Mol Gen Genet (1999) 261: 558–566 

ORIGINAL PAPER

S. Gertz · S. Engelmann · R. Schmid
K. Ohlsen · J. Hacker · M. Hecker

# Regulation of $\sigma^B$-dependent transcription of *sigB* and *asp23* in two different *Staphylococcus aureus* strains

Received: 17 November 1998 / Accepted: 27 January 1999

**Abstract** The alkaline shock protein Asp23 was identified as a $\sigma^B$-dependent protein in *Staphylococcus aureus*. In *Bacillus subtilis*, the *asp23* promoter from *S. aureus* is regulated like other $\sigma^B$-dependent promoters, which are strongly induced by heat and ethanol stress. However, almost no induction of *asp23* expression was found after heat or ethanol stress in *S. aureus* MA13 grown in a synthetic medium, where the basal expression level of *asp23* is high. Under the same experimental conditions the $\sigma^B$ gene itself showed a similar expression pattern: it was highly expressed in synthetic medium but not induced by heat or ethanol stress. In contrast, $\sigma^B$ activity was increased by heat stress when the cells were grown in a complex medium. The constitutive expression of *sigB* and $\sigma^B$-dependent stress genes in *S. aureus* MA13 grown in a synthetic medium is in a sharp contrast to the regulation of $\sigma^B$ activity in *B. subtilis*, and needs further investigation. A deletion of 11 bp in the *rsbU* gene, which encodes the phosphatase that acts on RsbV (the anti-anti-sigma factor), in *S. aureus* NCTC 8325-4 might be responsible for the failure of heat stress to activate $\sigma^B$ in complex medium, and thus reduce the initiation of transcription at $\sigma^B$-dependent promoters in this strain.

**Key words** *sigB* · *asp23* · *rsbU* · *Staphylococcus aureus* · *Bacillus subtilis*

Communicated by W. Goebel

S. Gertz · S. Engelmann · M. Hecker (✉)
Institut für Mikrobiologie und Molekularbiologie,
Ernst-Moritz-Arndt-Universität, D-17487 Greifswald, Germany
e-mail: hecker@microbio7.biologie.uni-greifswald.de
Tel.: +49-3834-864200; Fax: +49-3834-864202

R. Schmid
Fachbereich 5, Arbeitsgruppe Mikrobiologie,
Universität Osnabrück, D-49069 Osnabrück, Germany

K. Ohlsen · J. Hacker
Institut für Molekulare Infektionsbiologie
Universität Würzburg, D-97070 Würzburg, Germany

## Introduction

Bacteria have evolved highly efficient adaptive responses in order to meet the challenges of a changing environment and survive under diverse conditions of stress and starvation. The induction of stress proteins is an essential component of this adaptive network. Depending on their induction pattern, stress proteins fall into at least two groups: (i) stress- or starvation-specific proteins are induced by only one stimulus (e.g. heat shock, oxidative stress) and confer specific protection against a single stressor only, while (ii) general stress proteins are induced by a variety of stimuli and provide protection against multiple stresses in the non-growing cell, regardless of the specific stimulus applied (Hecker and Völker 1998).

In *B. subtilis*, the alternative sigma factor $\sigma^B$ is the master regulator of a large number of general stress genes induced in response to physiological stress and energy depletion (Boylan et al. 1993; Hecker et al. 1996; Bernhardt et al. 1997). The activity of $\sigma^B$ is regulated by two partner-switching modules of signal transduction, which integrate the response to two different types of challenge: environmental stress and energy restriction (Dufour and Haldenwang 1994; Kang et al. 1996; Völker et al. 1996; Yang et al. 1996; Völker et al. 1997). More than 60 genes belong to the $\sigma^B$-dependent stress and stationary-phase regulon in *B. subtilis* (Bernhardt et al. 1997). Because of their non-specific induction profiles, $\sigma^B$-dependent proteins are thought to make non-growing cells resistant to multiple stresses. This assumption is supported by the finding that *sigB* mutants fail to develop starvation-induced cross-resistance to oxidative stress (Engelmann and Hecker 1996) and are not able to survive at extremes of pH (Gaidenko and Price 1998). A similar physiological role has been postulated for the RpoS regulon in *Escherichia coli* (Hengge-Aronis 1993; Loewen and Hengge-Aronis 1994). Since *rpoS* mutants of gram-negative pathogens show significantly reduced virulence (Fang et al. 1992; Robbe-Saule et al. 1995) it may be speculated that in

pathogenic gram-positive species, the $\sigma^B$ regulon also plays a role in the ability of bacteria to interact with host defense mechanisms and persist during infection.

The alternative sigma factor $\sigma^B$ has also been found in other pathogenic gram-positive bacteria, including *Staphylococcus aureus* (Wu et al. 1996; Kullik and Giachino 1997). *S. aureus* is an important pathogen in humans. A variety of toxins, enzymes, and other extracellular or cell wall-associated proteins, which are postulated to be required for the establishment and maintenance of an infection, are produced and excreted during the late exponential or the stationary growth phase (Kornblum et al. 1990).

To address the question of whether the alternative sigma factor $\sigma^B$ is involved in the regulation of such genes, we have been interested in identifying $\sigma^B$-dependent proteins and studying their regulation under stress conditions in *S. aureus*. In this work, we have identified the alkaline shock protein Asp23 on two dimensional (2-D) gel profiles of protein extracts of *S. aureus*. The *asp23* gene is transcribed by the alternative sigma factor $\sigma^B$. Our results suggest that the regulation of $\sigma^B$-dependent stress induction may differ between the clinical isolate *S. aureus* MA13, which causes wound infections, and the reference strain *S. aureus* NCTC 8325-4.

## Materials and methods

### Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. The *E. coli* K12 strain DH5α was used for transformation and propagation of plasmids. The strain was routinely grown under vigorous agitation in LB at 37° C. *B. subtilis* strains were cultivated at 37° C in the synthetic medium described earlier (Stülke et al. 1993). *S. aureus* strains were grown in a synthetic medium with the following composition. The basic medium consisted of 10 mM $Na_2HPO_4$, 10 mM $KH_2PO_4$, 9.34 mM $NH_4Cl$, 8.55 mM NaCl, 0.81 mM $MgSO_4$, 0.142 mM citric acid, 40 mM MOPS pH 7.0, and 7.5 mM glucose. This was supplemented with amino acids (L-alanine, L-arginine, L-aspartic acid, L-cysteine, L-glutamic acid, L-glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan and L-valine, all at 160 mg/l), the vitamins 0.036 μM cyanocobalamine, 0.29 μM 4-aminobenzoic acid, 0.04 μM biotin, 0.81 μM nicotinic acid, 0.21 μM D-pantothenic acid (Ca salt), 0.62 μM pyridoxamine dihydrochloride, 0.29 μM thiamine dichloride and 0.26 μM riboflavin, and with 7.5 μM $FeCl_2$, 0.51 μM $ZnCl_2$, 0.5 μM $MnCl_2$, 0.097 μM boric acid, 1.46 μM $CoCl_2$, 0.015 μM $CuCl_2$, 0.1 μM $NiCl_2$, and 0.148 μM $Na_2MoO_4$.

