SigM-Responsive Genes of *Bacillus subtilis* and Their Promoters

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Promoters of nine *Bacillus subtilis* genes (berC, yaeK, ydaH, yfnL, yjbD, ypbG, ypuA, yraA, and ysxA), all responsive to artificially induced increases in the stress-responsive extracytoplasmic function sigma factor, SigM, were mapped by rapid amplification of cDNA ends-PCR. The resulting promoter consensus suggests that overlapping control by SigX or SigW is common.

Extracytoplasmic function (ECF) sigma factors M, W, and X are involved in the *Bacillus subtilis* cell’s response to environmental stresses. There is overlap between these sigma factors in terms of the envelope stress to which they respond, although each has some distinct specificity. Expression of the partially autoregulated *sigM* gene is induced by high salt, low pH, ethanol, bacitracin, vancomycin, phosphomycin, heat (25), and paraquat (8, 25). The expression of *sigW* and other sigma W-regulated genes is induced by alkali or high salt (28) and by vancomycin (9), and *sigW*-regulated genes are important in resistance to antimicrobial compounds produced by other *Bacillus* strains (4). The *sigX* operon and SigX-regulated genes are induced by heat (13), and also by some cationic antimicrobials (5), and *sigX* mutants display sensitivity to these stresses.

Published studies concerned with specific members of the *sigM* regulon are limited to the analysis of division (20), bcrC (6), and yqlL (8), as well as *tarA* of *B. subtilis* W23 (20, 21). In addition, a *sigM* mutation reduced the induction of transcription of bcrC, radC (ysxA), yjbC, and ypuA in response to the envelope-damaging mammalian cationic peptides LL37 (22). Comprehensive microarray data on the *sigM* regulon are not available, although Asai et al. (2), by comparing gene expression levels 2 h after artificial IPTG (isopropyl-β-D-thiogalactopyranoside) induction of a plasmid-borne copy of *sigM*, identified a large number of potentially regulated genes. Several candidate genes, including yrhI, bcrC (ywoxA), ysaA, yraA, and yjbD, were mentioned in a study by Thackray and Moir (25) as being SigM responsive, based on reporter data that are only presented as showing induction in response to increased expression by the *sigM* promoter consensus for SigM, transcriptional start points were determined using 5′ rapid amplification of cDNA ends (RACE)-PCR mapping of transcriptional start points in xylose-induced cells.

Expression of reporter fusions stimulated by ectopically induced SigM. A PCR fragment that includes the complete *sigM* gene, along with an improved consensus ribosome binding site and an ATG start codon, was cloned into pOR277 (23), a plasmid designed for construction of DNA insertions into the *amyE* locus. A copy of *xylR* and the *xylA* promoter, upstream of the cloning site in pOR277, allows xylose-inducible expression of the cloned gene (17). This construct was introduced into the *amy* locus of our laboratory wild-type *B. subtilis* strain 1604, yielding strain CWH001. The native *sigM* gene was inactivated by integration of pMUTKan, a derivative of pMUTIN4 in which the lacZ and *erm* regions have been replaced by a kanamycin resistance gene (J. Lindsay and S. J. Foster, unpublished data), and this mutation was introduced into CWH001 to generate CWH005 (*sigM::pMUTKan amyE::Pcryl-sigM cat*), in which the only functional SigM protein is produced under the control of *Pcryl*. Candidate genes for testing *sigM* dependence were chosen from an unpublished transcriptional array analysis (U. Zuber and M. Hecker, personal communication) and by exploiting the collection of mutant strains carrying *lacZ* transcriptional fusions (18), in which the gene of interest had been insertionally inactivated by integration of a pMUTIN-based plasmid (27). Chromosomal DNA carrying each of the selected fusion constructs was introduced into strain CWH005 by transformation, and *lacZ* expression was measured following xylose induction of early log-phase cells (25).

Figure 1 shows the specific activity of β-galactosidase in various *lacZ* fusion constructs. SigM-directed expression represents only a proportion of the expression of each gene, judging by the residual level of expression in uninduced cells. The genes expressed at the highest level overall are *yjbC* and *yjbD* (*spx*), already recognized as stress-induced genes. Most promoters responded rapidly to induction, but *yjbD* was particularly delayed, showing a significant induction only at late log phase. In others, too, there was increased SigM-dependent expression late in growth (e.g., *ypgB* and *ypuA*).

