aureus* and *L. monocytogenes* σ^B mutant cells showed diminished stress tolerance compared with wild-type cells (10, 35, 44, 65). Recent results concerning the involvement of σ^B in the virulence of these bacteria do not support the idea that σ^B plays a significant role in infection processes (10, 35, 44). However, the question arises of whether the infection models analyzed until now really reflect the natural situation in the host.

In order to elucidate the function of σ^B in the pathogenesis of *S. aureus*, it is necessary to know the genes which are under the control of this alternative sigma factor. Until now, only a few proteins have been identified that belong to the σ^B regulon in *S. aureus*, among them *asp23* and *coa* (25, 35, 43). It has also been demonstrated that the transcription of *sar*, encoding a global regulator which controls the synthesis of a variety of extracellular and cell surface proteins involved in the pathogenesis of *S. aureus*, is partly regulated by σ^B (17, 41). Therefore, it was very surprising that a *sigB* mutation is associated with an enhanced SarA level (13). The overproduction of alpha-hemolysin, thermonuclease, and some other extracellular proteins might be the consequence of the up-regulation of SarA in the mutant (13, 35). The role of σ^B in the regulation of SarA remains obscure and needs to be further analyzed.

The discovery and functional characterization of new σ^B-dependent proteins should improve our understanding of the physiological role of the σ^B regulon in *S. aureus*. High-resolution two-dimensional (2-D) protein gel electrophoresis is an excellent technique for visualizing a very large set of proteins synthesized by a bacterial cell. Looking for proteins that are no longer induced in a regulatory mutant is a good strategy with which to define the structure of regulons. In this study, we used 2-D protein gel electrophoresis and N-terminal sequencing of proteins to detect new members of the σ^B regulon to get a

* Corresponding author. Mailing address: Institut für Mikrobiologie und Molekularbiologie, Jahnstrasse 15, D-17487 Greifswald, Germany. Phone: 49 3834 864200. Fax: 49 3834 864202. E-mail: hecker@microbio7.biologie.uni-greifswald.de.

TABLE 1. Oligonucleotides used in this study

Designation[a]	Sequence (5′ → 3′)
CSB4F	AAACAAAGAAGACGCGGCTG
CSB4R	CTAATACGACTCACTATAGGGAGA-ACTTACCTTCGATTGCAGCG
CSB7F	CAAATGCCGTATAATTACAAG
CSB7R	CTAATACGACTCACTATAGGGAGA-ATATTTAATCTGTTCCAACCG
CSB9F	GGTTATAGGTGCTAATGGCG
CSB9R	CTAATACGACTCACTATAGGGAGA-CTTTAATGTCCTGATCACCAC
CSB10F	CCTACATGTGTCTATTGAGG
CSB10R	CTAATACGACTCACTATAGGGAGA-AATGCACCAAAGTTTTCCCC
CSB12F	TGTAGTAAATGACACTGGCG
CSB12R	CTAATACGACTCACTATAGGGAGA-CTAAGCTTTGGGACCTTTAG
CSB16F	TTATATGGCCGAGGCACTAC
CSB16R	CTAATACGACTCACTATAGGGAGA-TGTTACAGGTCGGTGATTGC
CSB22F	TGCTGATGTAATGGCAGAGC
CSB22R	CTAATACGACTCACTATAGGGAGA-AACACGACCTAAGCTTGACC
CSB28F	ACAAGAGGTACCGGGTTTAC
CSB28R	CTAATACGACTCACTATAGGGAGA-AACTCAACAGGTTGTCCTGC
CSB29F	TGGTGTCCTCTTTTACCATG
CSB29R	CTAATACGACTCACTATAGGGAGA-TCCAATTCATGCTATCACGC
CSB33F	TGTAGCAGAATATGCTGCTG
CSB33R	CTAATACGACTCACTATAGGGAGA-AAGCAAAGCGTGACGTAAAG
CSB35F (SarAF)	TAGGGAGGTTTTAAACATGG
CSB35R (SarAR)	CTAATACGACTCACTATAGGGAGA-GTTGTTTGGTTCAGTGATTC
CLPC2392F	CAATTAGAAACACCAAGACCG
CLPC2392R	CTAATACGACTCACTATAGGGAGA-ATCTAATGTACCGTCTTTGG
CLPC2161F	AAAAATAACACACAATATTC
CLPC2161R	CTAATACGACTCACTATAGGGAGA-CTCAACCGATAATTTGATGG

[a] Oligonucleotides with the letter R contain the recognition sequence for T7 at the 5′ end (29).

more comprehensive view of the physiological role of the general stress response of *S. aureus*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *S. aureus* strains used in this study were wild-type *S. aureus* COL and the isogenic *sigB* mutant (35). *S. aureus* strains were cultivated in LB (53) or in a synthetic medium described earlier (25). Heat stress conditions were provoked as follows. Cells were cultivated in LB to an optical density at 540 nm of 0.5 and transferred to 48°C. The time of the shift was regarded as zero. Samples were taken during exponential growth immediately prior to the shift or at the time indicated in the relevant figure legends.

Preparative 2-D gel electrophoresis and N-terminal microsequencing. For preparation of cell extracts, bacteria were grown in the synthetic medium mentioned above. At an optical density at 500 nm of 1.0, cells were harvested by centrifugation of 50 ml of the culture, washed twice with Tris-EDTA buffer, and resuspended in Tris containing 2 mM phenylmethylsulfonyl fluoride. After incubation for 10 min on ice with lysostaphin (50 μg/ml), cells were disrupted using a French press. The lysate was centrifuged (10 min, 10,000 rpm [Heraeus 12148]) at 4°C; the supernatant fluid was stored frozen. Preparative 2-D gel electrophoresis and N-terminal microsequencing of proteins were carried out as described earlier (56) by using immobilized ptt gradients of 4 to 7 and 3 to 10. For microsequencing, the Coomassie-stained protein spots were cut from several 2-D gels and the collected gel pieces were concentrated as previously described (50, 54). The proteins or peptides generated by treatment with cyanogen bromide were blotted onto a polyvinylidene difluoride membrane, stained, and sequenced as previously described (56).