Various stress conditions were imposed according to the following procedures. Cells were cultivated to an optical density of 0.5 (*B. subtilis*) or 1.0 (*S. aureus*) at 500 nm and transferred to 48° C (heat stress), or ethanol was added to a final concentration of 4% (v/v). *S. aureus* cells at an $OD_{500nm}$ of 1.0 were subjected to high pH stress by addition of NaOH to a final concentration of O.6N. The time of the shift was defined as time zero. Heat and alkaline stresses were applied for 1 h.

### Preparative 2-D gel electrophoresis and N-terminal microsequencing

Preparative 2-D gel electrophoresis and N-terminal microsequencing of proteins were carried out as described earlier (Schmid et al. 1997; Völker et al., in preparation). For microsequencing, the Coomassie-stained protein spots were cut from several 2-D gels and the gel pieces were concentrated as described (Schagger et al. 1987; Rider et al. 1995). The proteins or peptides generated by treatment with CNBr were blotted onto a polyvinylidene difluoride membrane, stained and sequenced as described (Schmid et al. 1997). Database searches were carried out using the BLAST program (Altschul et al. 1990).

### Analysis of transcription

Total RNA was isolated from exponentially growing or stressed cells of the *B. subtilis* strains BGH15 and BGH17 and of the

**Table 1** Bacterial strains and plasmids

| Strain | Genotype/features | Source/reference |
|---|---|---|
| *E. coli* | | |
| DH5α | F⁻ (ϕ80 lacZΔ*M15*) Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17* ($r_K^-$,$m_K^+$) *supE44, thi-1, gyrA69* | Sambrook et al. (1989) |
| *B. subtilis* | | |
| 168 | *trpC2* | Bacillus Genetic Stock Center |
| BSM51 | *trpC2 sigB*::Δ*Hin*dIII-*Eco*RV::*cat rsbX*::*spc* | U. Völker, Marburg |
| BGH15 | *trpC2 amyE*::*cat* (*asp23-'lacZ*) | This study |
| BGH17 | *trpC2 amyE*::*cat* (*asp23-'lacZ*) *sigB*::Δ*Hind*III-*Eco*RV::*cat* | This study |
| *S. aureus* | | |
| NCTC 8325-4 | | Novick (1967) |
| BB225 | | Berger-Bächi (1983) |
| MA13 | Clinical isolate, wound infection | Ohlsen et al. (1998) |
| MA19 | Clinical isolate, wound infection | Ohlsen et al. (1998) |
| MRSA17 | Clinical isolate, wound infection | Ohlsen et al. (1998) |
| **Plasmids** | **Description** | |
| pBluescript KS+ | Cloning vector, $Ap^r$ | Stratagene |
| pDH32M | Vector for construction of transcriptional *lacZ* fusions at the *amyE* site of *B. subtilis*, $Ap^r$, $Cm^r$ | Kraus et al. (1994) |
| pKSASP23 | pBluescriptII KS containing a 720-bp fragment of *asp23* from *S. aureus*, generated by PCR | This study |
| pDHASP23 | pDH32M containing a 720-bp fragment of *asp23* from *S. aureus*, generated by PCR | This study |

*S. aureus* strains NCTC 8325-4 and MA13 using the acid-phenol method described by Majumdar et al. (1991) with the modifications described previously (Völker et al. 1994). Instead of being treated with lysozyme, *S. aureus* cells were incubated with lysostaphin (50 μg/ml) for 10 min on ice.

For quantification of specific mRNAs, serial dilutions of total RNA were transferred onto a positively charged nylon membrane by slot blotting and hybridized with gene-specific, digoxigenin-labeled RNA probes according to the manufacturer's instructions (Boehringer Mannheim). Chemiluminescent signals were detected with a Lumi-Imager (Boehringer Mannheim) and quantified using the program LumiAnalyst (Boehringer Mannheim).

Northern blot analyses were carried out as described earlier (Wetzstein et al. 1992).

The probe for *asp23* transcripts was digoxigenin-labeled RNA synthesized in vitro from the linearized plasmid pKSASP23 using T7 RNA polymerase. This plasmid was derived from pBluescriptII KS. The plasmid pKSASP23 contains a 720-bp fragment of *asp23* amplified by PCR using chromosomal DNA of *S. aureus* NCTC 8325-4 as template. The primers ASP1 (5′-GAAGAATTCACAAGACTCTACACAACAAGTG-3′) and ASP2 (5′-CCGGGATCCATTCTTTTTGAGTCATTACATC-3′) were used for amplification. The PCR product was cut with *Eco*RI and *Bam*HI and ligated with the vector pBluescriptII KS digested with the same enzymes.

The *sigB*-specific RNA probe was prepared by in vitro transcription with T7 polymerase using a fragment of *sigB* DNA as template. The *sigB* fragment was generated by PCR using chromosomal DNA of *S. aureus* NCTC 8325-4 together with the oligonucleotide primers SASIGBF (5′-AAATAATGGCGAAAGAGTCG-3′) and SASIGBR (5′-CTAATACGACTCACTATAGGGAGACATAATGGTCATCTTGTTGC-3′). The oligonucleotide SASIGBR contains the T7 recognition sequence (Jorgensen et al. 1991) at its 5′ end.

Primer extension analysis was performed with a synthetic oligonucleotide complementary to the sequence encoding the N-terminal region of *asp23* (ASPP: 5′-ATTGTCGTATGCTTGTTTTGC-3′) and labeled at the 5′ end with [$\gamma$-$^{32}$P]ATP. For sequencing of the *asp23* promoter region the plasmid pKSASP23 was used as the template. Sequencing was performed according to the method of Sanger et al. (1977).

### Construction of the *B. subtilis* strains BGH15 and BGH17

In order to analyze the regulation of *asp23* transcription in *B. subtilis*, we constructed a *B. subtilis* strain containing the *asp23* gene inserted at the *amyE* locus. The *Eco*RI + *Bam*HI-digested 720-bp PCR-fragment of *S. aureus* NCTC 8325-4 DNA was ligated with the *Eco*RI + *Bam*HI-digested plasmid pDH32M (Kraus et al. 1994). The resulting plasmid, pDHASP23, was linearized with *Pst*I and transformed into competent *B. subtilis* 168 cells. Chloramphenicol-resistant colonies were selected on agar plates containing 5 μg/ml chloramphenicol. The insertion of *asp23* and *cat* at the *amyE* locus was veryfied by testing the amylase activity on starch plates. The resulting strain was designated BGH15.