Promoter analysis by RACE-PCR. Of the genes described above as showing induction in response to increased expression of *sigM*, the promoters of only *bcrC* and *yjbC* have previously been described. One of the three *yjbC* promoters (1) is recognized by SigW (15); given the similarity between this promoter and those identified here, it is likely that it is also recognized by SigM. To test for the direct involvement of SigM in the regulation of the rest of these genes, and to build up a stronger promoter consensus for SigM, transcriptional start points were determined using 5′ RACE-PCR. RNA was isolated from a culture of CWH001, which carries the xylose-inducible *sigM* at the *amy* in addition to the normal *sigM* operon. To obtain freshly growing cells, overnight cultures grown at 30°C in Oxoid nu-
trient broth (NB) were diluted 50-fold into fresh NB and grown to an optical density at 600 nm (OD 600) of 0.2. This culture was then further diluted to an OD 600 of 0.05 in fresh NB containing 40 mM xylose. When the culture reached an OD 600 of 0.6, 15-ml samples were taken, rapidly chilled, pelleted by centrifugation for 2 min at 4°C, and resuspended in 100 μl RNAsecure reagent (Ambion Inc.). RNA was then extracted using the FastRNA blue kit (Qbiogene). 5’/3’ RACE-PCR was carried out using a 5’/3’ RACE-PCR kit from Roche, using 1.5 μg total RNA as the template. Full details of gene-specific primers are available on request. PCR products were separated by gel electrophoresis and sequenced. If the product was unexpectedly large, additional oligonucleotide primers for cycle sequencing were designed using sequence closer to the 5’ end. The bcrC gene was included as a positive control, and the published promoter (6) was confirmed.

The 5’ ends of transcripts were defined for another eight genes (Fig. 2). Most were located within 100 bases upstream of the translational start point of the gene. An unexpected result was that two of the sigM-dependent promoters, for yacK and
yraA, lie within the coding regions of upstream genes sms and maf, respectively. These have been described as sigM induced in an array experiment (2), but neither gene would be expressed as a complete open reading frame; instead, a transcript with a long untranslated leader would be generated, raising the possibility of further regulation.

All of these promoters contain −35 and −10 regions that are generally similar to the sigma M-specific autoregulatory P sigM promoter of the sigM operon. It is therefore likely that they are directly regulated by sigma M, though none have the diagnostic C in the −35 region, and there are considerable variations, particularly in the −10 region, for yraA and yjbD. There was one clear exception among the apparent sigM-inducible genes tested—the only transcriptional start point for yjbD that was detected by RACE-PCR was the sigma A promoter, corresponding in position to that already reported (19), and it is therefore not included in Fig. 2.

In total, 14 experimentally derived sequences (Fig. 2) are available for comparison to generate a possible consensus sequence for a SigM-dependent promoter, TGAAAC-N_{17}-CGTC, but such a promoter would not be uniquely recognized by SigM, so its value as a consensus is limited. It would be very similar to that of other promoters reported as recognized by SigV, SigW, and SigX. Additional sequence elements must define which of these ECF sigma factors will be able to recognize a specific promoter in vivo with a functionally significant binding efficiency. The only uniquely recognized SigM-responsive promoter, that of sigM itself, has one base difference from this consensus sequence in each of the −35 and −10 regions, and these bases are not found in the same positions in any of the other promoters characterized.

Confirmation of yraA and yraA promoter regions. The atypical yraA and yraA promoters were chosen for detailed analysis. PCR fragments containing P_{yraA} (bp −142 to bp +95, relative to the measured transcriptional start point) or P_{yra} (bp −176 to bp +128) were cloned, using EcoRI and BamHI sites engineered into the fragments, into pPD1661, which is designed to create transcriptional fusions to lacZ at the amy locus, and were introduced into a wild-type genome by a double crossover (10). Strains AJ024 (P_{yraA}-lacZ) and AJ018 (P_{yra}-lacZ) are the other isogenic parents of strains in which the fusion constructs were introduced into different ECF sigma mutant backgrounds. Expression from P_{yraA} was reduced markedly in a sigM mutant, and the remaining expression was strictly dependent on sigX (Fig. 3A). Similarly, expression of bcrC in un-stressed cells requires SigX and SigM (6), and the tarA promoter of B. subtilis W23 is under SigX and SigM control (20). The twofold-higher induction of yraA expression by salt is dependent on sigM, as it is absent in a sigM mutant (Fig. 3B).
and this increase in expression was strictly dependent on SigM (Fig. 4A and B). Sigma B is not important to expression under these conditions (data not shown).

