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# Regulation of the *Bacillus subtilis bcrC* Bacitracin Resistance Gene by Two Extracytoplasmic Function σ Factors

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**Bacitracin resistance is normally conferred by either of two major mechanisms, the BcrABC transporter, which pumps out bacitracin, or BacA, an undecaprenol kinase that provides $C_{55}$-isoprenyl phosphate by de novo synthesis. We demonstrate that the *Bacillus subtilis bcrC* (*ywoA*) gene, encoding a putative bacitracin transport permease, is an important bacitracin resistance determinant. A *bcrC* mutant strain had an eightfold-higher sensitivity to bacitracin. Expression of *bcrC* initiated from a single promoter site that could be recognized by either of two extracytoplasmic function (ECF) σ factors, $\sigma^X$ or $\sigma^M$. Bacitracin induced expression of *bcrC*, and this induction was dependent on $\sigma^M$ but not on $\sigma^X$. Under inducing conditions, expression was primarily dependent on $\sigma^M$. As a consequence, a *sigM* mutant was fourfold more sensitive to bacitracin, while the *sigX* mutant was only slightly sensitive. A *sigX sigM* double mutant was similar to a *bcrC* mutant in sensitivity. These results support the suggestion that one function of *B. subtilis* ECF σ factors is to coordinate antibiotic stress responses.**

Bacitracin, a mixture of related cyclic peptide antibiotics, was isolated almost 60 years ago from a type of "growth-antagonistic" *Bacillus* strain from the Presbyterian Hospital of New York (15). Bacitracin is produced by some strains of *Bacillus licheniformis* and *Bacillus subtilis* and functions as an inhibitor of cell wall biosynthesis (1, 14). By binding to the $C_{55}$-isoprenyl pyrophosphate in the presence of divalent cations (most efficiently with $Zn^{2+}$), bacitracin prevents $C_{55}$-isoprenyl pyrophosphate dephosphorylation and thus interrupts the recycling of $C_{55}$-isoprenyl pyrophosphate to $C_{55}$-isoprenyl phosphate (26, 27). $C_{55}$-isoprenyl pyrophosphate functions as a lipid carrier for the transport across the membrane of the disaccharide-pentapeptide subunits of the peptidoglycan cell wall.

Bacitracin is a potent antibiotic used clinically in combination with other antimicrobial drugs. Two major mechanisms of bacitracin resistance have been studied, the BcrABC transporter, which pumps out bacitracin (20), and BacA, an undecaprenol kinase that generates $C_{55}$-isoprenyl phosphate by de novo synthesis (2). The BcrABC transporter provides immunity to the bacitracin-producing strain *B. licheniformis*. In this system, the BcrB and BcrC proteins form a transmembrane channel, while two BcrA proteins function as ATPases to provide energy for transport (19). Recently, the two-component regulatory system BacRS was identified as a regulator of *bcrABC* expression (18).

The bacitracin resistance gene *bacA* was first identified in *Escherichia coli* (2). BacA homologs were also identified in *Streptococcus pneumoniae* and *Staphylococcus aureus* and are important for resistance to bacitracin, presumably by increasing the synthesis of $C_{55}$-isoprenyl phosphate (6). In some gram-negative bacteria, mutations that block the synthesis of exopolysaccharides also lead to bacitracin resistance, presumably by increasing the supply of the common $C_{55}$-isoprenyl phosphate carrier (22).

We are interested in the physiological roles of the seven extracytoplasmic function (ECF) σ factors (8) in *Bacillus subtilis* that were revealed by sequencing of the genome (16). We have pursued two parallel strategies: identification of target genes for each σ factor, and characterization of stimuli that activate each σ regulon. Previous studies of two of these σ factors, $\sigma^X$ and $\sigma^W$, indicated that one function of the ECF σ factors is to coordinate antibiotic stress responses and cell envelope homeostasis (3, 5, 12). For example, $\sigma^W$ controls the *fosB* fosfomycin resistance gene in *B. subtilis* (3). Studies using DNA microarrays and reporter fusions have revealed that cell wall biosynthesis inhibitors (e.g., vancomycin, D-cycloserine, cephalosporin, and tunicamycin) strongly induce the expression of both *sigW* and the $\sigma^W$ regulon. During the course of these studies, we found that vancomycin also induces $\sigma^M$ and several candidate $\sigma^M$-controlled genes as well as increasing expression of two other ECF σ factors, $\sigma^Y$ and $\sigma^V$ (5).

The *bcrC* (formerly *ywoA)* gene, identified as a component of the vancomycin stimulon, encodes a putative bacteriocin permease similar to the BcrC component of the bacitracin immunity system of *B. licheniformis* (21). In this report, we demonstrate that *bcrC* is important for bacitracin resistance and is controlled by both $\sigma^X$ and $\sigma^M$. In addition to vancomycin, expression of *bcrC* is inducible by bacitracin, and this induction is dependent on $\sigma^M$ but not on $\sigma^X$.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** All *B. subtilis* strains used were derivatives of CU1065 (W168 *trpC2 att*SPβ). The strains HB7007 (*sigX*::*spc*), HB0020 (*sigW*::*MLS*), HB0031 (*sigM*::*kan*), HB7022 (wild type, Px-*cat-lacZ*), and HB7023 (*sigX* Px-*cat-lacZ*) have been described previously (3, 5, 10). *E. coli* strain DH5α was used for standard cloning procedures.