For the construction of the corresponding *sigB* mutant strain, BGH17, chromosomal DNA of *B. subtilis* BSM51 was introduced into competent *B. subtilis* BGH15 cells. Colonies were selected on agar plates containing 5 μg/ml chloramphenicol and 150 μg/ml spectinomycin. Deletion of *sigB* was verified by PCR.

### General methods

Plasmid isolation, restriction enzyme analysis, transformation of *E. coli*, ligation of DNA fragments, and filling in of recessed 3′ termini with the Klenow fragment of DNA polymerase I were performed according to standard protocols (Sambrook et al. 1989). Transformation of naturally competent *B. subtilis* cells was carried out as described by Hoch (1991).

PCR was carried out with chromosomal DNA purified with the Wizard genomic DNA purification kit, according to the manufacturer's (Promega) protocol. For detection of the 11-bp deletion in *rsbU*, the primers IK43M (5′-TATCTACCAATCTTTGATAATC-3′) and IK52M (5′-GCTCTAGAGTTCAAGACATTAG-3′) were used (Kullik and Giachino 1997).

## Results

### Identification of Asp23 on 2-D protein gels and regulation of its synthesis

Protein extracts of two different *S. aureus* strains before and after heat stress were fractionated on 2-D protein gels, protein spots were then cut from the gel and N-terminal sequences were determined. This procedure resulted in the identification of the alkaline shock protein Asp23 (N-terminal sequence: SLINKEILPFTA; Kuroda et al. 1995) as one of the most abundant proteins in extracts of *S. aureus* MA13 (Fig. 1). The level of this protein seems to increase in response to alkaline stress (Fig. 1). Surprisingly, Asp23 was not found in unstressed *S. aureus* NCTC 8325-4 cells and was not induced by alkaline stress in this strain (Fig. 1).

To verify the induction pattern of Asp23, the levels of *asp23* mRNA were determined before and after the exposure to various stresses. The specific mRNA was detected by slot-blot analysis of total RNA prepared either from exponentially growing cells or from bacteria harvested at different times after imposition of stress conditions. In agreement with the results obtained by 2-D gel analysis, we found *asp23* to be highly expressed during exponential growth in *S. aureus* MA13 and only weakly expressed in *S. aureus* NCTC 8325-4 (Fig. 2). Almost no induction was observed after heat (Fig. 2) or ethanol stress (not shown) in either strain. In Northern blot experiments, two *asp23* transcripts (1.5 kb and 0.7 kb) could be detected, as described previously by Kuroda et al. (1995). There was no change in the level of either transcript after exposure of the cells to heat or ethanol stress (data not shown).

Primer extension experiments to map the *asp23* promoter were carried out with RNA from *S. aureus* MA13 and *S. aureus* NCTC 8325-4 before and 6 min after initiation of heat and ethanol stress. A putative 5′ end was mapped 103 bp upstream from the translational start site, in agreement with the data of Kuroda et al. (1995). The primer extension studies confirmed the finding that *asp23* is only weakly transcribed in *S. aureus* NCTC 8325-4 (Fig. 3A). The 5′ end is preceded by the sequence 5′-GTTTAA-$N_{14}$-GGGTAT-$N_{10}$-3′, which is similar to the consensus for $\sigma^B$-dependent promoters known in *B. subtilis* (Fig. 3C). Therefore, we assumed that *asp23* is transcribed by the $\sigma^B$-containing holoenzyme of RNA polymerase.

To verify the dependence of *asp23* transcription on $\sigma^B$, we analyzed the expression of the *S. aureus asp23* gene in *B. subtilis* (for strain construction see Materials and methods). Primer extension experiments showed that in *B. subtilis* BGH15 transcription initiated from the same position as in *S. aureus* (Fig. 3B). Transcription

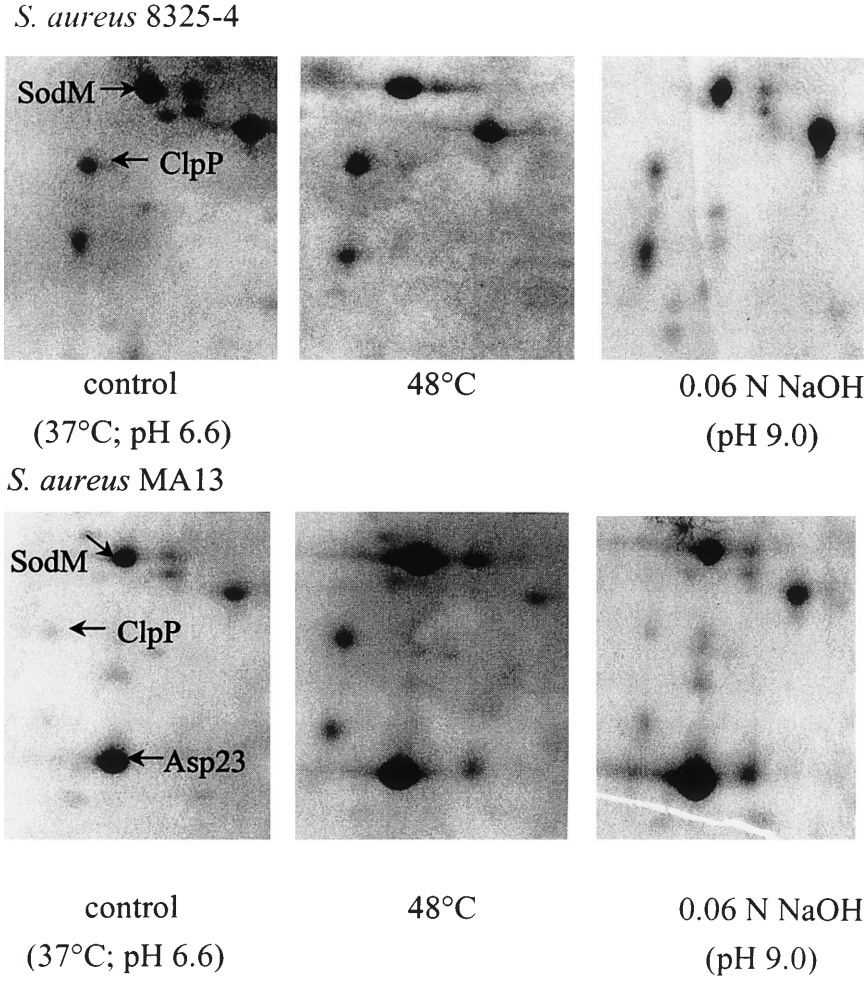


**Fig. 1** Sectors of 2-D gels including the region where Asp23 (pI = 5.13, MW = 19.19 kDa) is located. Protein extracts of the *S. aureus* strains MA13 and 8325-4 grown in the synthetic medium, prepared before (control) and after the imposition of different stress conditions (exposure to 48° C or 0.6 N NaOH for 1 h) were fractionated. The proteins were stained with Coomassie-Blue. Note that Asp23 is not detectable in *S. aureus* 8325-4

from this site was strongly increased in response to heat and ethanol stress in the wild type, but not in the *sigB* mutant BGH17. This observation confirms the suggestion that *asp23* expression is actually dependent on the alternative stress sigma factor $\sigma^B$.

