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A Bacitracin-Resistant *Bacillus subtilis* Gene Encodes a Homologue of the Membrane-Spanning Subunit of the *Bacillus licheniformis* ABC Transporter

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Bacitracin is a peptide antibiotic nonribosomally produced by *Bacillus licheniformis*. The *bcrABC* genes which confer bacitracin resistance to the bacitracin producer encode ATP binding cassette (ABC) transporter proteins, which are hypothesized to pump out bacitracin from the cells. *Bacillus subtilis* 168, which has no bacitracin synthesizing operon, has several genes homologous to *bcrABC*. It was found that the disruption of *ywoA*, a gene homologous to *bcrC*, resulted in hypersensitivity to bacitracin. Resistance to other drugs such as surfactin, iturin A, vancomycin, tunicamycin, gramicidin D, valinomycin and several cationic dyes were not changed in the *ywoA* disruptant. Spontaneous bacitracin-resistant mutants (Bcr-1 and -2) isolated in the presence of bacitracin have a single base substitution from A to G in the ribosome binding region. Northern hybridization analysis and determination of the expression of *ywoA-LacZ* transcriptional fusion gene revealed that the transcription of the *ywoA* gene was dependent on extracytoplasmic function (ECF) σ factors σ^M and σ^X. Preincubation of wild-type cells in the presence of a low concentration of bacitracin induced increased resistance to bacitracin about two- to threefold, although the mechanism of this induction has not yet been elucidated. It has been reported that a commercially available bacitracin is a mixture of several components and also contains impurity. Bacitracin A was purified by reverse phase high-performance liquid chromatography (HPLC). Similar results were obtained with bacitracin A as those with crude bacitracin, indicating that contaminating substances were not responsible for the results obtained in this study.

Bacitracin is a dodecapeptide antibiotic produced by some strains of *Bacillus licheniformis* and *Bacillus subtilis* (2, 11). The synthesis is nonribosomally catalyzed by a multienzyme complex composed of three subunits, BacA, BacB, and BacC, whose genes have been cloned and sequenced (6, 9, 12, 18, 22). Bacitracin has potent antibiotic activity against gram-positive bacteria (30). The inhibition of peptidoglycan biosynthesis is the best-characterized bactericidal effect of bacitracin (27). It forms a complex mediated by a metal ion (Zn^{2+}) with the lipid C_{55}-isoprenyl pyrophosphate (IPP) (24, 26), which is a carrier of a peptidoglycan unit or a disaccharide with pentapeptide across the membrane. Bacitracin, by binding to IPP, inhibits the conversion of IPP to C_{55}-isoprenyl phosphate, which is catalyzed by a membrane associated pyrophosphatase (25).

B. licheniformis, a bacitracin producer, has an ABC transporter system which is hypothesized to pump out bacitracin for self-protection (19). The transporter is composed of two membrane proteins, BcrB and BcrC, and two identical ATP-binding subunits, BcrA. Neumüller et al. recently reported that in *B. licheniformis*, *bcrABC* genes are localized about 3 kb downstream of the bacitracin biosynthetic operon *bacABC* (14). Between the *bacABC* operon and *bcrABC* genes, they also identified *bacR* and *bacS* genes which encode proteins with high homology to response regulator and sensory kinase of two-component regulatory systems and are involved in the regulation of *bcrA* expression.

The *B. subtilis* genome project determined the entire DNA sequence of strain 168 and found that there are two operons which encode nonribosomal peptide antibiotic synthetase complexes (13). One is a surfactin synthetase operon (4), and the other is a plipastatin (fengycin) synthetase operon (29, 31). There is no bacitracin synthetase operon in *B. subtilis* 168. *B. subtilis* 168 is more sensitive to bacitracin than *B. licheniformis*, a bacitracin producer. Heterologous expression of *bcrABC* transporter in *B. subtilis* results in an increase of bacitracin resistance to a level similar to that observed in a bacitracin producer (5, 19, 20). A homology search reveals that in *B. subtilis* 168, there are several homologues of BcrA, -B, and -C of *B. licheniformis.* In this study we showed that the disruption of the *ywoA* gene, which encodes the BcrC homologue, resulted in a marked decrease of resistance to bacitracin. We also reported that the transcription of the *ywoA* gene was dependent on extracytoplasmic function (ECF) σ factors, σ^M and σ^X.