Bacteria were grown in Luria-Bertani (LB) medium at 37°C with vigorous shaking. Antibiotics were added to the growth medium when appropriate: 100 μg of ampicillin per ml for *E. coli*; 100 μg of spectinomycin, 10 μg of kanamycin, 8

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μg of neomycin, and 1 μg of erythromycin plus 25 μg of lincomycin (MLS [for macrolide-lincomycin-streptogramin B resistance]) per ml for *B. subtilis*.

**Construction of *bcrC* and *sigX sigM* mutants.** To construct the *bcrC* mutant, primers 679 (5′-GCGGAATTCATGTGCTATACGCAGGCA) and 680 (5′-GCGGGATCCGAGGCTGCCCAATACATCT) were used to amplify an internal fragment of the *bcrC* gene (~310 bp) from the *B. subtilis* chromosomal DNA. The PCR fragment was digested with *Eco*RI and *Bam*HI and cloned into pMUTIN4 (28) to generate plasmid pSL02. The plasmid was transformed into *B. subtilis* wild-type strain CU1065 and inserted into the *bcrC* locus through Campbell integration, selecting for MLS$^r$. The generated strain (*bcrC*::pMUTIN) was designated HB0106.

A *sigX sigM* double mutant (HB0097) was constructed by transforming chromosomal DNA from HB0031 (*sigM*::*kan*) into HB7007 (*sigX*::*spc*) and selecting for Kan$^r$ and Spc$^r$.

**Construction and analysis of *bcrC* and *sigM* transcriptional fusions.** The *bcrC* promoter region was amplified from the *B. subtilis* chromosomal DNA by PCR using primers 683 (5′-CCAAGCTTCAGAATCCCCCCAGAAA) and 684 (5′-CGGGATCCGTGATGAAGACCAT). The resulting fragment was digested with *Hin*dIII and *Bam*HI and cloned into pJPM122 (25) to generate plasmid pMC100 ($P_{bcrC}$-*cat-lacZ*). The cloned sequence of the promoter region was verified by DNA sequencing (Cornell DNA sequencing facility). The promoter fusion was introduced into SPβ prophage by a double-crossover event, in which plasmid pMC100 was linearized with *Sca*I and transformed into *B. subtilis* strain ZB307A (25) with selection for neomycin resistance. SPβ lysates were prepared by heat induction and used to transduce various recipient strains: CU1065 (wild type), HB7007 (*sigX*::*spc*), HB0020 (*sigW*::*MLS*), and HB0031 (*sigM*::*kan*). The generated strains were designated HB0108 (CU1065, $P_{bcrC}$-*cat-lacZ*), HB0109 (*sigX* $P_{bcrC}$-*cat-lacZ*), HB0110 (*sigW* $P_{bcrC}$-*cat-lacZ*), and HB0111 (*sigM* $P_{bcrC}$-*cat-lacZ*).

Primers 331 (5′-CCCAAGCTTGGGTATATTCCATTGTGCCA) and 436 (5′-CGGGATCCCAGTAAGTCTTCAGCAAGATG) were used to amplify the promoter region of *sigM*. The $P_M$-*cat-lacZ* fusion was constructed following the procedure described above. The generated strains were designated HB0069 (CU1065, $P_M$-*cat-lacZ*) and HB0070 (*sigM* $P_M$-*cat-lacZ*). Note that the constructed *sigM* promoter region includes both the $\sigma^A$- and $\sigma^M$-dependent promoter sites.

To analyze gene expression, the β-galactosidase assay was performed by the procedure of Miller (17).

**Overproduction of $\sigma^X$ and $\sigma^M$ in *B. subtilis* with a xylose-inducible system.** The *sigX* open reading frame (ORF) was PCR amplified with primers 790 (5′-GCGGGATCCAAGTGAACGGAGGGGTTTCA) and 791 (5′-GCGGAATTCCCATCGTCAGCCGCTTGTAA), and the *sigM* ORF was amplified with primers 792 (5′-GCGGGATCCTATAACATAGAGGGGAGAA) and 793 (5′-GCGGAATTCTGGTCGCTCATTTCCCCATT). The two resulting fragments were digested with *Bam*HI and *Eco*RI and cloned into pXT (a derivative of pDG1731 in which the cloned gene is controlled by a xylose-inducible promoter, $P_{xylA}$ [T. Msadek, personal communication]). The generated plasmids were designated pRA01 ($P_{xylA}$-*sigX*) and pRA02 ($P_{xylA}$-*sigM*), respectively. The cloned sequences were verified by DNA sequencing (Cornell DNA sequencing facility).

pRA01 and pRA02 were linearized with *Sca*I and transformed into *B. subtilis* strain CU1065 with selection for Spc$^r$. The transformants were screened for MLS$^s$ and threonine auxotrophy. The resulting strains were designated HB0150 (CU1065, with $P_{xylA}$-*sigX* at the *thrC* locus) and HB0151 (CU1065, $P_{xylA}$-*sigM* at the *thrC* locus). For overproduction of $\sigma^X$ and $\sigma^M$, 50% xylose solution was added to the growth medium to a final concentration of 1%.

**Bacitracin MIC assay.** Overnight cultures were diluted 1:100 into fresh LB medium in the presence of serial twofold dilutions of bacitracin. After incubation at 37°C for 5 h with shaking, the optical density at 600 nm ($OD_{600}$)was measured. Bacitracin zinc salt (70,000 U/g) was purchased from Sigma.