Analysis of transcription. Total RNA of the *S. aureus* strains was isolated from exponentially growing or stressed cells by the acid phenol method described by Majumdar et al. (40) with modifications described previously (25, 61).

Northern blot analyses were carried out as described earlier (64). Chemiluminescent signals were detected by the Lumi-Imager from Boehringer Mannheim and analyzed by using the program LumiAnalyst (Boehringer Mannheim).

The specific RNA probes were prepared by in vitro translation with T7 polymerase and with the appropriate PCR fragments as templates. The PCR fragments were generated by using chromosomal DNA of *S. aureus* COL which was purified with a chromosomal DNA isolation kit in accordance with the protocol of the manufacturer (Promega) and the oligonucleotides listed in Table 1.

The oligonucleotides complementary to the C-terminal region of the genes contain the T7 recognition sequence (29) at the 5′ end (25).

Sequence analyses. Preliminary sequence data was obtained from The Institute for Genomic Research (TIGR) through the website at http://www.tigr.org. Database searches were carried out using the BLAST program (3).

RESULTS

Identification of proteins belonging to the σ^B regulon on 2-D protein gels. First, we looked for conditions that allowed induction of σ^B-dependent stress proteins only in the wild type. Because σ^B is active in cells growing in a synthetic medium (25), the protein synthesis patterns of exponentially growing cells of *S. aureus* COL and its isogenic *sigB* mutant cultivated in a synthetic medium were compared. This allowed us to identify 27 proteins belonging to the σ^B regulon (Fig. 1A and B). These proteins, designated Csb (controlled by sigma B), were not or hardly detectable in the *sigB* mutant and might be under the positive control of σ^B. The N-terminal sequences of 18 of these proteins were determined, and they are listed in Table 2. By using the uncompleted DNA sequence of *S. aureus* COL kindly provided by TIGR (updated May 1999 and August 1999), we were able to find the open reading frames coding for the majority of the proteins (Table 2). A protein database search was done with the deduced amino acid sequences of the newly identified σ^B-dependent genes (Table 3).

Only three proteins, Csb3, Csb9, and Csb35, have been described in *S. aureus* so far. Whereas Csb3 and Csb9 are identical to so far hypothetical proteins of *S. aureus* (8, 38), the N-terminal sequence of Csb35 resembles regulatory protein SarA of *S. aureus* (12) (Fig. 1B; Table 3). The transcription of the *sar* locus was already reported to be partly controlled by σ^B (17, 41). In our experiments, we showed that the amount of SarA is diminished in a *sigB* mutant (Fig. 1B). Unfortunately, the function of Csb3 and Csb9 is not known. However, it is interesting that Csb3 is similar to YfkM in *B. subtilis*, which is also regulated by σ^B (46).

The putative functions of nine of the newly identified proteins were derived from similarities to known proteins of other organisms. However, we did not confirm the physiological function of any of these proteins by experiments. Among them are three with similarities to various dehydrogenases: Csb22,