MATERIALS AND METHODS

Bacterial strains and plasmids. *B. subtilis* 168 (*trpC*2 *glt*) was used as a wild-type strain. The pMutin insertion mutants constructed by members of the Japan and European Union consortia of *B. subtilis* functional genomics were used as a disrupted mutant of the following genes: *ycbN*, *yhcH*, *yfiL*, *yxlF*, *yhcI*, *ydbk*, and *ywoA*. In these mutants, the 5′-upstream region of the genes disrupted is transcriptionally fused to the *lacZ* gene (33). For construction of the YWOAd mutant, the 174-bp fragment (position +22 to +195 relative to the translational

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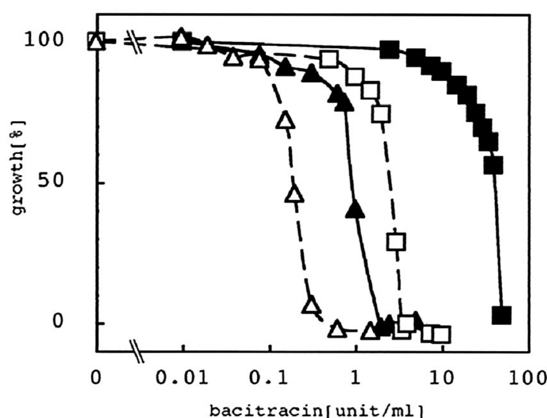

FIG. 1. Bacitracin sensitivity of *ywoA* disruptant (YWOAd). *B. subtilis* wild-type 168 and YWOAd were grown in L medium to early log phase. A 0.1-ml culture was added to a 96-well titer plate containing 0.1 ml of L medium with various concentrations of bacitracin. Growth was monitored by measuring OD_{540} after 5 h as described in Materials and Methods. Growth at various concentrations of bacitracin was expressed as the percent increase in the OD_{540} over that observed in the absence of bacitracin. Squares, wild-type strain 168; triangles, YWOAd. Open symbols, L medium with 40 μg/ml $ZnSO_4$.

start point of the *ywoA* gene) was cloned into pMutin3, and the resulting plasmid was transformed into *B. subtilis* 168. The transformant was selected by erythromycin resistance, to give the YWOAd mutant. In order to eliminate the possibility of secondary genetic changes, chromosomal DNAs were prepared from these disruptants and transformed into *B. subtilis* 168. The transformants were selected by erythromycin resistance and were named YCBNd and YHCHd, etc. The correct insertion of pMutin was confirmed by PCR.

The pMutin insertion mutants SIGMd, SIGXd, SIGId, and SIGVd, were constructed as follows. An internal fragment of the *sigM*, *sigX*, *sigI*, and *sigV* genes was amplified by PCR (primers used: sigMd_F, 5′-AAGAAGCTTACCA AATGTACATGAATG; sigMd_R, 5′-GGAGGATCCTTTATCTCTACCTGA TGG; sigXd_F, 5′-AAGAAGCTTTCAATTATTATATGATACATATC; sigXd_R, 5′-GGAGGATCCGAGTATGCGCTGTCTG; sigId_F, 5′-AGAAGCTTCC AGTGCTTAGCCTTTTG; sigId_R, 5′-GGAGGATCCATTTTGTGCGCTTC TGGC; sigVd_F, 5′-AAGAAGCTTGCTTGTCACATGCATAAC; sigVd_R, 5′-GGAGGATCCTTGTAATGGTCTTCC). The amplified DNA fragments were digested with *Hin*dIII and *Bam*HI and then cloned into pMutin3 digested with the same restriction enzymes. The resulting plasmids were transformed into *B. subtilis* 168 to obtain insertional disruptants through a single-crossover event (33). The *lacZ* gene of SIGMd was inactivated through the insertion of a tetracycline resistance gene of pBEST307 (10) between the 132nd and 1,012th codons, and the resulting strain was named SIGMd/*lacZ*::*tet*. The chromosomal DNA of this strain was prepared and transformed into YWOAd, SIGXd, and SIGVd. Transformants selected by tetracycline and erythromycin resistance yielded double mutants, YWOAd/*sigMd*, SIGXd/*sigMd*, and SIGVd/*sigMd*. The DNA region encompassing the structural gene for sigM and its ribosomal binding sequence (RBS) was amplified by PCR (primers used: sigM_F, 5′-GTCGT CGACGTGTATAACATAGAGGGG; sigM_R, 5′-GCAGCATGCAGTCATT TCCTGGTCG) and were cloned into pDG148 (28), resulting in pDG148-*sigM*. The fidelity of PCR was confirmed by sequencing.