**Primer extension assay.** RNA was prepared from mid-logarithmic-phase cells ($OD_{600} \approx 0.4$) with the Qiagen RNeasy mini kit. Then 100 μg of total RNA and 2 pmol of end-labeled reverse primer 684 were mixed for each primer extension experiment following the procedures described previously (10). The PCR-amplified *bcrC* promoter region (with primers 683 and 684) was sequenced with the same end-labeled reverse primer 684, and the reaction products were electrophoresed adjacent to the primer extension products.

**Probe preparation and RNA slot blot analysis.** A PCR fragment containing the *sigX* ORF (described above) was digested with *Mse*I and purified with the Qiagen PCR purification kit. The resulting ~440-bp fragment (which was deleted in the *sigX* mutant) was labeled by the 3′ fill-in method with Klenow fragment (3′→ 5′ exonuclease negative) (New England Biolabs), dTTP, and [α-$^{32}$P]dATP (New England Nuclear; 3,000 Ci/mmol, 10 mCi/μl). The PCR fragment containing the *sigM* ORF (described above) was digested with *Sac*I and *Cla*I and purified. The resulting ~320-bp fragment (which was deleted in the *sigM* mutant) was labeled by filling in with dCTP and [α-$^{32}$P]dGTP (New England Nuclear; 3,000 Ci/mmol, 10 mCi/μl).

Primers 683 and 691 (5′-AAGAATTCGAAGAAAACAAGAGAT) were used to amplify the complete *bcrC* gene, and primers 689 (5′-AAGGATCCCGTTATGTAAAAA) and 690 (5′-CAGAATTCCTCTTGAATTGACAGA) were used to amplify the complete *yubB* gene. Probe *bcrC* was labeled by digestion with *Hin*dIII, followed by filling in with [α-$^{32}$P]dATP, and probe *yubB* was labeled by digestion with *Bam*HI, followed by filling in with dGTP and [α-$^{32}$P]dATP.

Total RNA was prepared from 3 ml of *B. subtilis* cell culture (from four individual strains, CU1065, HB7007, HB0031, and HB0097) with and without bacitracin treatment. Bacitracin was added to the cell cultures to a final concentration of 7 U when the $OD_{600}$ reached 0.4, and samples were collected 2, 5, 10, and 30 min after induction by addition of 1 ml of a chilled phenol-ethanol (5:95) mixture and centrifugation at 5,000 rpm for 1.5 min at 4°C. The cell pellets were resuspended in 100 μl of lysis buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 3 mg of lysozyme, and 10 U of RNasin RNase inhibitor [ Promega]) and incubated at 37°C for 5 min. The Qiagen RNeasy mini kit was used to extract total RNA from the cell lysates.

The RNA samples were denatured by dissolution in 250 μl of ice-cold 10 mM NaOH–1 mM EDTA buffer and applied to a Zeta-Probe blotting membrane (Bio-Rad) with the Bio-Dot SF microfiltration apparatus (Bio-Rad). The blotted membrane was prehybridized at 55°C for more than 1 h, and then labeled probe (heated at 95°C for 10 min) was added to the hybridization tube. Hybridization was performed at 55°C overnight. The blot was washed twice with a low-stringency buffer, 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaH_2PO_4$, and 1 mM EDTA [pH 7.7]), at room temperature, followed by two high-stringency washes (0.1× SSPE) at 55°C. The blot was then wrapped in plastic wrap and exposed to a Phosphor screen (Molecular Dynamics). To quantify the signals, a Storm imaging system (Storm 840; Molecular Dynamics) was used.

For qualitative analysis, 1 μg of total RNA from each preparation was applied to each slot. To measure the induction of *bcrC* and *sigM* quantitatively, we applied RNA samples prepared from the wild-type strain on the blot in a serial twofold dilution (2, 1, 0.5, and 0.25 μg of RNA). The slot was hybridized with probes *sigM* and *bcrC*, respectively, and the resulting signals were quantified with the ImageQuant data analysis software.

## RESULTS

**Identification of *bcrC* by ROMA and vancomycin-induction microarray analysis.** We have developed a runoff transcription/macroarray analysis (ROMA) technique to identify promoter regions recognized by different alternative σ factors (4). In this technique, a restriction digest of *B. subtilis* chromosomal DNA is transcribed in vitro with RNA polymerase core enzyme, with or without supplementation with the σ factor of interest, to generate a radiolabeled population of runoff RNA molecules. Hybridization of this probe RNA with a DNA macroarray (Sigma/GenoSys) identifies genes that are downstream of promoters that are active in vitro.

Previously, we reported the use of ROMA to aid in the definition of the *B. subtilis* $\sigma^W$ regulon (4), and we have recently extended these studies to include $\sigma^X$. The *ywoA* gene was identified as an in vitro target for transcription with the $\sigma^X$ holoenzyme but was not found in the RNA samples generated with either core alone or $\sigma^W$ holoenzyme (Fig. 1A). In separate studies, we found that *ywoA* is induced by vancomycin (3.4-fold induction after 3 min of treatment and 5-fold after 10 min of treatment) (5). YwoA is 26% identical to the *B. licheniformis* bacitracin ABC transporter BcrC subunit. Since *ywoA* is also important for bacitracin resistance in *B. subtilis* (see below), we renamed *ywoA bcrC*.

