Identification and transcriptional analysis of new members of the $\sigma^B$ regulon in *Bacillus subtilis*

Anja Petersohn, Haike Antelmann, Ulf Gerth and Michael Hecker

Author for correspondence: Michael Hecker. Tel: +49 3834 864201. Fax: +49 3834 864202. e-mail: hecker@microbio7.biologie.uni-greifswald.de

*Bacillus subtilis* responds to various stimuli (heat, ethanol and salt stress, energy starvation) with the induction of general stress proteins (GSPs). Most of them belong to the stress and stationary-phase regulon controlled by the alternative sigma factor $\sigma^B$. The majority of $\sigma^B$-dependent proteins are thought to provide a precautionary general stress resistance in stressed or starved cells. In this report, the identification and transcriptional analysis of nine new members of the $\sigma^B$ regulon are described. The biochemical function was not determined for any of the proteins encoded by the nine new $\sigma^B$-dependent stress genes, however, similarities to proteins in the databases allowed a distinction between proteins with putative (i-iv) and unknown (v) function. The putative functions of BmrU, YcdF, YdaD, YdaP, YhdN and YocK underline the suggested protective role of $\sigma^B$-dependent GSPs and also elucidate new areas where $\sigma^B$ might play an important role. (i) The finding that the *bmrUR* operon is under $\sigma^B$ control indicates that the elimination of multidrug compounds might be a new function in multiple stress resistance. (ii) YcdF and YdaD resemble NAD(P)-dependent dehydrogenases. Both proteins could be involved in the generation of NAD(P)H and therefore in the maintenance of the intracellular redox balance under stress. (iii) The *ydaP* gene might belong to the increasing number of $\sigma^B$-dependent genes whose orthologues are under the control of $\sigma^B$ in *Escherichia coli*, indicating that both regulons may fulfil similar functions. (iv) YhdN shows weak similarities to potassium ion channel proteins and YocK shows resemblance to the DnaK suppressor protein DksA. (v) Three new $\sigma^B$-dependent genes (*ydaE*, *ydaG* and *yfkM*) encoding proteins with still unknown functions were also described. Further analyses of corresponding mutants might allow a first prediction of their function within the framework of the general stress regulon.

**Keywords:** *B. subtilis*, new $\sigma^B$-dependent genes

**INTRODUCTION**

As revealed by two-dimensional (2-D) PAGE analyses, more than 60 general stress proteins (GSPs) are induced after heat shock, ethanol and salt stress or after glucose starvation in *Bacillus subtilis* (Antelmann et al., 1997a; Bernhardt et al., 1997). Many genes encoding GSPs are controlled by the alternative stress and stationary-phase sigma factor $\sigma^H$ (Haldenwang & Losick, 1980; Boylan et al., 1993a; Haldenwang, 1995; Hecker et al., 1996).

**Abbreviations:** 2-D, two-dimensional; GSP, general stress protein.
supported by the finding that the orthologues of the σB-dependent genes katE, dps, opuE and osmC (Engelmann et al., 1995; Antelmann et al., 1997b; von Blohn et al., 1998) were under the control of the stress and starvation sigma factor σB in E. coli (Almiron et al., 1992; Altuvia et al., 1994; Loewen & Hengge-Aronis, 1994; Mellies et al., 1995; Schellhorn, 1995; Gordia & Gutierrez, 1996).

One essential function of the σB-dependent general stress response is to provide resistance against oxidative stress in stationary-phase cells of B. subtilis. This requires the presence of the non-specific DNA-binding and -protecting protein Dps in B. subtilis (Antelmann et al., 1997b). A dps deletion mutant fails to develop starvation-induced non-specific resistance to lethal concentrations of hydrogen peroxide (Antelmann et al., 1997b). Other products of σB-dependent genes such as KatE and TrxA, as well as SmS and YacK encoded by the fifth and sixth genes of the clpC operon, may also be involved in the protection from oxidative damage of membranes, proteins or DNA (Engelmann et al., 1995; Krüger et al., 1997; Scharf et al., 1998). σB-dependent proteins also seem to participate in the adaptation to acid, alkaline, osmotic and heat stress, including the renaturation or degradation of aberrant proteins (Krüger et al., 1994; Gaidenko & Price, 1998; Gerth et al., 1998; Spiegelhalter & Bremer, 1998).

In summary, the products of σB-dependent general stress proteins are expected to provide a wide ranging and prospective stress resistance to non-growing B. subtilis cells in anticipation of future stress (for a review see Hecker & Völker, 1998). To give additional evidence for these proposed functions and to understand the physiological role of the entire σB regulon, new σB-dependent genes were identified and their expression was studied.

