SigM, an Extracytoplasmic Function Sigma Factor of *Bacillus subtilis*, Is Activated in Response to Cell Wall Antibiotics, Ethanol, Heat, Acid, and Superoxide Stress

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The extracytoplasmic function sigma M of *Bacillus subtilis* is required for normal cell growth under salt stress. It is expressed maximally during exponential growth and is further induced by the addition of 0.7 M NaCl. The promoter region of the *sigM* operon contains two promoters; one (P_A) is sigma A dependent, and the other (P_M) is sigma M dependent. These have been placed separately at the *amy* locus, directing expression of a *lacZ* reporter gene. Only the P_M fusion responded to salt induction. This promoter, which was responsive to the level of active sigma M in the cell, was also induced by 5% ethanol, by vancomycin, bacitracin, or phosphomycin (inhibitors of cell wall biosynthesis; 2 μg per ml), and by heat shock of 50°C for 10 min. It was very strongly induced by acid (pH 4.3) and 80 μM paraquat, but after a 15- to 30-min delay. There was no induction by alkali (pH 9), 5 mM H_2O_2, the detergents 0.1% Triton X-100 and 0.1% Tween 20, or 50 μM monensin. In addition to their reduced tolerance to salt, null mutants of sigM were unable to grow at pH 4.3 and lysed after exposure to 5% ethanol. Genes regulated by SigM were also tested for their response to pH 4.3, 5% ethanol, and 2 μg of vancomycin per ml. Expression of the genes may have been activated by increased levels of sigma M, but at least some were also subject to additional controls, as they responded to one type of stress but not another. Expression of *yraJ*, which encodes a cytochrome P450/NADPH reductase, was induced in response to acid and vancomycin. *yraA* expression was acid, ethanol, and vancomycin induced, whereas *yjbD* showed only ethanol induction. YraA protein was extremely important to acid survival—a mutation in *yraA*, like a *sigM* mutation, resulted in the failure of *B. subtilis* to grow at pH 4.3. Sigma M is therefore involved in maintaining membrane and cell wall integrity in response to several different stresses in exponential growth phase and is activated by such stresses.

Bacteria respond to diverse growth-limiting stresses by producing a large set of general stress proteins. In *Bacillus subtilis* and related gram-positive pathogens, this response is governed by the sigma B transcription factor. *B. subtilis* also encodes seven potential extracytoplasmic function (ECF) RNA polymerase sigma factors (9) that also contribute to stress resistance, but in a rather different fashion. There is evidence that all (σ^M, σ^N, σ^W, σ^X, σ^Y, and YlaC) are expressed in *B. subtilis* (12), and several have been shown to contribute to stress resistance.

The best-characterized ECF sigma factors of *B. subtilis* are σ^W and σ^X. Sigma W is expressed maximally late in growth (12) and switches on a large regulon (3, 15), including a large fraction of the genes that are most strongly induced in response to alkali shock (29). The *sigX* gene is also switched on in late logarithmic phase, and *sigX* mutants are impaired in the ability to survive at high temperature and oxidative stress (14).

The *sigM* gene is cotranscribed with *yhdL* and *yhdK*, which negatively regulate SigM activity; these are predicted to be membrane associated and, by analogy with other ECFs, may represent membrane-bound anti-sigma factors which release σ^M in response to particular extracytoplasmic-inducing cues (7). Expression of *sigM* in nutrient broth has been shown to be maximal during the early to mid-exponential growth phase, with a sharp decline at the end of logarithmic-phase growth. Transcription is initiated from two promoters: P_A, which is recognized by the major vegetative sigma factor, SigA, and P_M, which is recognized by SigM itself—hence, expression of the *sigM* operon, like that of other ECFs, is positively autoregulated (11).

If cultured in nutrient broth with an additional 0.35 to 0.7 M NaCl, *sigM* mutant cells become swollen, and many lyse (11). These observations are consistent with severe defects in cell wall synthesis or stability, indicating that SigM may be required for maintaining cell envelope integrity under these conditions.