**$P_{bcrC}$ is controlled by two ECF σ factors, $\sigma^X$ and $\sigma^M$.** The *bcrC* gene is located between the *nrgAB* operon and the convergent *ywnJ* gene (Fig. 1B) and is therefore likely to be a

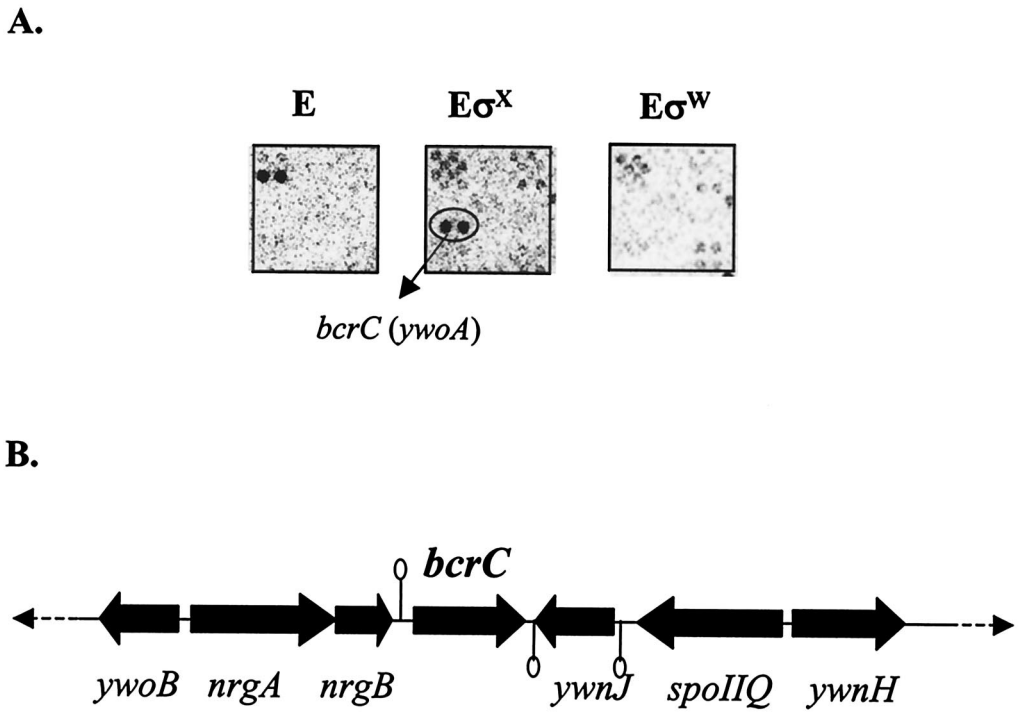


FIG. 1. (A) Identification of the *bcrC* (*ywoA)* gene by ROMA. Total *B. subtilis* chromosomal DNA was digested with *Eco*RI and transcribed in vitro with core alone (E), core with an excess of $\sigma^X$ ($E\sigma^X$), or core with an excess of $\sigma^W$ ($E\sigma^W$). The *bcrC* transcript is apparent in experiments with $E\sigma^X$ (oval), suggesting that *bcrC* is a new candidate for the $\sigma^X$ regulon. The location of *bcrC* on the Sigma/GenoSys macroarray is 1G16:4. (B) The *bcrC* region of the chromosome is illustrated. Predicted rho-independent transcription terminators are indicated by stem-loops.

monocistronic transcription unit. Inspection of the *nrgB-bcrC* intergenic region identified a candidate promoter element (TGAAACtttt-$N_{13}$-aGTCta; lowercase indicates less highly conserved bases) similar to those recognized by other characterized *B. subtilis* ECF σ factors (4, 9, 11–13). It has also been reported that *bcrC* is a target for $\sigma^M$ (A. Moir, personal communication).

To investigate the roles of $\sigma^X$, $\sigma^W$, and $\sigma^M$ in the expression of *bcrC*, we integrated a $P_{bcrC}$-*cat-lacZ* reporter fusion ectopically at SPβ. The resulting reporter fusion was transduced into the wild-type strain CU1065 or mutant strains altered in *sigX* (HB7007), *sigW* (HB0020), or *sigM* (HB0031), and β-galactosidase activity was measured throughout growth. The expression of *lacZ* was generally very low in the wild-type strain, with maximal expression during late logarithmic growth (Fig. 2A). Consistent with the results of ROMA, expression in the *sigX* mutant strain was reduced ca. threefold. In contrast, expression was slightly higher than in the wild-type strain in a *sigW* mutant, consistent with our previous observation that $\sigma^X$ and $\sigma^W$ are mutually antagonistic (11). We conclude that $\sigma^X$ plays a significant role in driving expression from the $P_{bcrC}$-*cat-lacZ* reporter fusion.

Unexpectedly, however, expression was reduced to background levels in the *sigM* mutant strain, suggesting that $\sigma^M$ also directs transcription from the cloned promoter region and that, in this strain, $\sigma^X$ activity at this promoter site was reduced. This is not a general effect on $\sigma^X$ activity, since other $\sigma^X$-dependent promoters were still active in the *sigM* mutant strain (data not shown).