In order to insert the *ywoA* gene at the *amyE* locus, pDLd2 was used. PDLd2 is a *lacZ* deletion derivative of pDL which has a multicloning site and a *lacZ* gene and a chloramphenicol resistance gene between the *amyE* front and *amyE* back regions (34). The *ywoA* gene from the promoter to the ρ-independent transcriptional terminator was amplified by PCR (primers used: ywoA_F, 5′-TGCACA GAATCCCCCCAGAAA; ywoA_R, 5′-TGGCGAAGCGAAGAAAACAAG) using chromosomal DNA of the wild type or BcrR mutant as a template and cloned into PCR2.1-Topo vector (Invitrogen). After the nucleotide sequence of the insert was confirmed by sequencing, a 0.8-kb *ywoA* fragment obtained by *Eco*RI digestion was cloned into the *Eco*RI site of the pDLd2 vector. The resulting plasmid was linearized by *Nde*I digestion and transformed into YWOAd, and then chloramphenicol-resistant colonies were selected. The correct insertion of the *ywoA* gene at the *amyE* locus was confirmed by PCR. The resulting strain was named YWOAd/*amyE*::*ywoA*.

DNA sequence analysis. DNA sequencing was carried out with an ABI Prism 310 genetic analyzer (PE Biosystems). In order to confirm the fidelity of the PCR amplifications, the DNA sequences of the inserts in all the recombinant plasmids were determined.

Northern hybridization analysis. Total RNA was extracted from cells grown in L or DSM medium with hot phenol as described by Aiba et al. (1). Total RNA (5 μg) was electrophoresed on a 1.5% formaldehyde-agarose gel and blotted onto a nylon membrane. A 180-bp DNA fragment containing the *ywoA* cording region was used as a probe. The labeling reactions were performed with [α-^{32}P]dCTP and the Multiprime labeling system (Amersham Pharmacia Biotech). Northern hybridization was carried out at 42°C as described previously (16). As an internal control for the Northern blot hybridization, the same membrane was reprobed with a 319-bp PCR fragment containing the *rrnA* gene. The intensity of the mRNA band was normalized with that of the 16S rRNA band.

Primer extension analysis of RNA. Two picomoles of XRITC (rhodamine X-isothiocyanate)-labeled oligonucleotide primer (5′-XRITC-AGGGCATAGG CGACAATGGC) complementary to the sequence localized 86 to 106 bp downstream from the putative initiation codon of the *ywoA* gene was annealed with 15 μg of total RNA at 47°C. The first strand cDNA was synthesized by Superscript II RNaseH$^-$ reverse transcriptase (Invitrogen) according to the procedure recommended by the manufacturer and was subjected to 8 M urea–6% polyacrylamide gel electrophoresis.

β-Galactosidase assays. At various growth phases, cells were harvested by centrifugation and the resulting pellet was frozen at −25°C. Frozen cells were resuspended in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$) (15) containing DNase (10 μg/ml) and lysozyme (100 μg/ml) and were incubated at 37°C for 20 min. Cell extract obtained by centrifugation was used for β-galactosidase assay in Z buffer containing *o*-nitrophenyl-β-D-galactopiranoside (1 mg/ml) and 38 mM β-mercaptoethanol. One unit of β-galactosidase activity was defined as the amount of enzyme that catalyzed the production of 1 μmol of *o*-nitrophenol/h/optical density at 530 nm (OD_{530}) unit

Assay of drug-resistant phenotype. *B. subtilis* cells were grown in L medium to early log phase (OD_{530}, 0.3). A 0.1-ml culture was added to a 96-well titer plate that contained 0.1 ml of L medium with serially diluted concentrations of a given drug. Growth was traced by measuring the OD_{540} after 5 h. Growth at various concentrations of the drug was expressed as the percent increase in OD_{540} observed in the absence of the drug. In order to calculate relative resistance of

TABLE 1. Bacitracin resistance of various strains

Expt	Strain	Mutation site	Relative resistance[a] in medium	
			L	L + $ZnSO_4$ [40 μg/ml]
1	168	aaaaggtg[b]	1	1
	YWOAd		0.034	0.065
	BcrR-1	aaaGggtg	2.7	2.4
	BcrR-2	aaGaggtg	2.8	2.5
2	168		1	1
	YWOAd/*amyE*::*ywoA*(w)		0.87	0.94
	YWOAd/*amyE*::*ywoA*(BcrR-1)		2.0	1.9

[a] Relative resistance was determined by dividing the IC_{50} for mutant strains by the IC_{50} for control strain 168.
[b] RBS of the *ywoA* gene (http://genolist.pasteur.fr/SubtiList/). Only mutated nucleotides are shown in uppercase letters.