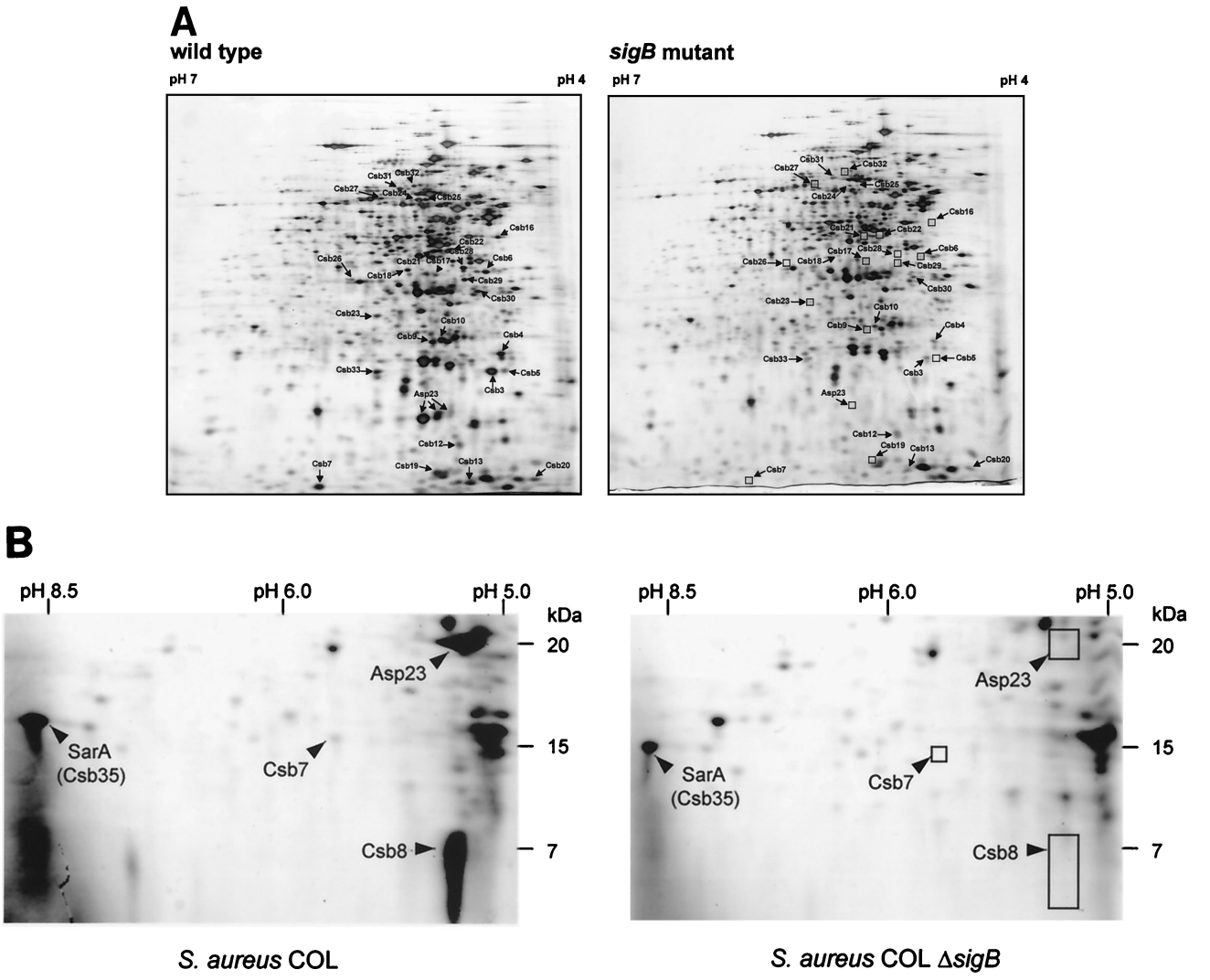


FIG. 1. σ^B-dependent proteins of *S. aureus*. (A) 2-D pattern of cytoplasmic proteins from *S. aureus* COL and its isogenic *sigB* mutant. The proteins from 100 (A) or 500 (B) μg of crude cell extract of exponentially growing cells were separated by preparative 2-D polyacrylamide gel electrophoresis. Proteins were stained with silver nitrate (A) or Coomassie blue (B). The protein spots identified are indicated by arrows. Comparison of the protein synthesis patterns of wild-type and *sigB* mutant *S. aureus* COL grown in synthetic medium allowed the identification of proteins belonging to the σ^B regulon. These proteins, designated Csb (controlled by sigma B), were not or hardly detectable in the mutant strain. (B) Sectors of 2-D gels covering the region where SarA (Csb35) is located. Cytoplasmic protein extracts of wild-type *S. aureus* COL and of its isogenic *sigB* mutant grown in synthetic medium were separated.

Csb24, and Csb28 (Table 3). Interestingly, Csb24 and Csb28 shared similarities with proteins described to be σ^B dependent in *B. subtilis* (46, 47) (Table 3).

Csb10 resembles various ATP-binding cassette transport (ABC transporter) proteins, and Csb29 is very similar to σ^B-dependent BmrU in *B. subtilis*. Proteins encoded by the *bmrRU* operon in *B. subtilis*, such as Bmr (transporter) and BmrR (regulator), are known to be responsible for the drug resistance (1). However, BmrU itself did not show any significant similarities to any known proteins in the database and its function remains a matter of speculation.

For the Csb4 protein, we observed weak similarities to YckG of *B. subtilis* (22), which might encode a hexulose-6-phosphate synthase. Proteins Csb7, Csb8, Csb12, and Csb19 did not display significant similarities to proteins with known functions in the database. Open reading frames coding for proteins Csb5 and Csb13 could not be identified in the databases, and their N termini showed no similarities to known proteins.

Promoter characterization and transcriptional analyses of the newly identified σ^B-dependent genes. In *B. subtilis*, the recognition sequences for the σ^B-containing RNA polymerase are strongly conserved and a consensus of all of the σ^B-dependent promoter sequences currently available was derived: GTTTaa and GGG(A/T)A(A/T) for the −35 and −10 regions, respectively, which are separated by 13 to 15 nucleotides (47). The known σ^B-dependent promoters in *S. aureus* are very similar to the consensus sequence in *B. subtilis* (17, 25, 34, 67). Recently, we have shown that the σ^B promoter of *asp23* is recognized by Eσ^B in *B. subtilis* (25). Therefore, we used the consensus of *B. subtilis* to search for σ^B-dependent promoters in front of the identified genes. As a result, we could find similar promoter structures immediately upstream of the translational start codon of 15 of these genes (Table 2).

For transcriptional analyses, we selected 11 genes and in all cases we confirmed their σ^B dependency by Northern blots. These results implied that the transcription of the newly iden-