**$\sigma^X$ and $\sigma^M$ initiate transcription of *bcrC* from the same promoter site.** To further clarify the roles of $\sigma^X$ and $\sigma^M$ in *bcrC* transcription, we mapped the *bcrC* transcriptional start site with primer extension of RNA extracted from the wild type, the *sigX* mutant, the *sigM* mutant, and the *sigX sigM* double mutant strain. Although 100 μg of total RNA was used in each reaction, we observed a very faint transcript only in the wild type, consistent with the low-level expression observed with the reporter fusion. Transcription initiated with an A residue 6 nucleotides downstream from the predicted AGTC −10 element (Fig. 2B). The same transcript was obtained in both the *sigX* and *sigM* single mutants but was not detectable in the *sigX sigM* double mutant. This suggests that either $\sigma^X$ or $\sigma^M$ can recognize this promoter element in vivo.

To further explore the roles of $\sigma^X$ and $\sigma^M$ in directing transcription of *bcrC*, we overproduced either $\sigma^X$ or $\sigma^M$ with a xylose-inducible promoter system and prepared RNA for primer extension mapping. The *bcrC* transcript was significantly more abundant in strains containing either the $P_{xylA}$-*sigX* or $P_{xylA}$-*sigM* fusion (Fig. 2B), even in the absence of xylose induction (presumably due to leaky expression from the xylose-inducible promoter). We did not detect any other transcripts in this assay under conditions that would detect transcripts initiating anywhere within 150 bases upstream of the start codon. Thus, expression of *bcrC* appears to be dependent on both $\sigma^X$ and $\sigma^M$, and these two σ factors activate the same promoter element.

Alignment of the *bcrC* promoter with the *sigX*, *sigM*, and *sigW* autoregulatory promoters revealed similarities in both the