**METHODS**

**Bacterial strains and culture conditions.** The bacterial strains used in this study were B. subtilis 168 (trpC2; Anagnostopoulos & Spizizen, 1961); ML6 (trpC2 sigB::xΔHindIII-EcoRV::cat; Igo et al., 1987) and E. coli DH5α (Hanahan, 1983). DH5α was routinely grown in a complex medium and used as the host for transformation and propagation of plasmids. B. subtilis strains were cultivated under vigorous agitation at 37 °C in a synthetic medium containing 15 mM (NH₄)₂SO₄, 8 mM MgSO₄, 7H₂O, 27 mM KCl, 7 mM sodium citrate, 2H₂O, 50 mM Tris/HCl pH 7.5, supplemented with 0.6 mM KH₂PO₄, 2 mM CaCl₂, 2 H₂O,

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<td>Cloning vector; Ap'</td>
<td>Stratagene</td>
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**Primers**

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<td>ydaP-PE</td>
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<td>yocK-reverse</td>
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* The restriction sites of the restriction enzymes BamHI (5' GGATCC 3') and HindIII (5' AAGCTT 3') are underlined.
Expression of six new genes described here is shown to no longer be induced in Bacillus subtilis upon starvation for glucose (Völker et al., 1994; Antelmann et al., 1997a; Bernhardt et al., 1997). For most of these new genes we first confirmed their σ^B^-dependence by Northern analyses, then found consensus σ^B^-recognition sequences preceding them. These results strongly suggest that the new genes are directly transcribed by σ^B^, containing RNA polymerase in vivo. For a subset of these genes (ydaDE and ydaG), we further demonstrated by primer extension experiments that the 5' end of their messages lay 9–10 nt downstream of the proposed σ^B^-recognition sequences. The σ^B^-dependency of bmrU and ydaF, which show typical σ^B^-promoters, was also confirmed by Northern analyses as well as primer-extension experiments. Lastly, for two of the new genes (ycdF and yfkM), we conducted no additional experimental work beyond the 2-D gel analysis, but we did note typical σ^B^-recognition sequences preceding their coding regions. Genetic and biochemical analyses of the function of these newly described GSPs were beyond the scope of this study. Therefore, physiological functions were not defined for any of the nine new proteins. However, similarities to proteins in the databases may indicate putative functions for six of them. The proteins were listed in two main groups with putative (i–iv) and unknown function (v) in alphabetical order by the current gene designation.

(i) The bmr locus had already been sequenced and characterized by Ahmed et al. (1994) and is located at 212.9° on the B. subtilis chromosome. Proteins encoded by the bmrUR operon such as Bmr (transporter) and BmR (regulator) are involved in multidrug resistance (Ahmed et al., 1994). However, BmrU, which is also part of the operon, showed no significant similarity to any known protein in the databases.

Transcription of bmrU was very low during exponential growth but increased severalfold after heat stress, exposure to ethanol and salt as well as after glucose starvation. Several transcripts were detected after stress in wild-type cells but not in the sigB mutant (Fig. 2a, b). The bmrU gene was transcribed as a monocistronic mRNA (about 1.0 kb) as well as cotranscribed with either bmr (about 2.5 kb) or with bmr and bmrR (approx. 3.8 kb) as earlier suggested by Neyfakh (see accession no. L25604) (Fig. 2b). Since the ygiW gene, which precedes the bmrUR operon, is transcribed in the opposite direction it should be expected that bmrU is not cotranscribed with ygiW. A σ^B^-dependent promoter consensus sequence (GTGTTG-N_14-GGGGAT) was found in front of bmrU and the corresponding transcriptional start site of the RNA polymerase was detected by primer extension experiments in wild-type but not in sigB mutant cells (Fig. 2c).

(ii) The YcdF protein (Gsp74; Fig. 1a, b), which is similar to Bacillus megaterium glucose 1-dehydrogenases (Table 2), showed the typical σ^B^-dependent induction profile and a σ^B^-consensus promoter sequence in front of the ycdF gene (GTTTTC-N_14-GGGTAT). The translated amino acid sequence of ydaD showed a similarity to proteins encoded by the paralogous genes.
Fig. 1. For legend see facing page.
Fig. 1. Protein synthesis pattern of B. subtilis 168 during exponential growth (a) and 10 min after treatment with 4% ethanol (b). Bacteria were grown in synthetic medium and labelled with L-[35S]methionine as described in Methods. For a better orientation a few σ^B-dependent GSPs as well as the proteins ClpC, ClpP, AhpC, AhpF, SodA and TrxA are labelled (see Antelmann et al., 1997a). The newly identified stress proteins are marked with boxes.

yhdF, yhxC and yhxD of B. subtilis, a glucose dehydrogenase in embryos of Hordeum vulgare (Alexander et al., 1994) as well as glucose dehydrogenase isoenseymes of B. megaterium (Nagao et al., 1992; Table 2). The presence of a short-chain dehydrogenase/reductase family signature (Joernvall et al., 1995) suggests that YdaD might be involved in the generation of NAD(P)H.