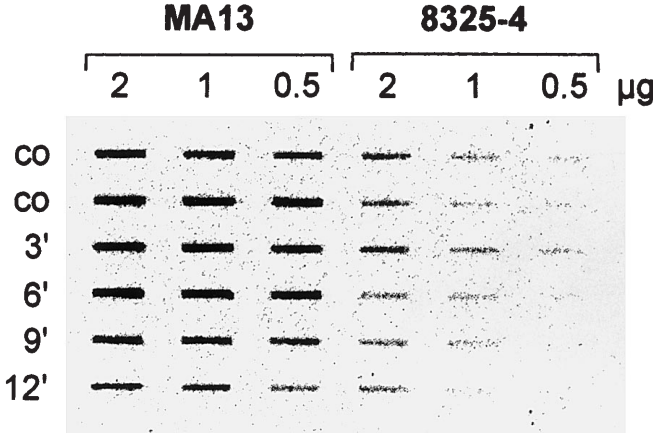


**Fig. 2** Transcriptional analysis of *asp23* in *S. aureus*. RNA was isolated from the *S. aureus* strains 8325-4 and MA13 grown in the synthetic medium. The indicated amounts of total RNA isolated before (co) and at different times after exposure to heat stress (48° C) (3, 6, 9, and 12 min) were blotted onto a positively charged nylon membrane and hybridized with a digoxygenin-labeled RNA probe specific for *asp23*

## Transcriptional analysis of the *sigB* operon in various *S. aureus* strains

The presence of different $\sigma^B$ activities in *S. aureus* MA13 and the reference strain *S. aureus* NCTC 8325-4 might be responsible for the differences observed in *asp23* expression. Therefore, we analyzed the regulation of *sigB* itself in both strains. By slot-blot analysis we found an extremely low amount of *sigB* mRNA in *S. aureus* NCTC 8325-4, which increased only slightly under heat shock conditions (data not shown). In *S. aureus* MA13, however, *sigB* is highly expressed. As was shown previously, the *sigB* operon consists of four genes, *rsbU*, *rsbV*, *rsbW* and *sigB*, preceded by a $\sigma^A$-like promoter (Wu et al. 1996; Kullik and Giachino 1997). An internal $\sigma^B$-like promoter is postulated to be located upstream of *rsbV*, suggesting a rather complex mode of regulation (Wu et al. 1996; Kullik and Giachino 1997). In order to analyze transcriptional regulation, Northern experiments were carried out using RNA from exponentially growing and heat-stressed cells of both *S. aureus* strains

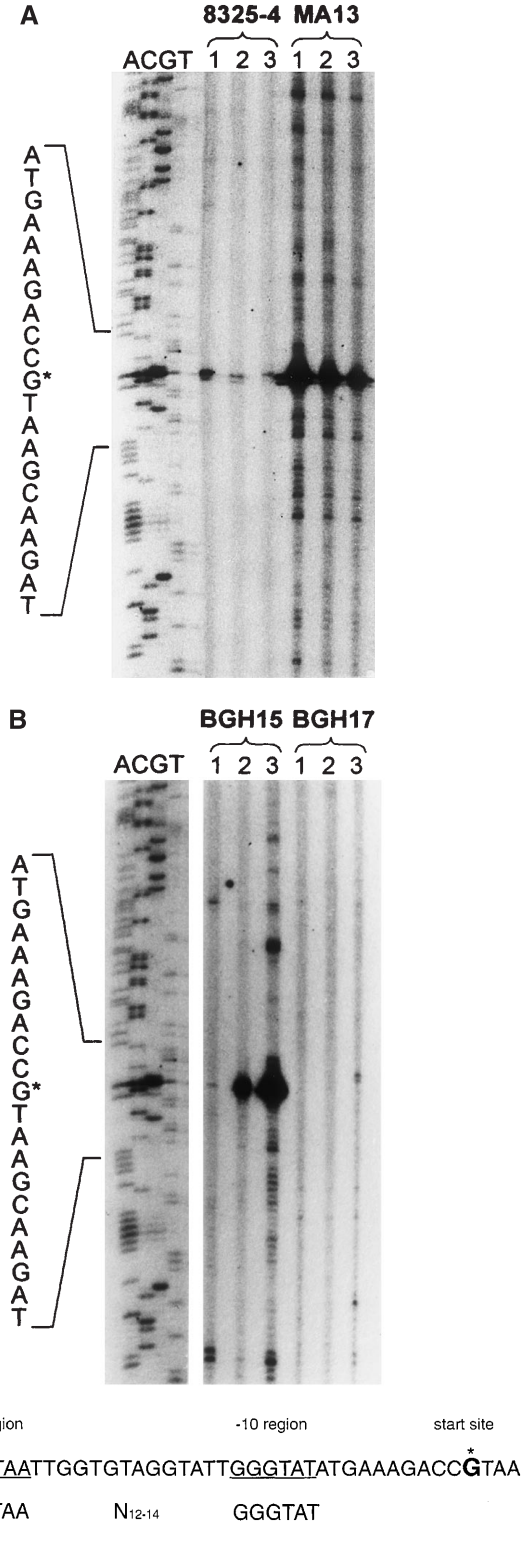


**Fig. 3** **A** Mapping of the 5′ end of *asp23* mRNA by primer extension analysis. RNA was isolated from *S. aureus* 8325-4 and MA13 before (lane 1) and 9 min after heat stress (lane 2) or ethanol stress (lane 3). Equal amounts of total RNA (10 µg) were used. The transcription start site is marked with an *asterisk*. Lanes A, C, G, and T show the dideoxy sequencing ladder obtained with the equivalent primer. **B** Mapping of the 5′ end of *asp23* mRNA by primer extension analysis. RNA was isolated from *B. subtilis* BGH15 and BGH17 before (lane 1) and 9 min after heat stress (lane 2) or ethanol stress (lane 3). Equal amounts of total RNA (10 µg) were used for the analysis. **C** Nucleotide sequence around the promoter region of *asp23*. The potential −10 and −35 regions are *underlined*. The consensus sequence for the −10 and −35 regions of $\sigma^B$-dependent promoters in *B. subtilis* are shown *below*

(Fig. 4A). The major transcript (1.6 kb), which is initiated at the $\sigma^B$-dependent promoter in front of *rsbV*, was found in *S. aureus* MA13 but not in *S. aureus* NCTC 8325-4. However, there was no heat induction at this promoter. In addition to this transcript, two minor transcripts were detected in both strains. The larger one (3.6 kb) was obtained under conditions of heat shock (Fig. 4A and 4B). Using an RNA probe specific for *orf1* (the gene upstream of *rsbU*) a transcript of the same size was detected (data not shown). Since there is no obvious terminator structure between *orf1* and *rsbU*, we assume that this minor band corresponds to an *orf1-rsbU-rsbV-rsbW-sigB* transcript. The minor 2.5 kb transcript might originate from a point directly upstream of *rsbU* (Fig. 4A and 4B).