TABLE 2. New σ^B-dependent proteins in *S. aureus*

Protein (length [amino acids])	N-terminal sequence	Molecular mass (kDa); pI[b]	Distance (bp) from −10 to ATG[d]	Suggested −35 promoter sequence[d]	No. of intervening nucleotides	Suggested −10 promoter sequence[d]
Csb3 (171)	TKKVAIILANEFEDIEYSSP	18.6; 4.6	38	**GTTTAA**	14	GTCTAT
Csb4 (210)	MELQLAIDLLNKEDAA	22.4; 4.6	43	**GTTTAA**	14	**GGGAAA**
Csb5	TKLVA[a]					
Csb7 (14)	PYNYKKQNGELM	16.5; 5.6	184	**GTGTGA**	14	**GGGTAG**
Csb8 (64)	ADESLFEQAL	7.0; 5.2	42	**GTTTAG**	13	**GGGTAA**
Csb9 (222)	TNILVIGANGGVGSLXVQQL	24.0; 4.9	34	**GTTTTA**	14	**TGGTAT**
Csb10 (253)	ASGLEIKDLEVE	28.3; 4.9	109	**GTTTAA**	13	**AGGTAT**
Csb12 (135)	ADITNXNDTGEDRNA	15.1; 4.9	43	**GTTTTA**	14	**GGGTAA**
Csb13	MKVVTDVYI[a]					
Csb16 (407)	MTFSEKEQIQ	45.1; 4.6	36	**GTTTAA**	13	GTTTAT
Csb19 (140)	SNSQXIQAIENVLATSKVGVL	15.8; 4.6	25	**GTTTAG**	14	CGCTAT
Csb22 (360)	MKIAVGHGNGAVTAXV	40.7; 4.8	106	**GTTTAA**	14	GGTTAT
Csb24 (475)	MYDYTKQRLNGESA	52.0; 5.0	36	**GTTTAT**	14	**GGATAA**
Csb28 (293)	AAQDPKTKFK	31.7; 4.7	36	**GATTAA**	15	**GGGTAA**
Csb29 (305)	MENKYTH	33.6; 4.7	40	**GTATTA**	12	**GGGTAT**
Csb33 (199)	AMNILVFDNSQLVAEYAADI	22.3; 5.3	59	**GTTTGA**	14	**GGGTAT**
Csb34	MATTEKPEGNXGAL					
Csb35	AITKINDCFELLSMVT			**GTGATA**	14	**GGGTAT**[c]

[a] No corresponding DNA sequence was found in the unfinished *S. aureus* COL database.
[b] The theoretical molecular mass and pI were computed by using Expasy tools.
[c] The promoter sequence was published previously (5).
[d] The translational start sites and promoter sequences were determined by sequence analyses. The consensus sequences of σ^B-dependent promoters in *B. subtilis*, with 13 to 15 intervening nucleotides, are as follows: −35, GTTTAT; −10, G**G**GTA**T**. Boldface letters indicate nucleotides essential for promoter activity.

tified genes really depends on σ^B-containing RNA polymerase in vivo. In all cases, the synthesis of the σ^B-dependent transcripts was heat inducible in complex medium and the induction failed in the *sigB* mutant. Furthermore, we can distinguish between genes controlled solely by σ^B and genes regulated in a more complex way.

Only *csb7*, *csb9*, and *csb16* are transcribed solely by σ^B under the conditions tested so far (Fig. 2). For the genes *csb7* and *csb16*, we detected one monocistronic transcript, respectively. Both genes were heat inducible. In the case of *csb9*, two main heat-inducible transcripts were found (0.73 and 2.4 kb), which are synthesized in a σ^B-dependent manner. While the 0.73-kb transcript is a monocistronic transcript of *csb9*, the 2.4-kb transcript contains, in addition to the *csb9* message, the mRNA of the open reading frame downstream of *csb9*, whose product (Csb9-1) is very similar to ManA (mannose-6-phosphate isomerase) in *B. subtilis* (49) (Table 4).

Transcription of *csb4*, *csb10*, *csb12*, *csb22*, *csb28*, *csb29*, *csb33*, and *csb35* (*sar*) is only partly regulated by σ^B (Fig. 3A and B). Transcription still occurred in the mutant, indicating that, in addition to σ^B, a second sigma factor is involved. However, with the exception of *csb22*, in all cases, the main contribution to transcription was that of σ^B. Only a low basal level of transcription was found in the *sigB* mutant which is not heat induced.

According to the sizes of their transcripts, *csb12*, *csb28*, *csb29*, and *csb35* (*sar*) seem to be monocistronically transcribed by σ^B (Fig. 3A). In contrast, the detected σ^B-dependent transcripts of the genes *csb4*, *csb10*, *csb22*, and *csb33* should contain additional messages of open reading frames located downstream of the genes (Fig. 3B). These data indicate that additional genes belong to the σ^B regulon whose products are very similar to subunits of ABC transporters (Csb10-1 and Csb10-4); to NifS (Csb10-2) and NifU (Csb10-3) in *B. subtilis* (36); to *nhaC* in *S. carnosus*, which might encode an Na^+/H^+ antiporter protein; and to YckF in *B. subtilis* (Csb4-1).

Transcriptional regulation of two different *clp*-like genes in *S. aureus*. Recently, we have started a second approach in order to identify σ^B-dependent genes in *S. aureus* by looking for genes homologous to σ^B-dependent genes of *B. subtilis*. By this approach, we found at least two genes homologous to *clpC* in *B. subtilis*. The corresponding gene products show 44% (Clp2161) and 69% (Clp2392) identity to *B. subtilis* ClpC. Both proteins contain two nucleotide binding regions that are highly conserved among the Clp ATPases and are separated by a spacer of 60 amino acids in Clp2161 and 67 amino acids in Clp2392. The lengths of the spacers are typical for ClpC proteins (58). The gene order of the *clp2392* operon supports the assumption that *clp2393* encodes a ClpC protein. The other ClpC-related protein, Clp2161, also shows similarities to ClpE in *Lactococcus lactis* (54% identical amino acids); however, there is no putative zinc binding domain typical for ClpE proteins.