The ydaD gene was cotranscribed with ydaE as a bicistronic mRNA. The protein coding sequence of the ydaE gene showed only a weak similarity to an unknown protein of Morganella morganii (accession no. L34345; Gallo & Mortlock, 1991). A stress-inducible transcript of about 1.6 kb was detected only in wild-type cells using ydaD- and ydaE-specific antisense RNA probes (Fig. 3a, b). The strongest induction occurred after cells were exposed to ethanol and salt. A weaker but significant induction also occurred after heat shock. A putative termination structure is located downstream of ydaE. Two additional less-induced transcripts of about 2.8 kb and 3.3 kb were detected after ethanol stress, presumably caused by a read-through of the potential termination structure (see Figs 3a, b and 7a, b).

As shown in Fig. 3(c), a stress-inducible transcript was detected starting 30 nt upstream of the translational-start codon of ydaD in the wild-type but not in the sigB mutant. The identified RNA polymerase-recognition sequence of ydaD (GTTTAT-N_6-AGGTAC) differed in two positions (underlined) from the consensus sequence of σ^B-dependent promoters (Haldenwang, 1995; Hecker et al., 1996).

(iii) A weak resemblance of YdaP was observed to bacterial pyruvate oxidases like PoxB of E. coli (Chang et al., 1994) and SpxB of Streptococcus pneumoniae (Spellerberg et al., 1996; Table 2). These enzymes catalyse the conversion of pyruvate into acetate or acetyl phosphate, respectively. E. coli PoxB, which is under the control of σ^B, seems to be responsible for the generation of C_2 units from pyruvate during the transition state
Table 2. Comparison of new σ^A-dependent B. subtilis proteins with proteins identified by BLAST searches

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<td>BmrU (297 aa)</td>
<td>Hypothetical protein YegS (299 aa)</td>
<td>E. coli</td>
<td>28% (83/296 aa)</td>
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<td>Hypothetical protein YerQ (303 aa)</td>
<td>B. subtilis</td>
<td>26% (77/293 aa)</td>
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<td>YcdF (258 aa)</td>
<td>Glucose 1-dehydrogenase I</td>
<td>B. megaterium</td>
<td>50% (128/256 aa)</td>
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<td>Glucose 1-dehydrogenase II</td>
<td>B. megaterium</td>
<td>50% (129/255 aa)</td>
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<td>Glucose dehydrogenase (Gdh)</td>
<td>B. subtilis</td>
<td>48% (124/255 aa)</td>
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<td>YdaD (286 aa)</td>
<td>Hypothetical protein YhdF (289 aa)</td>
<td>B. subtilis</td>
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<td>Glucose dehydrogenase</td>
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<td>35% (88/246 aa)</td>
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<td>YxbF (310 aa)</td>
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<td>YocK (209 aa)</td>
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<td>36% (44/121 aa)</td>
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<td>DnaK suppressor DksA</td>
<td>E. coli</td>
<td>25% (29/116 aa)</td>
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* The number of identical residues over the total length of the alignment is shown in parentheses.

from aerobic to anaerobic growth because pyruvate dehydrogenase and pyruvate formate-lyase are not fully functional under these conditions (Chang et al., 1994). The ydaP gene was transcribed as a 1.8 kb monocistronic mRNA. The stress-induction profile and the absence of the transcript in the sigB mutant confirmed that ex-
pression of ydaP is $\sigma^B$ dependent (Fig. 4a, b). Primer-extension experiments revealed that the upstream sequence of ydaP (GTTTTA-N$_{-1}$TGGTAT) resembles the consensus sequence of $\sigma^B$-dependent promoters, deviating only in one position in the $-10$ region (Fig. 4c). If ydaP really encodes a pyruvate oxidase it might be the fifth example of a gene (besides dps, katE, opuE and osmC) homologous to $\sigma^B$-dependent genes of E. coli that is under the control of $\sigma^B$ in B. subtilis.