Apparently, there is no $\sigma^B$-dependent stress response in *S. aureus* cultivated in synthetic medium. Because $\sigma^B$-dependent induction of *sigB* by heat stress in complex medium has been described previously (Kullik and Giachino 1997), we looked for *sigB*-specific transcripts in cells grown in LB before and after heat stress. The amount of the 1.6-kb transcript starting in front of *rsbV* was strongly increased after heat shock in *S. aureus* MA13 grown in LB medium (Fig. 5A). The transcript could not be detected in *S. aureus* NCTC 8325-4, regardless of whether it was grown in synthetic or complex medium (Fig. 5B). By comparing the $\sigma^B$-dependent expression of *sigB* in the two media in *S. aureus* MA13, we found the 1.6-kb transcript already to be induced in exponential-phase cells growing in synthetic medium (Fig. 5).

## Characterization of *rsbU* in the different *S. aureus* strains

Our results indicate that there are drastic differences in $\sigma^B$ activity between the two *S. aureus* strains used in this study. This might be due to differences in the cascade that activates this sigma factor. Since 11 bp of the *rsbU* sequence are deleted in *S. aureus* BB255 (Kullik and Giachino 1997), which is isogenic to *S. aureus* NCTC 8325, we looked by PCR for this deletion in *rsbU* in *S. aureus* NCTC 8325-4 and in *S. aureus* MA13, as well as in two other clinical isolates (MA19 and MRSA17). Using oligonucleotides that flank the deletion site in *S. aureus* BB255 we obtained a 77-bp fragment from strain NCTC 8325-4 and an 88-bp fragments from the clinical isolates MA13, MA19 and MRSA17 (Fig. 6). This deletion in *S. aureus* NCTC 8325 and in all derivatives of this strain (BB255, NCTC 8325-4) might prevent the synthesis of the RsbV phosphatase, RsbU,

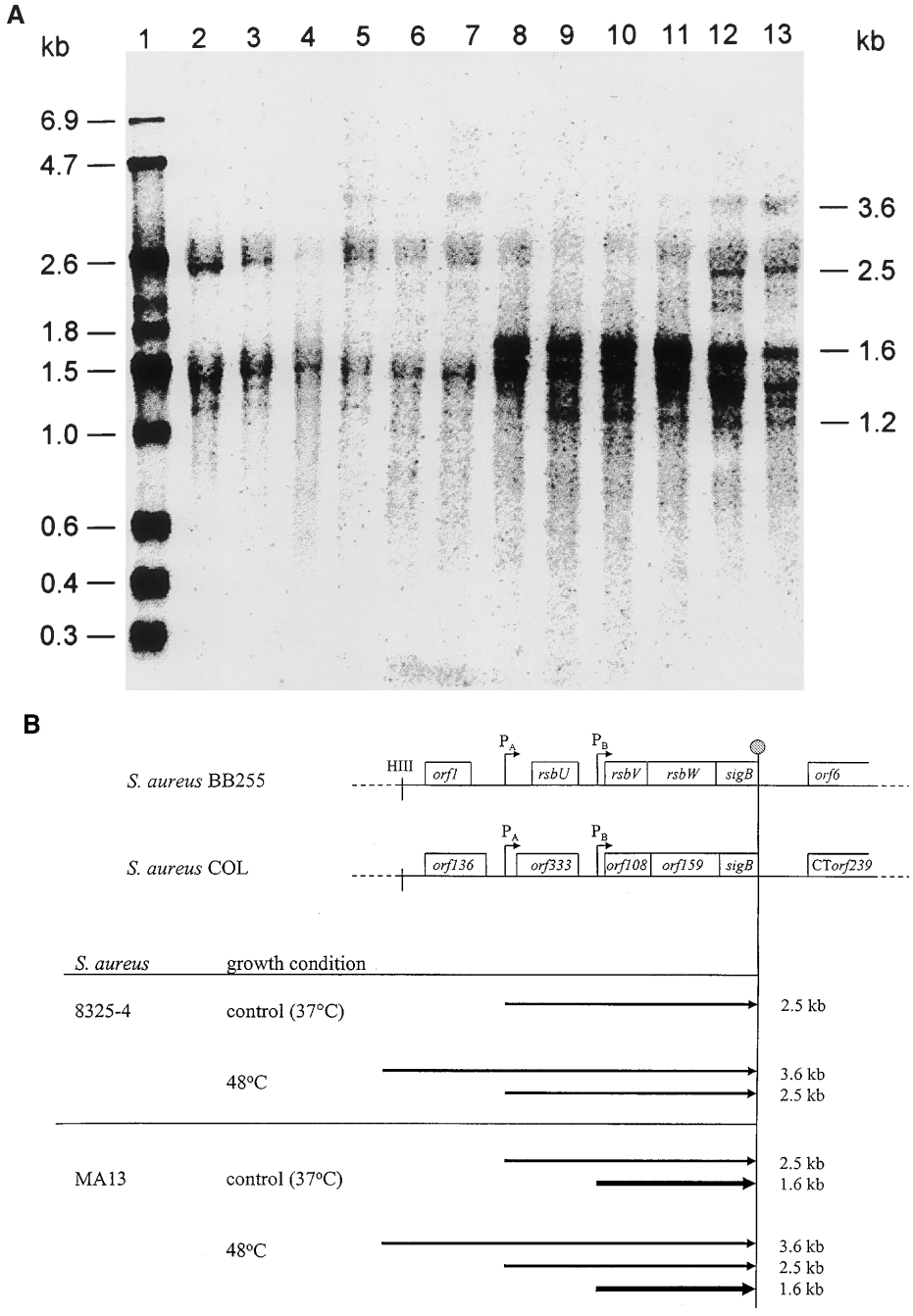


**Fig. 4** **A** Northern analysis of *sigB* induction by heat shock. RNA was isolated from *S. aureus* 8325-4 (lanes 2–7) and *S. aureus* MA13 (lanes 8–13) grown at 37° C (lanes 2, 3, 8 and 9), and 3, 6, 9, and 12 min after shifting to 48° C (lanes 4–7 and 10–13). The membrane was hybridized with a digoxygenin-labeled *sigB* RNA probe. Relevant transcripts are indicated. The 1.5-kb and 1.2-kb bands detected may represent degradation products. Lane 1 contains the molecular weight standard. **B** Schematic representation of the *sigB* operon based on published sequences of *S. aureus* BB255 (Kullik and Giachino 1997) and *S. aureus* Col (Wu et al. 1996). The transcripts detected by Northern analysis are indicated

leading to permanent binding of the anti-sigma factor RsbW to $\sigma^B$ and consequently to drastically reduced expression of $\sigma^B$-dependent genes in *S. aureus* NCTC 8325-4.