In Northern blot experiments for *clp2161*, we detected one heat-inducible transcript of about 2.3 kb, corresponding to the size of the proposed open reading frame only in the wild type and not in the *sigB* mutant. We found a promoter sequence very similar to the consensus of promoters recognized by σ^B in front of the gene (GTTTTA N_{14} TGGAAA). Computer analysis predicted the presence of a terminator structure at the end of the putative gene (Fig. 4). In contrast, the transcription of *clp2392* did not appear to be influenced by a mutation in *sigB*. We found a 4.5-kb heat-inducible transcript probably containing four genes homologous to *ctsR*, *yacH*, *yacI*, and *clpC* of *B. subtilis* (32, 33). Upstream of *ctsR*, we found a promoter region possibly recognized by the vegetative sigma factor σ^A. Furthermore, three CtsR boxes were localized around the promoter region.

DISCUSSION

Our data on the σ^B-dependent general stress regulon in *B. subtilis* suggested that this stress response might fulfill a physiological role similar to that of the σ^S-dependent response in *E. coli* and other gram-negative bacteria, thus providing nonspecific, multiple, and general stress resistance to nongrowing cells (26, 27, 39, 63). Moreover, some σ^S-dependent genes

TABLE 3. Similarities of σ^B-dependent *S. aureus* proteins to proteins in the database

Protein (length [amino acids])	Similar protein(s); function (length [amino acids])	% Identity (no. of amino acids identical/total)
Csb3 (171)	*S. aureus* Yly1; hypothetical 18.6-kDa protein (171)	100 (171/171)
	B. subtilis YraA; unknown (154)	56 (86/151)
	B. subtilis YfkM[a]; GS18, unknown (172)	49 (84/171)
Csb4 (210)	*B. subtilis* YckG; hexulose-6-phosphate synthase (210)	55 (115/207)
	Methanobacterium thermoautotrophicum MTH129; orotidine 5-phosphate decarboxylase (228)	28 (58/207)
	B. subtilis PyrF; orotidine 5-phosphate decarboxylase (239)	22 (41/185)
Csb7 (141)	*B. subtilis* YdfG; unknown (147)	36 (46/126)
	Dehalospirillum multivorans Orf1; hypothetical protein (177)	30 (28/93)
Csb8 (64)	*B. subtilis* YwmG[a]; unknown (62)	42 (20/47)
Csb9 (222)	*S. aureus* hypothetical protein (212)	99 (211/212)
	B. subtilis YhfK[a]; unknown (214)	42 (91/213)
Csb10 (253)	*B. subtilis* YurI; unknown, V296 vegetative protein, similar to ABC transporter (261)	80 (200/249)
Csb12 (135)	*B. subtilis* YtrI; unknown (167)	22 (20/87)
	B. subtilis YopM; unknown (66)	28 (13/46)
Csb16 (407)	*L. monocytogenes* DapE; succinyl-diaminopimelate desuccinylase (379)	42 (171/403)
	B. subtilis ArgE; acetylornithine deacetylase (436)	29 (99/337)
Csb19 (140)	*B. subtilis* YdaG[a]; GS26, unknown (140)	38 (54/139)
Csb22 (360)	*Arthrobacter* sp. ODH; opine dehydrogenase, norvalin dehydrogenase (359)	25 (90/351)
Csb24 (475)	*Staphylococcus xylosus* CudA; glycine betaine aldehyde dehydrogenase (497)	36 (175/477)
	B. subtilis YcnH[a]; unknown, similar to succinate-semialdehyde dehydrogenase (462)	37 (173/457)
	B. subtilis AldY[a]; aldehyde dehydrogenase (485)	33 (155/468)
Csb28 (293)	*B. subtilis* YhxD[a]	60 (176/289)
	B. subtilis YdaD[a]; GS39, unknown, similar to alcohol dehydrogenase (286)	60 (129/267)
	B. subtilis YhdF[a]; unknown, similar to glucose-1-dehydrogenase (289)	46 (129/279)
Csb29 (305)	*B. subtilis* BmrU[a]; multidrug resistance protein cotranscribed with Bmr (297)	32 (95/292)
Csb33 (199)	None found	
Csb35[b]	*S. aureus* RN450 SarA; staphylococcal accessory regulator (124);	100 (16/16)
	S. aureus RN6390 SarA; staphylococcal accessory regulator (113)	100 (16/16)

[a] Proteins are described as σ^B dependent in *B. subtilis* (2, 46, 47; Petersohn et al., submitted for publication).
[b] The N-terminal sequence of Csb35 (Table 2) was used for National Center for Biotechnology Information BLAST search.

control virulence genes of gram-negative bacteria (66). Therefore, it was tempting to speculate that σ^B is also involved in the control of virulence gene expression in gram-positive bacteria. This suggestion was supported by the finding that σ^B initiates transcription at one of the three promoters of *sarA* encoding one of the important regulators of virulence-associated genes in *S. aureus* (41).

Only a few genes under σ^B-control have been identified so far; among them is that for alkaline shock protein Asp23. However, the identification of Asp23 as a σ^B-dependent protein did not make any progress in the functional analysis of the regulon since nothing was known about the function of Asp23 itself (25, 35, 37). In order to obtain a more comprehensive picture of the role of σ^B in stress or starvation survival in general, including evidence for the suggested function of the σ^B regulon in virulence control, new members of the general stress regulon in *S. aureus* should be identified. The putative functions of these new proteins should allow a preliminary prediction of the physiological role of the entire regulon. In the present study; we showed that the synthesis of about 27 proteins visible on 2-D gels of crude protein extracts of *S. aureus* COL are under the positive control of alternative sigma factor σ^B. Eighteen of these proteins were identified by N-terminal sequencing, and the open reading frames coding for the proteins were localized on the uncompleted DNA sequence of *S. aureus* COL kindly provided by TIGR (updated May 1999 and August 1999). By transcriptional analyses of these new genes, about eight additional σ^B-dependent genes were found which are cotranscribed with the newly detected genes, forming operons. Comparison of all of the newly identified genes with the *B. subtilis* genome showed that 20 of them are homologous to *B. subtilis* genes, only 7 of which are known to be regulated by σ^B (Tables 3 and 4). The proteins belonging to the σ^B regulon in *S. aureus* but not in *B. subtilis* may provide evidence about additional functions of the regulon in gram-positive pathogens. Moreover, two of the genes (*csb12* and *csb22*) in *S. aureus* did not have any orthologues in *B. subtilis*. These genes are particularly interesting because they may form