(iv) YhdN showed a relatively weak similarity to a number of potassium ion-channel proteins in eukaryotic and prokaryotic organisms. The yhdN gene was transcribed in a $\sigma^B$-dependent manner under stressful conditions. In addition to the yhdN-specific 1.2 kb transcript, a larger ethanol-inducible transcript of 1.8 kb (probably containing yhdN and yhdO) was detected (Fig. 5a, b). Both transcripts were missing in the sigB mutant. The promoter region of yhdN (GTTTAA-N$_{-1}$GGGAAA) was in accordance with the consensus sequence of $\sigma^B$-dependent promoters.

Gsp16u was identified as YocK with relatively weak similarity to the Chlamydia trachomatis and E. coli DnaK suppressor protein DksA (Stephens et al., 1998; Kang & Craig, 1990; Table 2). Using a yocK-specific RNA probe, a monocistronic transcript of 0.5 kb was detected after stress in the wild-type but not in the sigB mutant (Fig. 6a, b). The putative $\sigma^B$-dependent promoter (GTTTTA-N$_{-1}$GGGAAA) deviates only in one position from the consensus sequence.

(v) In this section, new $\sigma^B$-dependent proteins with still unknown function are described that show similarity to hypothetical proteins in the databases. The ydaE gene is cotranscribed with ydaD as the promoter-proximal gene and was described in section (ii). The amino acid sequence of YdaG displayed no similarities to any known proteins in databases. Several stress-inducible ydaG-specific transcripts were found (0.47, 0.6 and 0.9 kb; Fig. 7a, b). The ydaG gene was additionally part of the 2.8 kb and 3.3 kb transcripts which were detected with ydaD- and ydaE-specific RNA probes (Fig. 3a, b). A heat-inducible 1.6 kb transcript was detected in the sigB mutant that probably comprises the genes ydaF, ydaG and the 0.5 kb region between ydaG and ydaH (Fig. 7a, b). The coding region of ydaG is preceded by a
Fig. 6. Analysis of the yocK gene. Details are as for Fig. 2, except g_{30} = 30 min, g_{60} = 60 min after entry into stationary phase due to glucose starvation.

Fig. 7. Analysis of the ydaG gene. Details are as for Fig. 2.

\( \sigma^B \)-dependent promoter consensus sequence (GTATA-N_{13}-TGGAAA) and the 5' end of the ydaG message was detected by primer extension experiments (Fig. 7c).

Gsp18 was identified as YfkM showing similarities to hypothetical proteins of E. coli and B. subtilis (Table 2). The search for \( \sigma^H \) promoters revealed the existence of such a sequence upstream of yfkM (GTATTAT-N_{13}-GGGTAG).

**DISCUSSION**

*B. subtilis* cells induce a large number of GSPs in response to different stressful conditions. The majority of these GSPs are induced by heat, osmotic, acid or ethanol stress as well as by energy depletion and they belong to the \( \sigma^B \)-dependent general stress regulon. A few years ago hardly anything was known about the physiological role of this regulon. By systematic studies relying either on a transposon-mutagenesis approach (Boylan *et al.*, 1991) or on proteomic studies (Völker *et al.*, 1994; Hecker *et al.*, 1996; Antelmann *et al.*, 1997a; Bernhardt *et al.*, 1997) first data on the function of the GSPs were presented. The \( \sigma^B \)-dependent general stress response might fulfill a similar physiological role as the \( \sigma^H \)-dependent response in *E. coli*, thus providing a non-specific, multiple and general stress resistance to non-growing cells (Gaidenko & Price, 1998; Hecker & Völker, 1998). The protection of DNA, proteins and membranes against oxidative damage might represent
an essential component within the complex stress response (Engelmann & Hecker, 1996; Antelmann et al., 1997b). To obtain additional experimental evidence for the suggested physiological role of the σ^B regulon, new GSPs were identified by N-terminal sequencing of proteins which were no longer stress-inducible in a sigB mutant. The putative functions of these new proteins provide additional information on the physiological role of the entire regulon in B. subtilis.

(i) New σ^B-dependent genes were found that may provide new information on the physiological role of the regulon. The finding that bmU as well as the bmU operon are under the control of σ^B indicates that the σ^B-mediated multiple stress resistance might be extended to the interaction with, and the elimination of, multidrug compounds in B. subtilis. The bmU operon encodes proteins that provide resistance against multidrug compounds. Bmr causes the efflux of various toxic compounds such as ethidium bromide, rhodamine 6G, tetraphenylphosphonium and fluoroquinoline antibiotics out of cells (Ahmed et al., 1994). The most downstream gene (bmrR) encodes BmrR which is able to bind these substances and to act as a positive transcriptional regulator for bmr transcription (Ahmed et al., 1994; Markham et al., 1996). A specific bmr induction is mediated by BmrR in response to toxic and antibiotic compounds, while non-specific bmr induction depends on σ^B under stressful conditions.