In this study, the response of SigM to an array of stresses was investigated in an attempt to further elucidate the function of SigM in the cell. The autoregulated *sigM* promoter P_M was separated from the P_A promoter so that the effects of stresses on the individual promoters could be assessed.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and medium.** The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *Bacillus subtilis* were routinely cultured in or on L-broth and L-sugar or Oxoid nutrient broth and nutrient agar, respectively, with the appropriate antibiotics (for *E. coli*, chloramphenicol at 30 μg ml^-1_; for *B. subtilis*, erythromycin and lincomycin at 1 and 25 μg ml^-1_; chloramphenicol at 5 μg ml^-1_; and kanamycin at 10 μg ml^-1_; 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used at 40 μg ml^-1_; 35 region of PM but

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TABLE 1. B. subtilis strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
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<tbody>
<tr>
<td>1604</td>
<td>amyE::pMUTkan</td>
</tr>
<tr>
<td>AM1601</td>
<td>sigM::pMUTkan</td>
</tr>
<tr>
<td>AM1602</td>
<td>amyE::Pma-lacZ</td>
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<tr>
<td>AM1616</td>
<td>amyE::Pma-bgaB</td>
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<td>amyE::Pma-lacZ sigmM::pMUTkan</td>
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</tr>
<tr>
<td>AM1615</td>
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</tbody>
</table>

RESULTS

Dissection of salt induction of sigM promoter region. The effect of increased NaCl levels on the expression of the promoters upstream of the sigM gene was investigated with strains AM1601 (P_x-lacZ), AM1602 (P_y-lacZ), and AM1603 (P_alpha-P_m- lacZ), in each of which the normal sigM region remains intact and the reporter fusions are at the amyE locus.

The complete P_x-P_m promoter region showed salt induction (ca. 2.5-fold) and a more gradual increase in expressed β-galactosidase through growth phase in the absence of salt (Fig. 1A). Expression from the P_m promoter was strongly salt induced (eightfold; Fig. 1B). The P_y promoter did not show salt induction, but it did provide a gradual increase in β-galactosidase throughout exponential growth in the absence of added salt (Fig. 1C). In these experiments, the cells were grown in the presence of high salt from the start of growth; the same results were obtained when exponential growth phase cells were exposed to salt shock (data not shown).

This effect was still observed in cells that were subcultured, after overnight growth but before salt shock, in 1 mM glycine betaine (10), an osmoprotectant; when the salt shock was imposed at an OD_600 of 0.1, the induction of SigM was still clearly visible (Fig. 1D). The control level of β-galactosidase was unusually high in the presence of betaine; this high level was also observed when the P_x-P_m lacZ strain was exposed to betaine and is therefore not thought to be due specifically to the induction of P_m.

The SigM dependence of the stress response at P_m was tested by introducing the P_m-lacZ construct into the sigM null mutant AM1447, and induction was carried out in nutrient broth with 0.7 M NaCl. The level of β-galactosidase expression in the sigM null mutant (50 to 100 units) was similar to the low endogenous levels in wild-type cells and was not increased by salt stress (Fig. 1E).

Induction of SigM by pH, oxidative, ethanol, and antibiotic stress and heat, and phenotype of a null mutant under stressed conditions. The possibility that sigM is upregulated in response to other environmental stresses was investigated. A marked increase in β-galactosidase expressed from the P_m promoter in AM1601 was seen in response to growth at pH 4.3 (10-fold; Fig. 2A), or addition of 80 μM paraquat (20-fold, Fig. 2B), but only after a 30-min delay. The 5% ethanol caused a fourfold induction of P_m expression (Fig. 2C). Several antibiotics which inhibit growth of the cell wall also caused induction of the SigM promoter — bacitracin (fivefold), vancomycin (threelfold), and phosphomycin (twofold) (Fig. 2D, E, and F, respectively). The expression levels achieved on vancomycin and phosphomycin induction were hard to quantitate, as the cells began to lyse. No induction was observed in a sigM null mutant for any of the above stresses (Fig. 2A to F). The increase in reporter activity from P_m on stress induction will reflect both activation of existing SigM, by release from its negative regulators and de novo synthesis of the products of the sigM operon. The P_m promoter was not recognized by any of the other ECF sigma factors under these conditions.