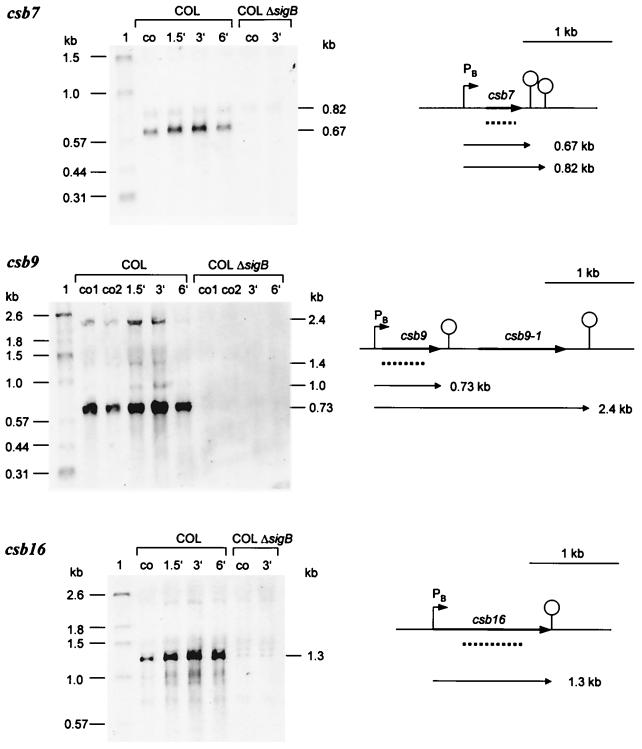


FIG. 2. Northern blot analyses of solely σ^B-dependent genes. RNA was isolated from *S. aureus* COL and its isogenic *sigB* mutant growing in LB at 37°C (lanes co) and at various times after a shift to 48°C. The membrane was hybridized with digoxigenin-labeled RNA probes for the respective genes. Relevant transcripts are indicated. Schematic representations of the gene loci based on sequences of *S. aureus* COL (TIGR, unpublished data) are shown (P_B, σ^B-dependent promoter). The broken lines represent the RNA probes used in the experiments whose results are shown. The operon structure of the *csb9* locus was verified by using an RNA probe specific for *csb9-1*.

a reservoir of σ^B-dependent genes whose products could interact in a specific manner with the host. Unfortunately, the function of these genes is still unknown.

For most of the genes described so far, at least a second sigma factor, in addition to σ^B, is involved in gene expression. Obviously, the corresponding proteins seem to be necessary even under conditions under which σ^B is not active. However, particularly in response to heat shock, the σ^B-dependent promoter seemed to be the strongest one in all of the cases tested so far. This complex regulation was also described for many σ^B-dependent proteins in *B. subtilis* (2, 19, 31, 55). Only *csb7*, *csb9*, and *csb16* seem to be transcribed solely by σ^B under the conditions tested so far.

The newly identified genes allow first conclusions on the physiological role of the σ^B regulon in *S. aureus*. Protection of starved *B. subtilis* cells against oxidative damage could be the most essential component of σ^B-mediated stress resistance (21). In accordance with this, a *sigB* mutant of *S. aureus* showed increased sensitivity to hydrogen peroxide (10, 35). Furthermore, maintenance of intracellular redox balance under stress and starvation might be very important and requires the reduction of oxidized biological molecules by using NAD(P)H. Therefore, a sufficient level of NAD(P)H seems to be a prerequisite for the cell to face oxidative stress. Among the newly identified genes, three are possible dehydrogenases which could be involved in the generation of reduction equivalents like NAD(P)H and FADH. In *B. subtilis*, the *nifS* gene product might contribute to NAD biosynthesis by generating the Fe-S clusters required for NadA activity (59). Interestingly, among the newly identified *S. aureus* genes there is one *nifS*-homologous gene.

Prokaryotic, as well as eukaryotic, organisms possess multidrug resistance efflux transporters whose expression is induced by various structurally divergent compounds such as antibiotics, inhibitors, and other toxic substances. In *B. subtilis*, the *bmrUR* operon, which encodes proteins that may contribute to

TABLE 4. Similarities of new σ^B-dependent *S. aureus* proteins whose genes are cotranscribed with the newly detected genes, forming operons