The complexity of regulation from ECF promoters. As shown for P_{yraA}, an ECF sigma factor that does not contribute to transcription under normal conditions of cell growth may come into play if the activity of the sigma factor increases, for example, as a result of release from anti-sigma factor control in response to stress. Induction of expression of bcrC and yjbC following envelope stress induced by antimicrobial peptides (22) was dependent on both sigW and sigM. Data obtained by artificially increasing the activity of individual ECF sigma factors (2) or from in vitro transcription experiments (7) also suggest a less stringent promoter specificity that may have some significance in vivo. Expression of the yglL gene, for example, appears from lacZ reporter fusions to be almost completely dependent on SigM, and yet its promoter was recognized by SigW and SigX in in vitro runoff transcription experiments (8). The bcrC and yprA genes were very strongly induced in a strain in which the ECF sigma factor SigV was released from anti-sigma factor control (29). The presence of multiple promoters for all the sigM-regulated genes examined, the potential for recognition of the same promoter sequence by multiple ECF sigma factors, and the potential for different levels of activity of such sigma factors under various stress conditions together generate a complexity of regulation that makes a specific and individual role for this sigma factor in cell growth and envelope maintenance difficult to define.

Salt-induced expression and mutant sensitivity among members of the σ^M regulon. A sigM mutant strain grows less well than the wild type in NB with added NaCl, in which many cells balloon towards the end of growth (12). A number of the gene fusion constructs demonstrated above as being sigM inducible were tested for salt inducibility by adding 0.7 M NaCl to NB. In addition to the ectopically located yxaA and yraA promoters described above, constructs involving fusions to bcrC, ypgB, yacK, yacL, ypuA, and yrhJ genes were also tested, and all showed inducibility (data not shown). As described previously (25), the complete absence of sigM resulted in an increased level of expression of yrhJ, even in the absence of stress, and the failure to detect a sigM-responsive promoter upstream of yrhJ may reflect complex, indirect effects of sigM on the expression of this gene.

The bcrC and yxaA mutants showed a lowered growth rate and eventual ballooning of cells in response to the addition of 0.7 M NaCl to NB. The bcrC (ywoA) gene encodes an undecaprenyl pyrophosphate phosphatase (3), important in the recycling of the lipid carrier during cell wall biosynthesis. YxaA's function is unknown, but the MreB and MreC proteins encoded downstream are important in the morphogenesis of the bacterial cytoskeleton; an in-frame deletion of yxaA would be required to define a specific phenotype. The salt stress phenotype of sigM mutants may be attributable to the cumulative reduction in expression of members of the regulon that are important for cell envelope stability during rapid growth in the

FIG. 3. Expression from the isolated P_{yraA} region, fused to lacZ, and relocated at amyE. (A) Cultures were grown in NB. All strains have the P_{yraA} fusion construct. ○, AJ024; □, AJ025 (sigM); ▼, AJ034 (sigX); ■, AJ028 (sigM sigW); ▽, AJ027 (sigM sigX). (B) Salt induction of P_{yraA-lacZ} in NB in AJ024 (circles) and AJ025 (sigM) (triangles). Cultures were grown to early exponential phase and then divided, and NaCl (0.7 M) was added (open symbols). The time of addition corresponds to the first open symbol.

FIG. 4. Expression of P_{ylha} lacZ under salt and acid stress. All strains have the P_{ylha} fusion construct. (A) β-Galactosidase levels in AJ018 in NB (○) and NB plus 0.7 M NaCl (□) and in AJ019 (sigM) in NB (■) and NB plus 0.7 M NaCl (▲). (B) Expression in AJ018 in NB (○) and in NB adjusted to pH 5 (□) and in AJ019 (sigM) in NB (▲) and NB at pH 5 (△). Dotted lines with the same small symbols represent the OD_{600}. The time of addition of salt or acid corresponds to the first open symbol.
presence of high salt concentrations, including these. In contrast, Steil and coworkers demonstrated that in minimal medium, and in a sigB mutant background, many genes of the sigma W regulon are switched on in response to salt shock (24), and their array data highlighted only one salt-induced gene that corresponds to a sigM-regulated gene described here (vrhJ). This may reflect the difference in the media used during the imposition of stress.

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