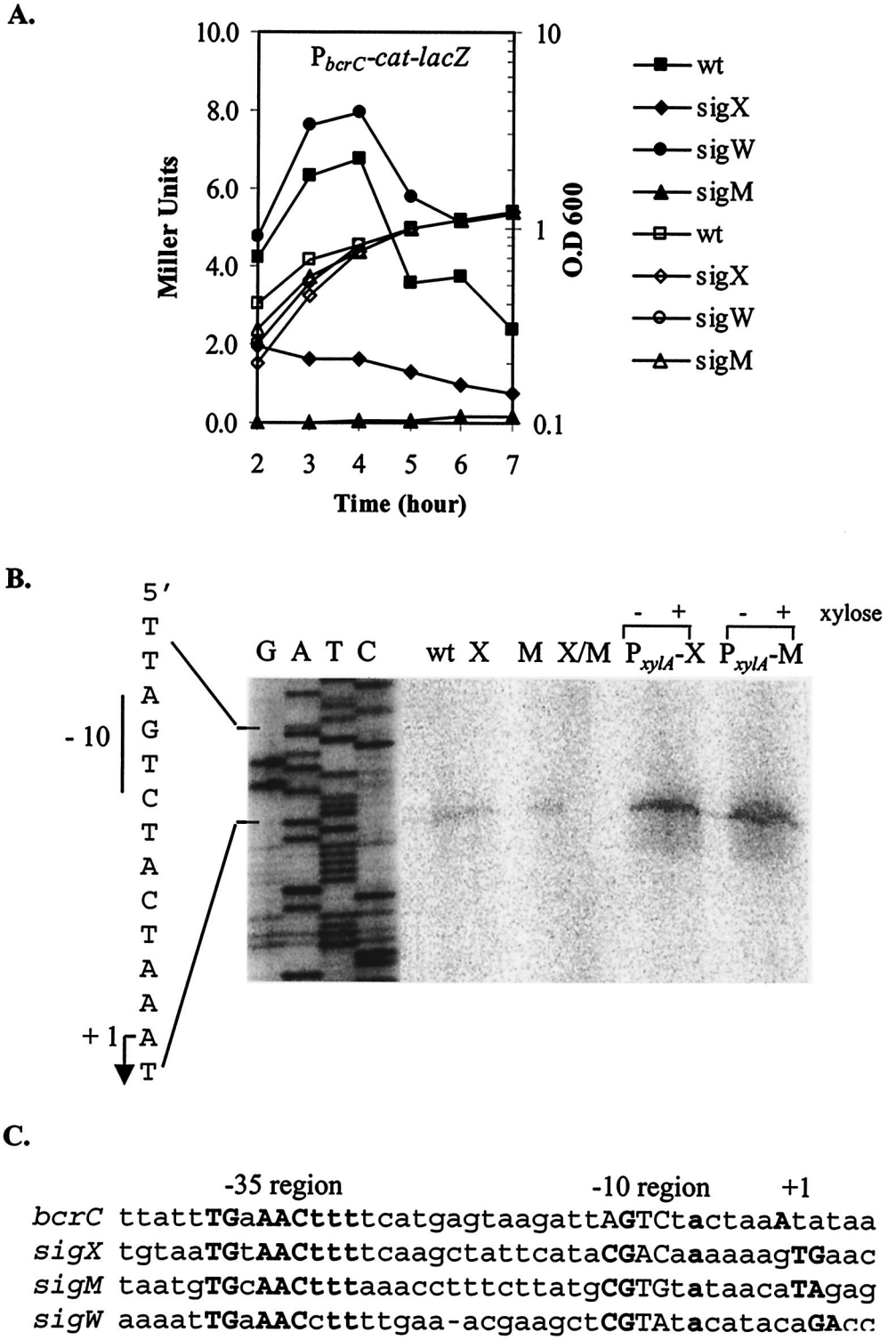


FIG. 2. (A) Expression of P$_{bcrC}$-*cat-lacZ* during growth in liquid culture. *B. subtilis* strains HB0108 to HB0111 were grown in LB medium, and β-galactosidase activities were determined at each time point. β-Galactosidase activities are illustrated by solid symbols, and growth curves are illustrated as open symbols. (B) Mapping of the transcription start site by primer extension. RNA was extracted from mid-logarithmic phase wild-type (wt), *sigX* (X), *sigM* (M), and *sigX sigM* (X/M) mutant cells, and the wild-type strain overproducing either $\sigma^X$ (P$_{xylA}$-X) or $\sigma^M$ (P$_{xylA}$-M) in the presence (+) or absence (−) of xylose. The transcription start site and the −10 element are indicated. The same primer was used to sequence the promoter region to index the reverse transcript (lanes AGCT). (C) Comparison of the *bcrC* promoter sequence with the promoters recognized by the *B. subtilis* ECF σ factors $\sigma^X$ (10), $\sigma^M$ (9), and $\sigma^W$ (11).

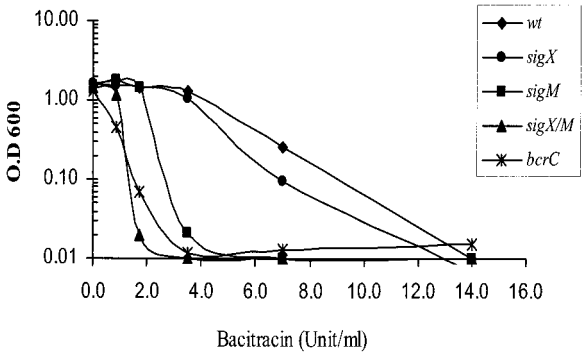


FIG. 3. Effects of bacitracin on growth of *B. subtilis* strains. All strains were grown for 5 h after dilution into LB medium containing the indicated concentration of bacitracin. This experiment was repeated three times, and the results shown are representative. wt, wild type.

−35 and −10 regions (Fig. 2C). Previously, we demonstrated that $\sigma^X$ and $\sigma^W$ have overlapping recognition specificity and that −10 elements with a CGTC motif tend to be recognized by both σ factors (23). In this case, both $\sigma^X$ and $\sigma^M$ recognized an AGTC motif in the −10 region. However, other sequence elements are also likely to participate in promoter discrimination.

***bcrC* and *sigM* mutants are sensitive to bacitracin.** To determine the physiological role of BcrC in *B. subtilis*, we constructed a *bcrC* null mutant by gene disruption with an integrational plasmid. The *bcrC* mutant strain was eightfold more sensitive to bacitracin than the wild-type strain (Fig. 3) but was unaffected in its sensitivity to a variety of other antibiotics (cephalosporin, D-cycloserine, fosfomycin, penicillin, ristocetin, tetracycline, tunicamycin, and vancomycin) and bacteriocins (e.g., bacilysin, colicin E, polymyxin, gramicidin, magainin, and nisin). The *sigM* mutant had a fourfold-increased sensitivity to bacitracin, while the *sigX* mutant was only slightly sensitive. The *sigX sigM* double mutant was as sensitive as the *bcrC*