Experiments to test the effects of alkali (pH 9), 5 mM H_2O_2, 50 μM monensin, 0.1% Triton X-100, and 1% Tween 20 on P_m were also carried out, but no induction of sigM was observed (data not shown).
Strain AM1616, containing the same P_M-lacZ construct at amy except that the reporter β-galactosidase produced is thermostable was exposed to a heat shock of 10 min at 50°C and then returned to 37°C. There was a rapid fourfold induction during the heat shock (Fig. 3), which declined after the cells were returned to 37°C.

To summarize, the kinetics and level of induction of the P_M promoter depended on the stress to which the cells were exposed. The most dramatic level of induction of SigM was observed under acid and paraquat stress, but the response was delayed for 15 to 30 min after exposure to the stress. The lower level of induction with heat, ethanol, bacitracin, vancomycin, and phosphomycin was more rapid. Induction in response to 0.7 M NaCl was seen after 15 min, but the level of induction was much lower than that seen for acid or paraquat stress.

B. subtilis 1604 (wild type) and AM1447 (sigM) cells were observed by light microscopy during growth phase under acid and ethanol stress. The wild-type cells were slowed in growth rate, but their morphology was the same as that of unstressed cells. The sigM null mutant showed no OD increase at pH 4.3, and the cells showed some elongation; in contrast, under ethanol stress, many of the mutant cells lysed—both cell debris and elongated cells were observed.

Stress induction of SigM-regulated genes. Several genes (yrhJ, ywoA, ysaA, yraA, and yjbD) whose expression is upregulated upon artificial induction of a xylose-controlled ectopic copy of sigM at the amyE locus (13) were tested for their expression in response to stresses shown to activate the P_M promoter.

Strains containing lacZ transcriptional fusions, resulting from insertional inactivation of cognate genes by pMUTIN4, were obtained from the collections of the European and Japanese sections of the Bacillus subtilis functional analysis program (28; http://locus.jouy.inra.fr; http://bacillus.genome.ad.jp).

The yrhJ, ywoA, ysaA, yraA, and yjbD fusions were tested for induction upon a shift from pH 7.0 to 4.3. The yrhJ gene showed strong induction at pH 4.3 after a 30-min delay (Fig. 4A), as was seen for the P_M promoter itself (Fig. 2A). This induction was SigM dependent, as yrhJ was not induced at pH 4.3 in a sigM null mutant (Fig. 4B). The yrhJ mutant was still viable at pH 4.3 and therefore YrhJ is not essential for growth at low pH. The yraA null mutant was unable to grow at all at
pH 4.3, suggesting that this gene is essential for acid stress resistance at this pH, but growth was possible at pH 5 and induction of yraA was observed (Fig. 4C). None of the other genes tested showed induction at acid pH.

The effect of adding 5% ethanol on yrhJ, ywoA, ysxA, yraA, and yjbD was also investigated. The yraA gene showed a two-fold induction (Fig. 5a) and yjbD showed a two- to threefold induction (Fig. 5B). The growth rate of a null mutant in either of these genes was the same as the wild type in 5% ethanol stress conditions. In contrast, the expression of yrhJ (Fig. 5C) and ywoA (Fig. 5D) appeared to be lowered in 5% ethanol; at a time when the level of active αM was higher (Fig. 2C), in the case of these genes, expression levels were not reflecting this increase. The reduction in expression was not observed in sigM null mutants (Fig. 5E and 5F), indicating that the stress regulation was under the control of SigM.