## Discussion

Studies on the mechanisms of pathogenicity in *S. aureus* have become a center of attention during the last few years because strains that show high levels of resistance to many antibiotics are widespread. Information regarding the mechanisms by which pathogenic bacteria cause disease is a prerequisite for finding new molecular targets associated with infection. *S. aureus* is a major human pathogen, causing infections which range from minor superficial skin abscesses to life-threatening diseases such as toxic shock syndrome, sepsis, and endocarditis (Iandolo 1990). The ability to survive sub-

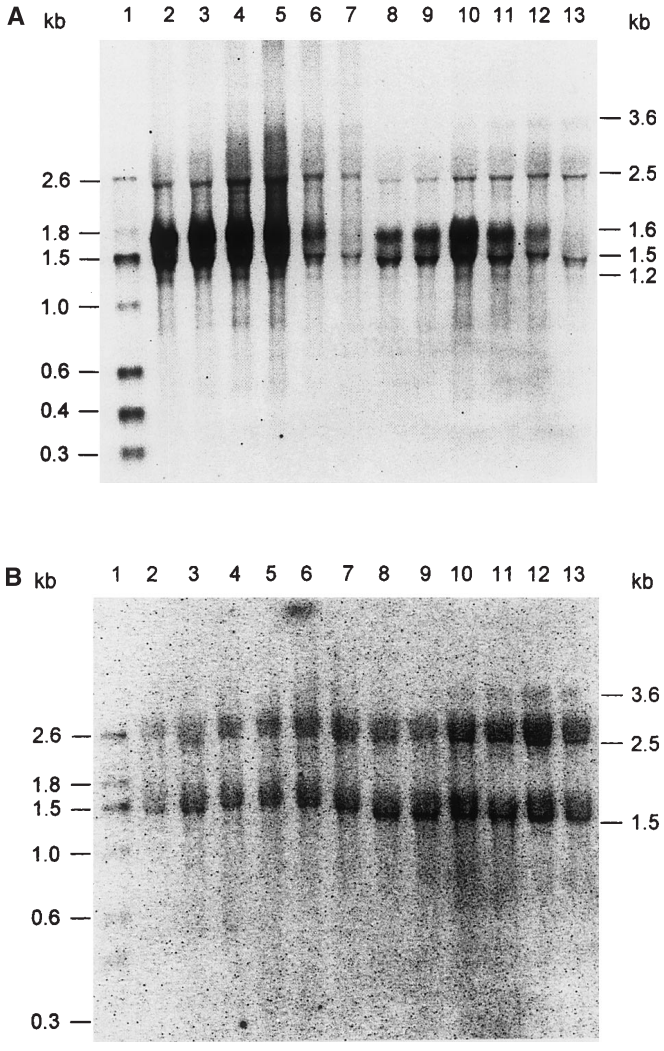


**Fig. 5A, B** Northern analysis of *sigB* expression in complex and synthetic media. RNA was isolated from *S. aureus* MA13 (**A**) and *S. aureus* 8325-4 (**B**). Cells were grown in synthetic medium (lanes 2–7) or in complex medium (lanes 8–13). The RNA samples were isolated during exponential growth (37° C) immediately prior to (lanes 2, 3, 8 and 9) or 3, 6, 9, and 12 min after a shift to 48° C (lanes 4–7, and 10–13). The membrane was hybridized with a digoxygenin-labeled *sigB* RNA probe. Relevant transcripts are indicated. Lane 1 contains the molecular weight standard

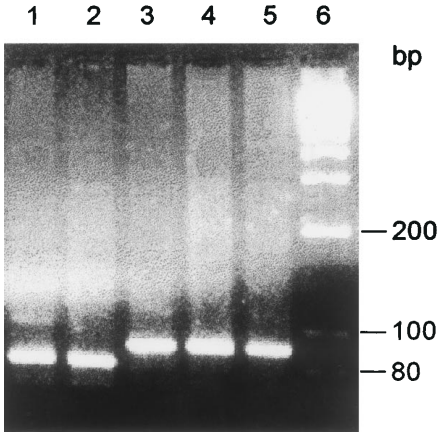


**Fig. 6** Detection of the 11-bp deletion in *rsbU* by PCR, using oligonucleotides complementary to sequences flanking the deletion in *rsbU* in *S. aureus* BB255 (Kullik and Giachino 1997). The following *S. aureus* strains: BB255 (lane 1), 8325-4 (lane 2), MA13 (lane 3), MA19 (lane 4) and MRSA 17 (lane 5) were tested. The resulting PCR products were separated on a 4% agarose gel. Lane 6 contains the molecular weight standard

optimal growth conditions in the host without any loss of viability represents a property which contributes significantly to the virulence of this organism and may be closely connected with the expression of stress proteins.

In *B. subtilis*, $\sigma^B$ is a global regulator of a very large general stress regulon (Boylan et al. 1993; see Haldenwang 1995 for review; Bernhardt et al. 1997). This alternative sigma factor also exists in *S. aureus* (Wu et al. 1996; Kullik and Giachino 1997); however, only the *sigB* operon (*rsbV*, *rsbW*, and *sigB*) and the *sar* gene have so far been shown to be regulated by $\sigma^B$ in this species (Wu et al. 1996; Deora et al. 1997; Kullik and Giachino 1997, Manna et al. 1998).

The N-terminal sequence of a protein that is synthesized at a strikingly high level in exponentially growing cells of *S. aureus* MA13 was identified as Asp23 (Kuroda et al. 1995). Upstream of the *asp23* gene, a promoter sequence that is very similar to $\sigma^B$-dependent promoters in *B. subtilis* was identified and was shown to be recognized by $E\sigma^B$. Very recently Kullik et al. (1998) have demonstrated that *asp23* expression is affected by deletion of *sigB* in *S. aureus*.

With respect to the expression of $\sigma^B$-dependent genes in *B. subtilis*, it was very surprising to find that *S. aureus* MA13 exhibits almost no increase in transcription of *asp23* after heat or ethanol stress. Therefore, we analyzed the regulation of *sigB* expression itself. We found a high *sigB* mRNA level even in slowly growing cells, which was not increased after heat stress. These experiments were done with slowly growing cells in a synthetic medium ($\mu = 0.5$). In contrast, in cells grown in complex medium ($\mu = 2.4$) the expression level was much lower but could, however, be enhanced by heat shock. These results indicate that high levels of $\sigma^B$ activity are present during slow growth in the synthetic medium used in this study.