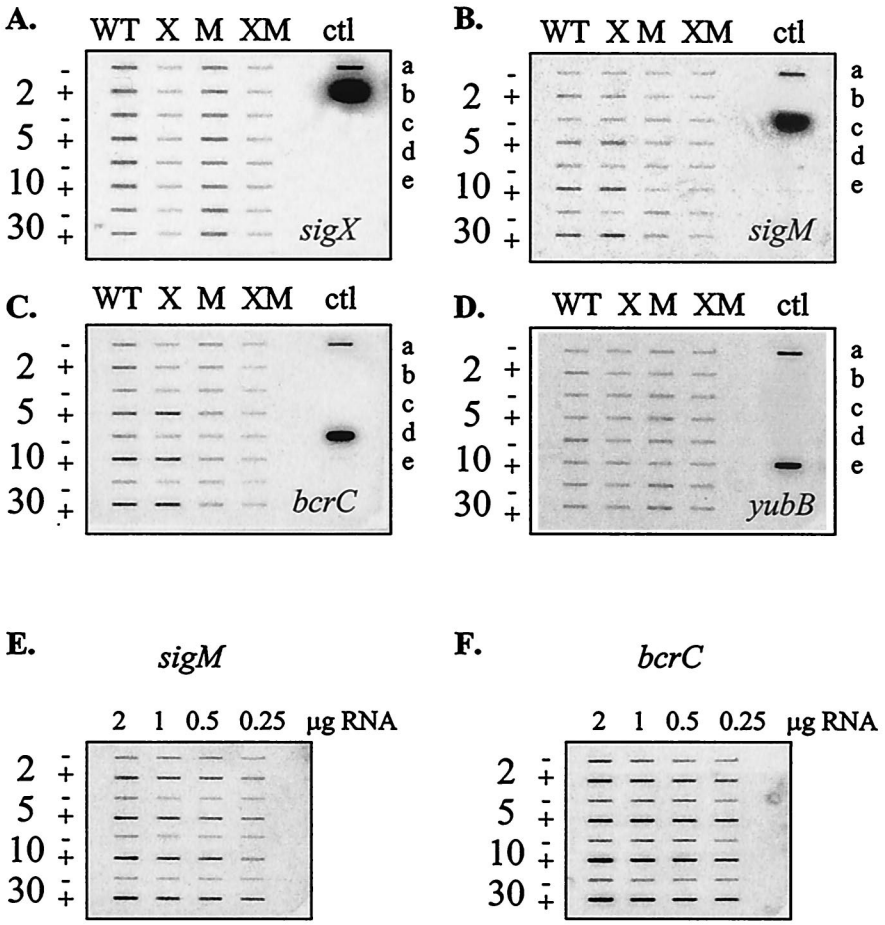


FIG. 4. Slot blot analyses of expression of *sigX* (A), *sigM* (B), *bcrC* (C), and *yubB* (D) in response to bacitracin treatment. WT, X, M, and XM indicate that RNA samples applied to the corresponding column were extracted from the wild-type, *sigX*, *sigM*, or *sigX sigM* mutant strain, respectively. The − and + symbols indicate that RNA samples in this row were extracted from cells not treated (−) or treated (+) with bacitracin after 2, 5, 10 or 30 min. A total of 1 μg of total RNA was applied to each slot in these semiquantitative assays. Positive controls were applied to the control (ctl) column. a, *B. subtilis* chromosomal DNA; b, PCR fragment of the *sigX* ORF; c, PCR fragment of the *sigM* ORF; d, PCR fragment of the *bcrC* ORF; and e, PCR fragment of the *yubB* ORF. To quantify the induction of *sigM* and *bcrC* expression by bacitracin, RNA samples extracted from the wild-type strain were applied to the blot in a serial twofold dilution series (2, 1, 0.5, and 0.25 μg). The blot was hybridized with probe *sigM* (E) or *bcrC* (F), and the resulting signals were quantified with the ImageQuant data analysis software.

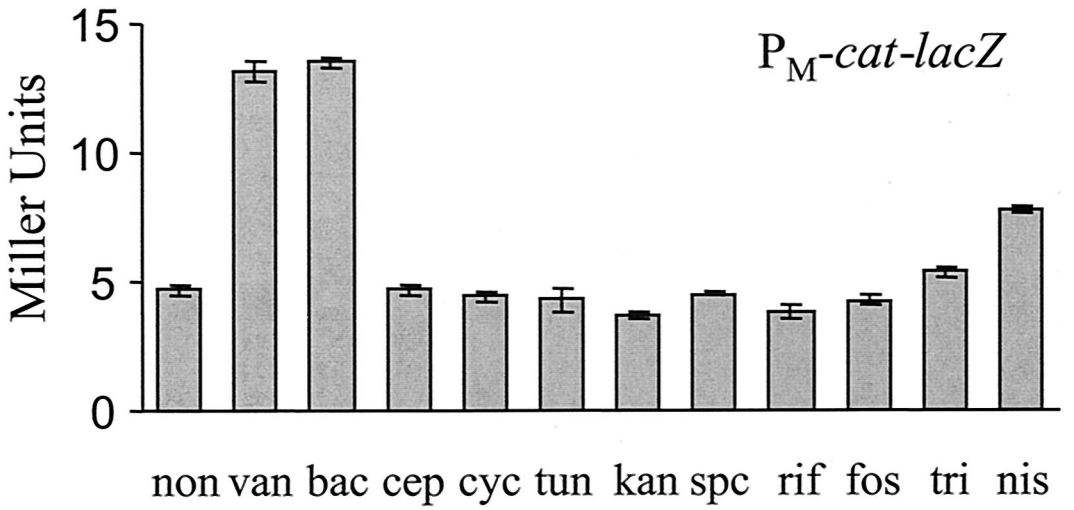


FIG. 5. Expression of $P_M$-*cat-lacZ* in response to various antibiotic treatments. *B. subtilis* strain HB0069 (wild type, $P_M$-*cat-lacZ*) was grown in LB medium to an $OD_{600}$ of 0.3. The cell culture was split and treated with antibiotics as indicated for 30 min. Cell pellets were immediately harvested by centrifugation and stored at −80°C. Antibiotics and their final concentrations (in micrograms per milliliter) in the medium were as follows: none (non); vancomycin (van), 1; bacitracin (bac), 100 (=7 U/ml); cephalosporin (cep), 1; D-cycloserine (cyc), 10; tunicamycin (tun), 50; kanamycin (kan), 100; spectinomycin (spc), 100; rifampin (rif), 20; fosfomycin (fos), 100; Triton X-100 (tri), 0.05%; and nisin (nis), 50. Error bars represent standard deviations from the average of three independent experiments.

mutant, consistent with the observation that *bcrC* expression was eliminated in the *sigX sigM* double mutant strain.

**Expression of *bcrC* is induced by bacitracin through $\sigma^M$.** We used slot blot analysis of RNA levels to investigate the ability of bacitracin to induce expression of *bcrC*, *sigX*, and *sigM* (Fig. 4). As expected, the *sigX* transcript was detected in both wild-type and *sigM* mutant strains, but only a background level of hybridization was observed in the *sigX* and *sigX sigM* mutants. Expression of *sigX* was not induced by bacitracin (Fig. 4A). In contrast, both *sigM* (Fig. 4B) and *bcrC* (Fig. 4C) were induced by bacitracin. Induction was apparent after 2 min of treatment and reached a plateau after 5 min. Quantitation of the RNA signals indicated that *sigM* was induced about three- to four-fold, while *bcrC* was induced two- to threefold (Fig. 4E and F; see Materials and Methods also). The induction of *bcrC* by bacitracin was $\sigma^M$ dependent and was readily apparent in both the wild-type and *sigX* mutant strains but not in the *sigM* or *sigX sigM* mutants. We also tested expression of *yubB*, encoding a possible BacA homolog (see Discussion), and found that this gene was expressed constitutively and was not induced by bacitracin (Fig. 4D).