Prokaryotic as well as eukaryotic organisms possess multidrug-resistance efflux transporters (for a review see Lewis, 1994) whose expression is induced by various structurally divergent compounds such as antibiotics, inhibitors and other toxic substances. In Candida albicans and Saccharomyces cerevisiae, expression of the multidrug ABC transporters CDR1 (Krishnamurthy et al., 1998), PDR5 and SNQ2 (Hirata et al., 1994; Miyahara et al., 1996) is stimulated by drugs, human steroid hormones and heat shock. The E. coliacrAB operon, encoding a drug-efflux pump, is induced by treatment with salt and ethanol, after transition into stationary phase (Ma et al., 1995), and also by drugs like acriflavine (Ma et al., 1993). These data underline the involvement of multidrug resistance in general stress resistance.

(ii) Our idea that σ^B-dependent GSPs might be involved in the maintenance of the intracellular redox balance under stress as well as in protection against oxidative damage (Hecker & Völker, 1998) might be strengthened by the identification of the σ^B-dependent proteins YcdF and YdaD. On the basis of their similarities to several NAD(P)-dependent dehydrogenases it might be possible that YcdF and YdaD are involved in the generation of NAD(P)H, which could play an important role as a reduction equivalent in the oxidative stress response (Farr & Kogoma, 1991; Dowds, 1994; Scharf et al., 1998). The maintenance of a constant redoxreduction state within the cell requires the reduction of oxidized biological molecules which may need NAD(P)H. The σ^B-dependent thioredoxin TrxA appears to be an essential protein in B. subtilis (Scharf et al., 1998) and might reduce oxidized thiol groups of proteins, leading to the restoration of active proteins. Oxidized TrxA becomes reduced by the thioredoxin reductase TrxB using NAD(P)H as a reduction equivalent. Another σ^B-dependent protein, NadE, participates in NAD synthesis (Antelmann et al., 1997c). Therefore, we suggest that a sufficient level of NAD(P)H seems to be a prerequisite for the cell to face oxidative stress. However, further experiments are necessary to provide evidence for this assumption.

(iii) New members of the σ^B regulon whose orthologous genes are under the σ^B-control in E. coli strengthen the suggestion that both regulons fulfill a similar physiological role. Besides katE and dps (Engelmann et al., 1995; Antelmann et al., 1997b), the orthologues of the σ^B-dependent genes prop and osmC in E. coli are under σ^B control in B. subtilis (von Blohn et al., 1997; Völker et al., 1998). In this paper, another gene that is regulated by σ^B is described, namely ydaP of B. subtilis. Whether YdaP is indeed an acetyl phosphate-producing enzyme remains to be investigated.

(iv) The induction of σ^B-dependent genes in response to high osmolarity led to the suggestion that some GSPs participate in osmoprotection. This assumption was supported by the finding that the proline uptake transporter OpuE is regulated by high osmolarity and σ^B (von Blohn et al., 1997; Spiegelhalter & Bremer, 1998). It is worth mentioning that YdhN, which is also σ^B-dependently expressed, resembles potassium ion-channel proteins. Potassium ion uptake is one of the first responses of cells after a sudden osmotic upshift (Csonka & Epstein, 1996). Furthermore, YocK, which shows weak similarity to the E. coli DksA protein, was identified as a σ^B-dependent protein. E. coli DksA was shown to suppress the temperature-sensitive and filamentous phenotype of dnaK mutations (Kang & Craig, 1990).

(v) The entire sequence of the B. subtilis genome shows that approximately 42% of the genes encode proteins with still unknown functions (Kunst et al., 1997). It is a big challenge for future research to obtain information on the physiological role of these unknown proteins. The allocation of these proteins to regulons with known functions is a strategy to obtain initial information on the function of these unknown proteins within the framework of the regulon. New proteins with still unknown functions were identified that belong to the σ^B-stress/starvation regulon. It is highly probable that these proteins are also somehow involved in non-specific and prospective protection against stress. However, this is only a preliminary and general prediction. To define the function of each single new member of this regulon, a detailed analysis of the stress response of corresponding mutants must be conducted.

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