Exposure to vancomycin induced the expression of yraA two- to threefold (Fig. 6A) and yrhJ twofold (Fig. 6B). This induction, at least for yrhJ, was SigM dependent (Fig. 6C); the yjbD, ysxA, and ywoA genes showed no induction in response to vancomycin (data not shown).
These five SigM-responsive genes responded very differently to conditions that increased the levels of active SigM, as judged by expression from P_M. This suggests that they are subject to additional levels of control.

**DISCUSSION**

The ECF sigma factor \( \sigma^M \) and genes regulated by SigM are switched on by a variety of stresses, including high salt, low pH, paraquat, ethanol, bacitracin, vancomycin, phosphomycin, and heat, most of which interfere with functions involved in control of cell membrane, envelope, and wall biosynthesis. There must be some specificity, however, as alkali, \( \text{H}_2\text{O}_2 \), monensin, Triton X-100, and Tween 20 did not elicit the same response, even though they were used at concentrations that reduce (or, in the case of \( \text{H}_2\text{O}_2 \), stop) growth.

The effects of the different stresses on the activity of the SigM-dependent promoter were studied by separating the individual promoters of the \( \text{sigM} \) operon (\( \text{PA} \) and \( \text{PM} \)) and following the kinetics and level of induction.

Salt induced expression from \( \text{PM} \) but not from \( \text{PA} \). When the same experiments were carried out in Luria broth rather than nutrient broth, no salt induction of \( \text{PM} \) or the \( \text{PAPM} \) region was observed, although the overall levels of uninduced expression were similar (data not shown). In addition, the reduction in the growth rate of a \( \text{sigM} \) null mutant seen in nutrient broth on addition of 0.35 M or 0.7 M salt was not seen in Luria broth medium. The reason for this medium-dependent effect on the salt stress/salt induction phenotype of \( \text{sigM} \) is not known, but a similar effect has been reported for \( \text{degU} \) (17).

Bacitracin, vancomycin, and phosphomycin, inhibitors of cell wall biosynthesis (2), activated SigM. This confirms the observation of Cao et al. (4) that \( \text{sigM} \), along with several other ECF sigma factors, is induced on exposure of the cells to vancomycin.

In the case of acid and paraquat stress, the response was only seen after 15 to 30 min, suggesting a response to the eventual loss of some cellular function rather than a direct response to the immediate pH or superoxide stress.

Under conditions of ethanol stress, it has been reported that membrane composition is dependent on ethanol concentration and cell physiological state (26). Ethanol was shown here to switch on \( \sigma^M \) activity, suggesting a possible function for the SigM regulon in maintaining cell membrane integrity, although the detergents Triton X-100 and Tween 20 did not induce \( \text{sigM} \).

SigM also showed very rapid induction upon heat shock at 50°C. *B. subtilis* activates the transcription of over 100 genes in response to heat stress. Many of these are members of the general stress response regulon under the control of SigB, while others are under the control of the heat shock regulators HrcA and CtsR. Helmann et al. (8) used DNA microarrays to monitor the global transcription response to heat shock. Several genes that they identified as responding to heat shock have also been shown to be regulated by SigM (13). These include \( \text{yacL}, \text{yjbC} \), and \( \text{yjbD} \)—genes that are also downstream of SigB-dependent promoters, \( \text{yacK} \), a class III heat shock gene, and \( \text{yrhJ} \), reported as a new member of the heat shock stimulon. SigM was not reported before (8) to be heat stress responsive.

The high level of expression of \( \text{sigM} \) is not always reflected in significantly higher expression of individual regulated genes, and in some cases the genes appear to have lowered expression in certain stress conditions. This indicates a further level of regulation in addition to \( \sigma^M \) for these genes.

Three of the SigM-responsive genes shown in this study to be induced by individual stresses have diverse possible functions, \( \text{yrhJ} \), whose expression was induced by 0.7 M NaCl, at pH 4.3, and in response to 2 \( \mu \)g of vancomycin per ml, is a homologue (58% amino acid identity) of the fatty acid monoxygenase cytochrome P-450BM-3 of *Bacillus megaterium*, which incorporates both a P-450 and an NADPH:P-450 reductase in proteolytically separable domains (1, 16, 23).