**Induction of *sigM* by other antibiotics.** Previously, we reported that *sigM* and several candidate $\sigma^M$-dependent genes were strongly induced by vancomycin treatment (5), and the results above indicate that *sigM* was also induced by bacitracin. To test whether other antibiotics could also induce *sigM*, we treated HB0069 (CU1065 $P_M$-*cat-lacZ*) with various antibiotics at subinhibitory concentrations and measured *lacZ* expression by β-galactosidase assays. As expected, both vancomycin and bacitracin induced $P_M$-*cat-lacZ* expression by more than two-fold. Expression of $P_M$-*cat-lacZ* decreased to background levels in the *sigM* mutant, and no induction was observed, consistent with the results from slot blot experiments. There was also a slight induction by nisin (Fig. 5). Although we did not measure induction of $P_M$ by cephalosporin, D-cycloserine, or tunicamycin in cells grown in liquid culture, we did observe apparent induction on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) plates, as judged by the formation of blue halos. This same phenomenon was observed previously in induction of the $P_W$-*cat-lacZ* fusion by cell wall-active antibiotics (5).

## DISCUSSION

We identified the *bcrC* (formerly *ywoA*) gene as a target gene for regulation by ECF σ factors based on two independent lines of evidence. First, *bcrC* was identified as an in vitro target for $\sigma^X$-directed transcription in studies of the $\sigma^X$ regulon with the ROMA technique (Fig. 1A). Second, *bcrC* was found to be a component of the vancomycin stimulon, which includes numerous members of both the $\sigma^W$ and $\sigma^M$ regulons (5). The results presented here demonstrate that *bcrC* is an important determinant of bacitracin resistance in *B. subtilis* and that its expression is controlled by both $\sigma^X$ and $\sigma^M$. Induction of *bcrC* by bacitracin (Fig. 4C) and vancomycin (5) was dependent on $\sigma^M$ and independent of $\sigma^X$ (Fig. 4C and data not shown).

Judging from the similarity to the BcrABC efflux system, we hypothesize that BcrC may form a dimeric channel that facilitates the efflux of bacitracin. The protein(s) that may function to energize such an efflux pump (analogous to BcrA) is currently not known. Similarly, *E. coli* encodes a BcrC homolog but does not encode obvious homologs of BcrA or BcrB, suggesting that BcrC may interact heterologously with another ABC transporter system to derive the energy to support active export (7). Precedence for such a heterologous interaction is provided by the *Staphylococcus epidermidis* erythromycin exporter MsrA, an ATP-binding protein which interacts with transmembrane proteins from *S. aureus* and *Staphylococcus* (24). Recently, Ohki and colleagues also reported that a *ywoA* mutant was hypersensitive to bacitracin (R. Ohki et al., Abstr.

102nd Annu. Meet. Am. Soc. Microbiol., abstr. 240, 2002). Furthermore, they reported that disruption of several genes encoding putative ATP-binding proteins (e.g., *yxlF*, *yfiL*, *yhcH*, and *ycbN*) did not have any effect on bacitracin resistance.

The bacitracin resistance gene *bacA* was first identified in *E. coli* and encodes a membrane-associated isoprenol kinase (2). BacA, which functions in the de novo synthesis of $C_{55}$-isoprenyl phosphate, also confers bacitracin resistance in *S. pneumoniae* and *S. aureus* (6). *B. subtilis* also encodes a BacA homolog: the product of the *yubB* gene is 44% identical to *E. coli* BacA. To investigate the possible role of YubB in bacitracin resistance, we sought to disrupt the gene by allelic replacement. However, several attempts were unsuccessful, indicating that the gene may be essential. Expression of *yubB* was not induced by bacitracin, nor was it affected by mutations in *sigX*, *sigM*, or both genes (Fig. 4D). Thus, the possible role of YubB in bacitracin resistance remains an open question.

The results presented here further strengthen the functional link between ECF σ factors and resistance to antimicrobial compounds. Previously, we demonstrated that the $\sigma^W$ regulon is strongly induced by vancomycin and other inhibitors of cell wall biosynthesis (5). At least one member of the $\sigma^W$ regulon, FosB, plays a direct role in the detoxification of an antibiotic (3), and others may also contribute to antibiotic resistance (12). The $\sigma^X$ regulon has been found to include both the *pssA-psd* operon and the *dlt* operon. The products of these operons both serve to decrease the net negative charge density in the cell envelope, and this contributes to increased resistance to cationic antimicrobial peptides (M. Cao and J. D. Helmann, unpublished results).

The discovery of $\sigma^M$ and the $\sigma^M$ regulon as a component of the vancomycin stimulon (5) and the finding that *bcrC* contributes to bacitracin resistance suggest that this σ factor also plays a significant role in antibiotic resistance. The functions of the remaining four ECF σ factors encoded in *B. subtilis* ($\sigma^V$, $\sigma^Y$, $\sigma^Z$, and $\sigma^{YlaC}$) are still unknown.

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