In *S. aureus* NCTC 8325-4 $\sigma^B$-dependent expression levels of *asp23* and *sigB* seemed to be extremely low relative to those in *S. aureus* MA13, indicating a strong defect in $\sigma^B$ activity in the strain NCTC 8325-4. In *B. subtilis*, the RsbU phosphatase controls the $\sigma^B$ activity in response to physical stress (Völker et al. 1995a, 1995b; Kang et al. 1996). A 11-bp deletion in the *rsbU* gene present in *S. aureus* NCTC 8325-4 and all other derivatives of *S. aureus* NCTC 8325 tested so far (Kullik et al. 1998) could be responsible for the low $\sigma^B$ activity. The deletion is located immediately downstream of the translational start codon and probably prevents the translation of RsbU phosphatase, causing permanent binding of the anti-sigma factor RsbW to $\sigma^B$.

The mechanism of heat induction of $\sigma^B$ in complex medium in the strain MA13 has to be elucidated. The "upstream module" of the $\sigma^B$ activation pathway (RsbS, RsbT, and RsbX) responsible for heat induction in *B. subtilis* (Kang et al. 1996; Yang et al. 1996; Akbar et al. 1997) has not yet been detected in *S. aureus* (Wu et al. 1996; Kullik and Giachino 1997). Before we can understand the heat induction mechanism of $\sigma^B$-dependent proteins it will be necessary to ascertain whether the upstream module actually exists in *S. aureus*.

From the physiological point of view the high activity of $\sigma^B$ in slowly growing cells, in the absence of stress or starvation, is a remarkable finding. It is tempting to speculate that the environmental conditions encountered outside and within the host trigger a constitutively high level of expression of the genes belonging to the $\sigma^B$ regulon. The reason for this high $\sigma^B$ activity during growth in synthetic medium is currently under investigation.

Development of a physiological state in which *S. aureus* cells can survive long periods of starvation is known to be induced under conditions of limitation of glucose or many other nutrients; depletion of phosphate or amino acid limitation, however, leads to a rapid loss of viability (Watson et al. 1998). There are indications that the *sigB* regulon of *S. aureus* is involved in making the starving cell resistant to oxidative stress (Chan et al. 1998; Kullik et al. 1998) but is not implicated in resistance to starvation itself (Chan et al. 1998). Data on the role of $\sigma^B$ in the pathogenicity of *S. aureus* and *Listeria monocytogenes* are still contradictory (Becker et al. 1998; Chan et al. 1998; Kullik et al. 1998; Wiedmann et al. 1998). Based on our knowledge of the function of the $\sigma^B$-regulon in *B. subtilis* (Engelmann and Hecker 1996; Antelmann et al. 1998; Gaidenko and Price 1998), it is very likely that $\sigma^B$-dependent proteins are also involved in the development of multiple stress resistance in starving *S. aureus* cells (Chan et al. 1998).

**Acknowledgements** S. Gertz and S. Engelmann contributed equally to this work. The authors are indebted to Eva Ng and Ute Hentschel for critical reading the manuscript and helpful comments. We are very grateful to Uwe Völker for providing the *B. subtilis* strain BSM51 and Reinhold Brückner for helpful discussion. Furthermore, we thank Renate Gloger for excellent technical assistance, Gertrud Schittek and Sabine Schade for preparing the photographs. This work was supported by grants from the Fonds der Chemischen Industrie to M. H.

## References

Akbar S, Kang CM, Gaidenko TA, Price CW (1997) Modulator protein RsbR regulates environmental signalling in the general stress pathway of *Bacillus subtilis*. Mol Microbiol 24:567–578

Altschul SF, Lipman DJ (1990) Protein database searches for multiple alignments. Proc Natl Acad Sci USA 875509–5513

Antelmann H, Engelmann S, Schmid R, Sorokin A, Lapidus A, Hecker M (1998) A stress and starvation-induced *dps/pexB* homologous gene is controlled by the alternative sigma factor $\sigma^B$ in *Bacillus subtilis*. J Bacteriol 179:7251–7256

Becker LA, Cetin MS, Hutkins RW, Benson AK (1998) Identification of the gene encoding the alternative sigma factor sigmaB from *Listeria monocytogenes* and its role in osmotolerance. J Bacteriol 180:4547–4554

Berger-Bächi B (1983) Insertional inactivation of staphylococcal methicillin resistance by Tn*551*. J Bacteriol 154:479–487

Bernhardt J, Völker U, Völker A, Antelmann H, Schmid R, Mach H, Hecker M (1997) Specific and general stress proteins in *Bacillus subtilis* – a two-dimensional protein electrophoresis study. Microbiology 143:999–1017

Boylan S, Redfield A, Price CW (1993) Transcription factor $\sigma^B$ of *Bacillus subtilis* controls a large stationary-phase regulon. J Bacteriol 175:3957–3963

Chan PF, Foster SJ, Ingham E, Clements MO (1998) The *Staphylococcus aureus* alternative sigma factor sigmaB controls the environmental stress response but not starvation survival or pathogenicity in a mouse abscess model. J Bacteriol 180:6082–6089.

Deora R, Tseng T, Misra TK (1997) Alternative transcription factor sigmaSB of *Staphylococcus aureus*: characterization and role in transcription of the global regulatory locus *sar*. J Bacteriol 179:6355–6359

Dufour A, Haldenwang WG (1994) Interactions between a *Bacillus subtilis* anti-sigma factor (RsbW) and its antagonist (RsbV). J Bacteriol 176:1813–1820

Engelmann S, Hecker M (1996) Impaired oxidative stress resistance of *Bacillus subtilis sigB* mutants and the role of *katA* and *katE*. FEMS Microbiol Lett 145:63–69

Fang FC, Libby SJ, Buchmeier NA, Loewen PC, Switala J, Harwood J, Guiney DG (1992) The alternative sigma factor KatF (RpoS) regulates *Salmonella* virulence. Proc Natl Acad Sci USA 89:11978–11982

Gaidenko TA, Price CW (1998) General stress transcription factor sigmaB and sporulation transcription factor sigmaH each contribute to survival of *Bacillus subtilis* under extreme growth conditions. J Bacteriol 180:3730–3733

Haldenwang WG (1995) The sigma factors of *Bacillus subtilis*. Microbiol Rev 59:1–30

Hecker M, Völker U (1998) Non-specific, general and multiple stress resistance of growth-restricted *Bacillus subtilis* cells by the expression of the $\sigma^B$ regulon. Mol Microbiol 29:1129–1137