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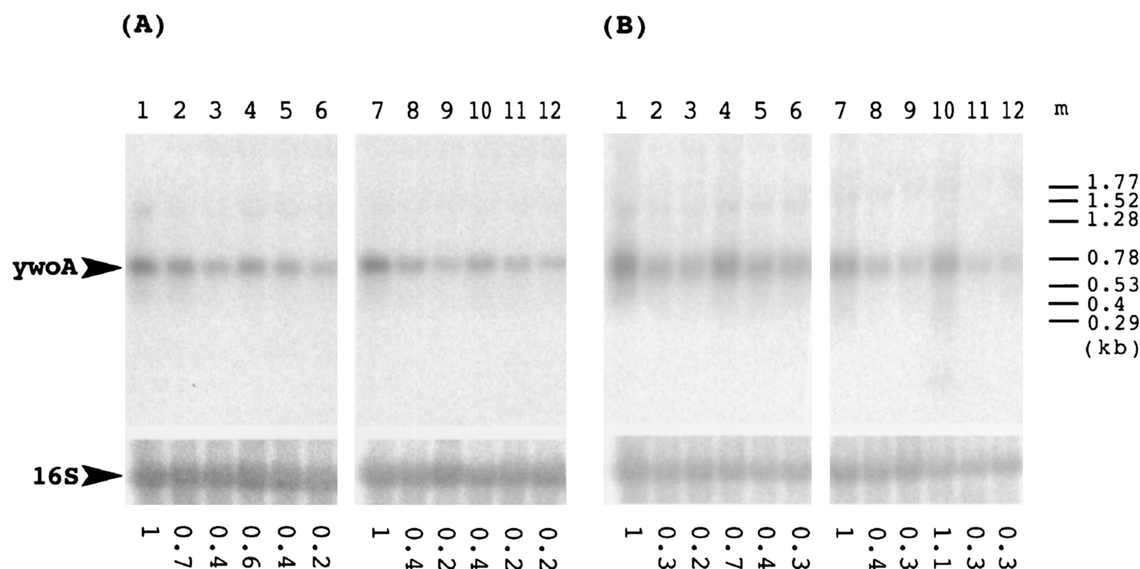

FIG. 2. Northern hybridization analysis of RNA isolated from wild-type strain 168, *sigM*-disrupted mutant, SIGMd (A), and strain 168 after preincubaton with bacitracin (B). *B. subtilis* 168 and SIGMd cells were grown in L and DSM media to early (OD_{530}, 0.5; lanes 1, 4, 7, and 10), middle (OD_{530},1.2; lanes 2, 5, 8, and 11), and late (OD_{530},2.0; lanes 3, 6, 9, and 12) log phases. Isolation of total RNA and Northern hybridization was carried out as described in Materials and Methods. 5 μg of RNA was loaded per lane. ^{32}P-labeled *ywoA* probe was used. The same membranes were reprobed with ^{32}P-labeled 16S rRNA fragment and the results shown in the lower part of the figure. The radioactivity of each band was quantified by using a BAS 2000 Imaging Analyzer (Fuji). The relative amounts of *ywoA* mRNA normalized with that of 16S rRNA are shown below. (A) Lanes: 1 to 3, 168 in L medium; 4 to 6, SIGMd in L medium; 7 to 9, 168 in DSM medium; 10 to 12, SIGMd in DSM medium. (B) Lanes: 1 to 3, L medium; 4 to 6, L medium containing bacitracin (10 U/ml); 7 to 9, DSM medium; 10 to 12, DSM medium containing bacitracin (10 U/ml); m, molecular size standard (0.16 to 1.77-kb RNA ladder; Invitrogen).

various mutant strains, the concentration of the drug that gave 50% inhibition of growth was expressed as a ratio of that required to inhibit growth by 50% in the control strain.

Chromatographic purification and mass spectrometry analysis of bacitracin A. Bacitracin (70 U/mg; Sigma) was purified by preparative high-performance liquid chromatography (HPLC) with a UV detector at 254 nm and reversed-phase C_{18} column (particle size, 10 μm; column dimensions, 19 mm by 30 cm; Waters) using a linear gradient from of acetonitrile-water-trifluoroacetic acid (TFA) from 0:100:0.1 to 50:50:0.1 (by volume) for 30 min at a flow rate of 10 ml/min. Bacitracin A fractions were combined and concentrated to about one-third of the initial volume under reduced pressure at 35 to 40°C. The residual solution was dialyzed against water overnight at 4°C, to remove traces of acetonitrile and TFA with Spectra/Pro CE (molecular weight cutoff, 500; Spectrum Medical Industries, Inc.) and subsequently lyophilized to give a white powder.