Protein (length [amino acids])	Similar protein(s); function (length [amino acids])	% Identity (no. of amino acids identical/total)
Csb4-1 (183)	*B. subtilis* YckF; hypothetical protein (185)	43 (76/176)
Csb9-1 (338)	*S. aureus* hypothetical protein (160)	93 (150/160)
	Bacillus halodurans mannose-6 phosphate isomerase (315)	41 (129/311)
	Streptococcus mutans ManA; mannose-6-phosphate isomerase (316)	44 (139/311)
	B. subtilis ManA; mannose-6-phosphate isomerase (316)	42 (134/312)
Csb22-1 (466)	*Staphylococcus carnosus* NhaC; putative Na^+/H^+ antiporter (231)	61 (130/210)
	Haemophilus influenzae YB07; hypothetical N^+/H^+ antiporter (468)	30 (139/458)
	B. subtilis YqkI; unknown, similar to Na^+/H^+ antiporter (468)	34 (160/460)
	B. subtilis YheL; unknown, similar to Na^+/H^+ antiporter (453)	31 (140/450)
Csb10-1 (435)	*B. subtilis* YurX; unknown, similar to unknown proteins (437)	56 (245/430)
	Synechocystis sp. hypothetical 52.8-kDa protein SLR0074, ABC transporter subunit (480)	24 (106/438)
	E. coli YnhE; hypothetical protein (508)	22 (103/450)
	B. subtilis YurU; unknown, similar to unknown proteins (465)	24 (101/419)
Csb10-2 (290)	*B. subtilis* YurW; similar to NifS protein homolog (406)	60 (175/290)
Csb10-3 (155)	*B. subtilis* YurV; similar to NifU protein homolog (147)	72 (101/140)
	Mycobacterium leprae nitrogen fixation homolog NifU (165)	38 (54/140)
Csb10-4 (466)	*B. subtilis* YurU; unknown, similar to unknown proteins (465)	84 (394/465)
	Synechocystis sp. hypothetical 52.8-kDa protein SLR0074, ABC transporter subunit (480)	43 (205/468)
	Guillardia theta ABC transporter (483)	43 (202/467)
	E. coli YnhE; hypothetical protein (508)	39 (190/478)

resistance to multidrug compounds, is regulated by σ^B. Very recently, it was shown that σ^B is activated in mycobacterial cells after exposure to rifampin, streptomycin, and cycloserine (42). In *S. aureus*, methicillin resistance is widespreaded; however, the mechanism of this phenomen is not fully understood. Interestingly, it was reported that the *sigB* mutant of *S. aureus* COL showed a drastic reduction in methicillin resistance (15). Among the newly identified σ^B-dependent proteins of *S. aureus*, we found proteins with significant similarities to Na^+/H^+ antiporters or ABC transporters. The Na^+/H^+ antiporters are widely distributed in cell membranes from bacteria to mammals. The antiporters play important roles in the Na^+ cycle across the cytoplasmic membrane of all living cells. In bacteria, the antiporter extrudes Na^+ or Li^+ in exchange for H^+. Besides their function in antibiotic resistance, they may play a role in (i) the establishment of an electrochemical potential of Na^+ across the cytoplasmic membrane, (ii) the extrusion of Na^+ and Li^+, (iii) intracellular pH regulation under alkaline conditions, and (iv) cell volume regulation (11, 28).

Besides the identification of σ^B-dependent genes by proteomics, we started a second approach. In *B. subtilis*, we know of more than 150 genes belonging to the regulon (Petersohn et al., submitted for publication); among them are Clp proteins with essential functions in stress resistance (24, 30).

Recently, ClpC was shown to be involved in the virulence of *L. monocytogenes* (52). Therefore, we looked for *clp*-homologous genes in the *S. aureus* genome sequence. As a result, we

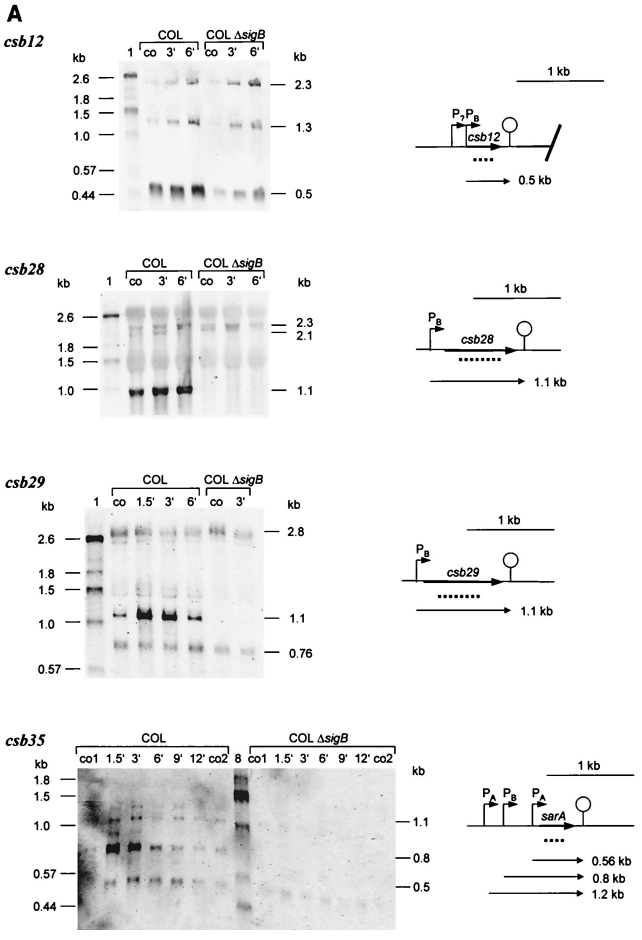
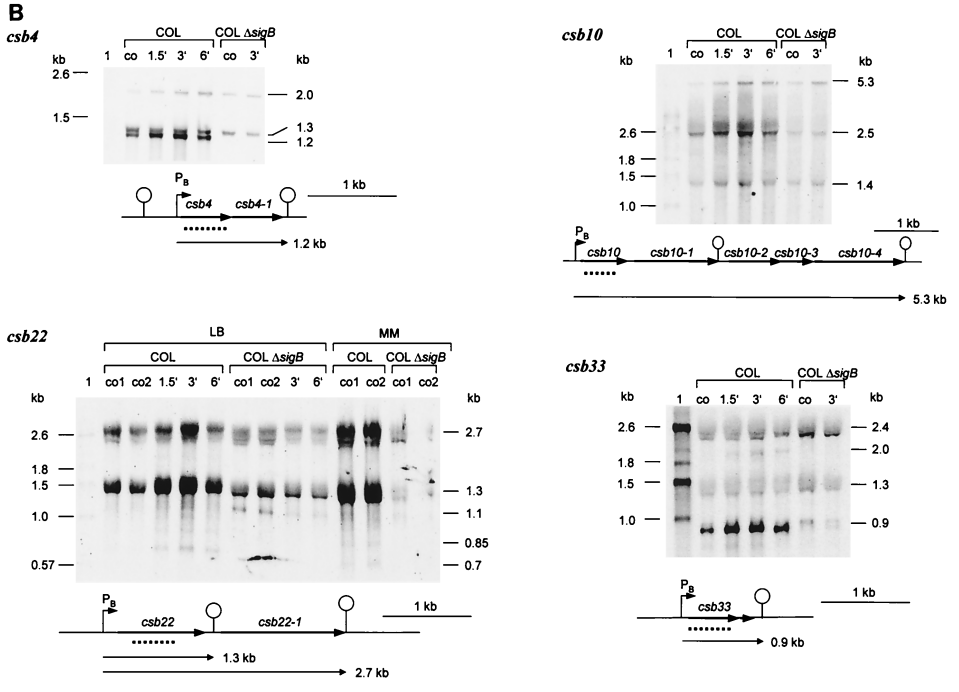