The \( \text{yjbD} \) and \( \text{yraA} \) genes were both induced by 5% ethanol, and \( \text{yraA} \) was also induced by 0.7 M NaCl, 2 \( \mu \)g of vancomycin per ml, and pH 5. \( \text{YjbD} \) (SpX) appears to be involved in com-
petence development in *B. subtilis*, as a null mutation in *yjbD* results in ClpX- and ClpP-independent competence development (18). Null mutants of *yjbC* and *yjbD* show reduced resistance to salt stress (21). *YjbD* is subject to proteolysis by Clp proteases, but this requires additional, as yet unrecognized factors (19). *YraA* is a paralogue of *YfkM* which was not essential for growth at pH 4.3 and was not induced at pH 4.3 (data not shown). *YfkM* is a recognized general stress protein which is SigB regulated (20). Both *YraA* and *YfkM* are homologues of an intracellular protease of *Pyrococcus* species (PH1704 and PfpI from *P. horikoshii* and *P. furiosus*, respectively). These are representatives of a class of protease that has no sequence homology to any other known protease family. PH1704 is probably a cysteine protease and forms a hexameric ring structure (5). *YraA* is the first protein, to our knowledge, that is essential for acid stress tolerance in bacilli.

Other ECF sigma factors, *σ^W^* and *σ^X^*, are hypothesized to play a role in cell envelope integrity. *SigW* is expressed in early stationary phase, and its regulon may function in the detoxification and production of antimicrobial compounds (15). *SigW* and members of its regulon are induced by alkali stress (29). *SigX* is expressed in late log phase, and its regulon is proposed to modulate cell surface properties. *SigX* null mutants show reduced survival at high temperature and oxidative stress (14), but *σ^X^* is not induced by heat shock (6, 22). As members of the *σ^W^* and *σ^X^* regulons have no obvious function in pH homeostasis or heat shock, respectively, the stress sensed may be indirect and rather related to cell wall impairment, leading to activation.

Cao et al. (4) have shown induction of *σ^W^* by antibiotics that inhibit cell wall biosynthesis, and the *σ^X^* and *σ^W^* responses are activated by mutations in genes that affect multidrug efflux pumps, sugar isomerases, or antimicrobial biosynthesis, but these mutations are located in different genes for the two sigma factors (27). Therefore, while both pathways appear to be involved in mediating adaptation to toxic compounds or membrane and cell wall alterations, the ECFs recognize distinct extracytoplasmic signals. *SigM* is required for cell maintenance under conditions of salt, acid, and ethanol stress, and so clearly has a related role.

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**FIG. 5.** Ethanol induction in nutrient broth of (A) *yraA*, (B) *yjbD*, (C) *yrhJ*, (D) *ywoA*, (E) *yrhJ* in a *sigM* null mutant, and (F) *ywoA* in a *sigM* null mutant. Solid symbols, β-galactosidase level; open symbols, OD<sub>600</sub>; triangles, plus stress; squares, control. Stress was applied at 0 min.
σM, σW, and σX react to a different but overlapping spectrum of inhibitors, which act at different locations in the cell envelope. As the ECF factors are most active at different growth phases of the cell, these overlaps may assure protection to the cell whatever its nutritional status. SigM may also play a role in cell wall homeostasis during normal growth or growth phase transitions, as it is most active in early to mid-log growth.

Stress induction of some genes (e.g., yrhD) can be mediated by several of these ECFs, but induction of others (e.g., yrhJ) is SigM specific. How SigM is activated in response to such a variety of different environmental stresses remains to be elucidated. The variation in kinetics of induction suggests that there may be more than one mechanism of activation.

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REFERENCES


24. Stoss, O., A. Mogk, and W. Schumann. 1997. Integrative vector for con-
constructing single-copy translational fusions between regulatory regions of \textit{Bacillus subtilis} and the \textit{bgaB} reporter gene encoding a heat-stable $\beta$-galactosidase. FEMS Microbiol. Lett. 150:49–54.