Hecker M, Schumann W, Völker U (1996) Heat-shock and general stress response in *Bacillus subtilis*. Mol Microbiol 19:417–428

Hengge-Aronis R (1993) Survival of hunger and stress - The role of *rpoS* in early stationary phase gene regulation in *Escherichia coli*. Cell 72:165–168

Hoch JA (1991) Genetic analysis in *Bacillus subtilis*. Methods Enzymol 204:305–320

Iandolo JJ (1990) The genetics of staphylococcal toxins and virulence factors. In: Iglewski BH, Clark VL (eds) Molecular basis of bacterial pathogenesis. Academic Press, New York, pp 399–426

Jorgensen ED, Durbin RK, Risman SS, McAllister WT (1991) Specific contacts between the bacteriophage T3, T7, and SP6 RNA polymerases and their promoters. J Biol Chem 266:645–651

Kang CM, Brody MS, Akbar S, Yang X, Price CW (1996) Homologous pairs of regulatory proteins control activity of *Bacillus subtilis* transcription factor $\sigma^B$ in response to environmental stress. J Bacteriol 178:3846–3853

Kornblum J, Kreiswirth BN, Projan SJ, Ross H, Novick RP (1990) *agr*: a polycistronic locus regulating exoprotein synthesis in *Staphylococcus aureus*. In: Novick RP (ed) Molecular biology of staphylococci. VCH, New York, pp 373–402

Kraus A, Hueck C, Gärtner D, Hillen W (1994) Catabolite repression of the *Bacillus subtilis xyl* operon involves a *cis* element functional in the context of an unrelated sequence, and glucose exerts additional *xylR*-dependent repression. J Bacteriol 176:1738–1745

Kullik I, Giachino P (1997) The alternative sigma factor sigmaB in *Staphylococcus aureus*: regulation of the *sigB* operon in response to growth phase and heat shock. Arch Microbiol 167:151–159

Kullik I, Giachino P, Fuchs T (1998) Deletion of the alternative sigma factor $\sigma^B$ in *Staphylococcus aureus* reveals its function as a global regulator of virulence genes. J Bacteriol 180:4814–4820

Kuroda M, Ohta T, Hayashi H (1995) Isolation and the gene cloning of an alkaline shock protein in methicillin resistant *Staphylococcus aureus*. Biochem Biophys Res Commun 207:978–984

Loewen PC, Hengge-Aronis R (1994) The role of the sigma factor $\sigma^S$ (KatF) in bacterial global regulation. Annu Rev Microbiol 48:53–80

Majumdar D, Avissar YJ, Wyche JH (1991) Simultaneous and rapid isolation of bacterial and eukaryotic DNA and RNA – a new approach for isolating DNA. Biotechniques 11:94–101

Manna AC, Bayer MG, Cheung AL (1998) Transcriptional analysis of different promoters in the sar locus in *Staphylococcus aureus*. J Bacteriol 180:3828–3836

Novick R (1967) Properties of a cryptic high-frequency transducing phage in *Staphylococcus aureus*. Virology 33:155–166

Ohlsen K, Ziebuhr W, Koller K-P, Hell W, Wichelhaus TA, Hacker J (1998) Effects of subinhibitory concentrations of antibiotics on alpha-toxin (*hla*) gene expression of methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* isolates. Antimicrob Agents Chemother 42:2817–2823

Rider MH, Puype M, Van Damme J, Gevaert K, De Boeck S, D'Alayer J, Rasmussen HH, Celis JE, Vanderkerchove J (1995) An agarose-based gel-concentration system for microsequence and mass spectrometric characterization of proteins previously purified in polyacrylamide gels starting at low picomole levels. Eur J Biochem 230:258–265

Robbe-Saule V, Coynault C, Norel F (1995) The live oral typhoid vaccine Ty21a is a *rpoS* mutant and is susceptible to various environmental stresses. FEMS Microbiol Lett 126:171–176

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Habor Labority, Cold Spring Harbor, N.Y.

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain termination inhibitors. Proc Nat Acad Sci USA 74:5463–5467

Schagger H, von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 166:368–379

Schmid R, Bernhardt J, Antelmann H, Völker A, Mach H, Völker U, Hecker M (1997) Identification of vegetative proteins for a two-dimensional protein index of *Bacillus subtilis*. Microbiology 143:991–998

Stülke J, Hanschke R, Hecker M (1993) Temporal activation of $\beta$-glucanase synthesis in *Bacillus subtilis* is mediated by the GTP-pool. J Gen Microbiol 39:2041–2045

Völker U, Engelmann S, Maul B, Riethdorf S, Völker A, Schmid R, Mach H, and Hecker M (1994) Analysis of the induction of general stress proteins of *Bacillus subtilis*. Microbiology 140:741–752

Völker U, Dufour A, Haldenwang WG (1995a) The *Bacillus subtilis rsbU* gene product is necessary for RsbX-dependent regulation of $\sigma^B$. J Bacteriol 177:114–122

Völker U, Völker A, Maul B, Hecker M, Dufour A, Haldenwang WG (1995b) Separate mechanisms activate $\sigma^B$ of *Bacillus subtilis* in response to environmental and metabolic stresses. J Bacteriol 177:3771–3780

Völker U, Völker A, Haldenwang WG (1996). Reactivation of the *Bacillus subtilis* anti-$\sigma^B$ antagonist, RsbV, by stress- or starvation-induced phosphatase activity. J Bacteriol 178:5456–5463

Völker U, Luo T, Smirnova N, Haldenwang WG (1997). Stress activation of *Bacillus subtilis* $\sigma^B$ can occur in the absence of the negative regulator RsbX. J Bacteriol 179:1980–1984

Watson SP, Clements MO, Foster SJ (1998) Characterization of the starvation-survival response of *Staphylococcus aureus*. J Bacteriol 180:1750–1758

Wetzstein M, Völker U, Dedio J, Löbau S, Zuber U, Schiesswohl M, Herget C, Hecker M, Schumann W (1992) Cloning, sequencing and molecular analysis of the *dnaK* locus from *Bacillus subtilis*. J Bacteriol 174:3300–3310

Wiedmann M, Arvik TJ, Hurley RJ, Boor KJ (1998) General stress transcription factor sigmaB and its role in acid tolerance and virulence of *Listeria monocytogenes*. J Bacteriol 180:3650–3656

Wu S, de Lencastre H, Tomasz A (1996) A Sigma-B, a putative operon encoding alternate sigma factor of *Staphylococcus aureus* RNA polymerase: molecular cloning and DNA sequencing. J Bacteriol 178:6036–6042

Yang X, Kang CM, Brody MS, Price CW (1996) Opposing pairs of serine proteine kinase and phosphatases transmit signals of environmental stress to activate a bacterial transcription factor. Genes Dev 10:2265–2275