A Micromass ZQ mass spectrometer (Waters) equipped with an electrospray ionization source was used in a positive-ionization mode for mass spectral confirmation of bacitracin A. Mass conditions were as follows: capillary voltage, 3.0 kV; cone voltage, 90 V; source temperature, 120°C; cone gas flow, 65 liters/h; desolvation gas flow, 215 liters/h.

RESULTS

Hypersensitivity of the *ywoA* disrupted mutant toward bacitracin. Homology search revealed that in *B. subtilis* 168, there are several homologues of BcrA, BcrB, and BcrC of *B. licheniformis* (13). YcbN, YhcH, YfiL, and YxlF showed 45, 37, 33, and 33% identity to BcrA, which is an ATP-binding subunit of the efflux transporter. YdbK and YhcI are homologues of BcrB, which is a membrane-spanning subunit, and both showed 24% identity to BcrB. YwoA is a homologue to BcrC and showed 28% identity to BcrC. We measured bacitracin resistance of these seven gene-disrupted mutants and found that only *ywoA* disruptants (YWOAd) showed hypersensitivity to bacitracin (Fig. 1). Increased sensitivity to bacitracin in the presence of $ZnSO_4$, which mediates binding between bacitracin and IPP, has been reported previously (24). In the presence of $ZnSO_4$ (40 μg/ml), which caused no growth inhibition on its own, the sensitivity was further increased in both wild type and YWOAd. The 50% inhibitory concentration (IC_{50}) of wild-type strain 168 was 2.45 and 39.9 U/ml in the presence or absence of $ZnSO_4$ (40 μg/ml). Relative resistance was calculated from the growth inhibition curve shown in Fig. 1, as a ratio of the IC_{50}s. Relative resistance of YWOAd decreased to 6.5 and 3.4% of that of the wild-type strain in the presence and absence of $ZnSO_4$, respectively (Table 1, experiment 1). In order to confirm that the hypersensitive phenotype is caused by the *ywoA* gene disruption, the wild-type *ywoA* gene was introduced into the *amyE* locus on the chromosome of YWOAd. Bacitracin resistance recovered to a level similar to that of the wild-type strain by complementation of the *ywoA* gene (Table 1, experiment 2).

Isolation of bacitracin-resistant mutants (BcrR). Wild-type cells grown in L medium were plated on L agar containing bacitracin at 10 U/ml and $ZnSO_4$ at 40 μg/ml. The number of colonies that appeared on this plate after overnight incubation at 37°C was less than $1/10^6$ of that on the L plate containing $ZnSO_4$ (40 μg/ml) alone. A single colony was inoculated into L medium containing bacitracin (10 U/ml) and $ZnSO_4$ (40 μg/ml). After being grown overnight, cells were plated on L agar containing bacitracin (20 U/ml) and $ZnSO_4$ (40 μg/ml). Two colonies which were larger than the rest were named BcrR-1 and -2. BcrR-1 and -2 showed increased resistance to bacitracin. An almost two- to threefold increase in relative resistance was obtained (Table 1, experiment 1). The chromosomal