FIG. 3. Northern blot analyses of genes partly regulated by σ^B. RNA was isolated from *S. aureus* COL and its isogenic *sigB* mutant growing in LB at 37°C (lanes co) and at various times after a shift to 48°C. The membrane was hybridized with digoxigenin-labeled RNA probes for the respective genes. Relevant transcripts are indicated. Schematic representations of the gene loci based on sequences of *S. aureus* COL (TIGR, unpublished data) are shown (P_B, σ^B-dependent promoter; P_A, σ^A-dependent promoter). The broken lines represent the RNA probe used in the experiments whose results are shown. (A) σ^B-dependent genes monocistronically transcribed. (B) σ^B-dependent operons. We verified the operon structures of the *csb10* and *csb22* loci by using additional probes (*csb10-1*, *csb10-4*, and *csb22-1*). MM, synthetic medium.



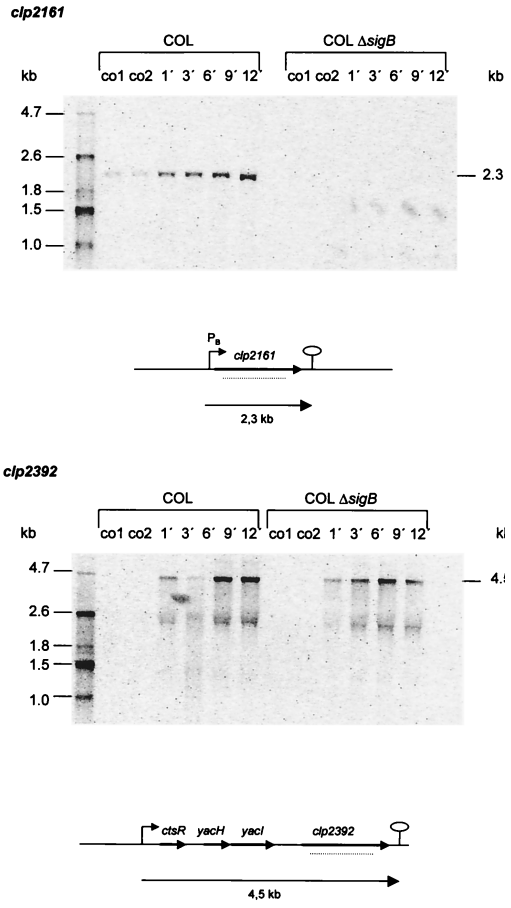


FIG. 4. Northern blot analyses of *clpC*-homologous genes in *S. aureus*. RNA was isolated from *S. aureus* COL and its isogenic *sigB* mutant growing in LB at 37°C (lanes co) and at various times after a shift to 48°C. The membrane was hybridized with digoxigenin-labeled RNA probes for *clp* on contig 2161 (236099. . .21954) and contig 2392 (2866. . .1966). Relevant transcripts are indicated. Schematic representations of the gene loci based on sequences of *S. aureus* COL (TIGR, unpublished data) are shown (P_B, σ^B-dependent promoter). The broken lines represent the RNA probes used in the experiments whose results are shown. We used additional probes to find out which genes are cotranscribed with *clp2392*.

localized two open reading frames possibly encoding ClpC proteins. It is a remarkable finding that at least one *clpC*-homologous gene seemed to be controlled solely by alternative stress sigma factor σ^B and the other is probably controlled by the global regulator of class III general stress genes CtsR; however, experimental evidence for this is still lacking (18, 33). The Clp proteins are involved in several physiological processes, such as proteolysis, stress tolerance, competence, cell division, and virulence. The presence of at least two *clpC*-homologous genes in *S. aureus* implies an essential role of the protein in the physiology of this organism.

The identification of new members of the σ^B regulon is a preliminary but essential step toward a more comprehensive understanding of the role of this large regulon in stress adaptation and virulence. No evidence has previously been presented for a role of σ^B in the infection process and virulence (10, 44). Analysis of the effects of promising mutations in individual σ^B-dependent genes on stress adaptation and infection is another approach to the problem of whether and to what extent σ^B-dependent proteins contribute to survival within the host. These studies will provide an essential contribution to the understanding of the cell physiology of *S. aureus*.

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