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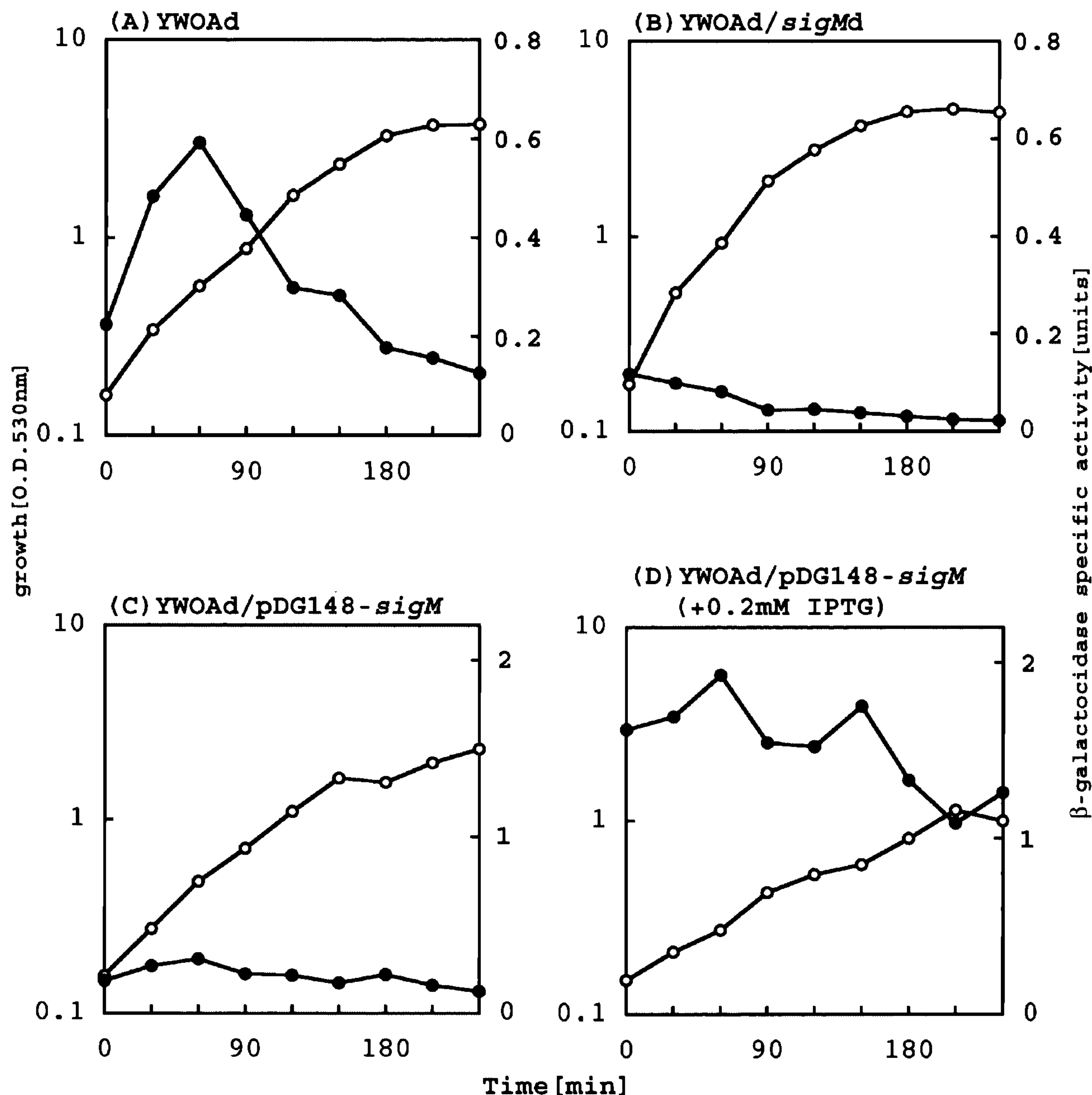

FIG. 3. σ^M-dependent expression of the *ywoA-lacZ* transcriptional fusion gene. YWOAd (A) and YWOAd/*sigMd* (B) mutant cells having a *ywoA-lacZ* transcriptional fusion gene were grown in DSM medium. YWOAd/pDG148-*sigM* cells were grown in DSM medium with (D) or without (C) 0.2 mM IPTG. β-Galactosidase activity was assayed at the times indicated. Open circles, growth (OD_{530}); closed circles, β-galactosidase activity (units).

DNAs of BcrR-1 and -2 were prepared and transformed into wild-type cells. The transformant showed levels of resistance similar to that of the parent strains. Determination of the nucleotide sequences of the *ywoA* gene of BcrR mutants revealed that only a single base substitution, A to G, occurred in the RBS region of BcrR mutants, as shown in Table 1. When the *ywoA* gene of BcrR mutant was complemented at the *amyE* locus of the YWOAd, the resistance recovered to a level similar to that of the parent BcrR mutant (Table 1, experiment 2), indicating that a base substitution in the RBS region results in the increased resistance of BcrR mutants. From these results, it is hypothesized that a base substitution in the RBS region resulted in the increased production of YwoA which contributed to the increased resistance in BcrR.

Identification of the *ywoA* transcript and the sigma factor dependency of the promoter. Northern hybridization analysis of total RNA isolated from *B. subtilis* 168 grown in L or DSM medium with a probe containing *ywoA* coding region identified a 0.6-kb band (Fig. 2A). A faint band around 1.5 kb is due to nonspecific hybridization to rRNA. The size of the transcript

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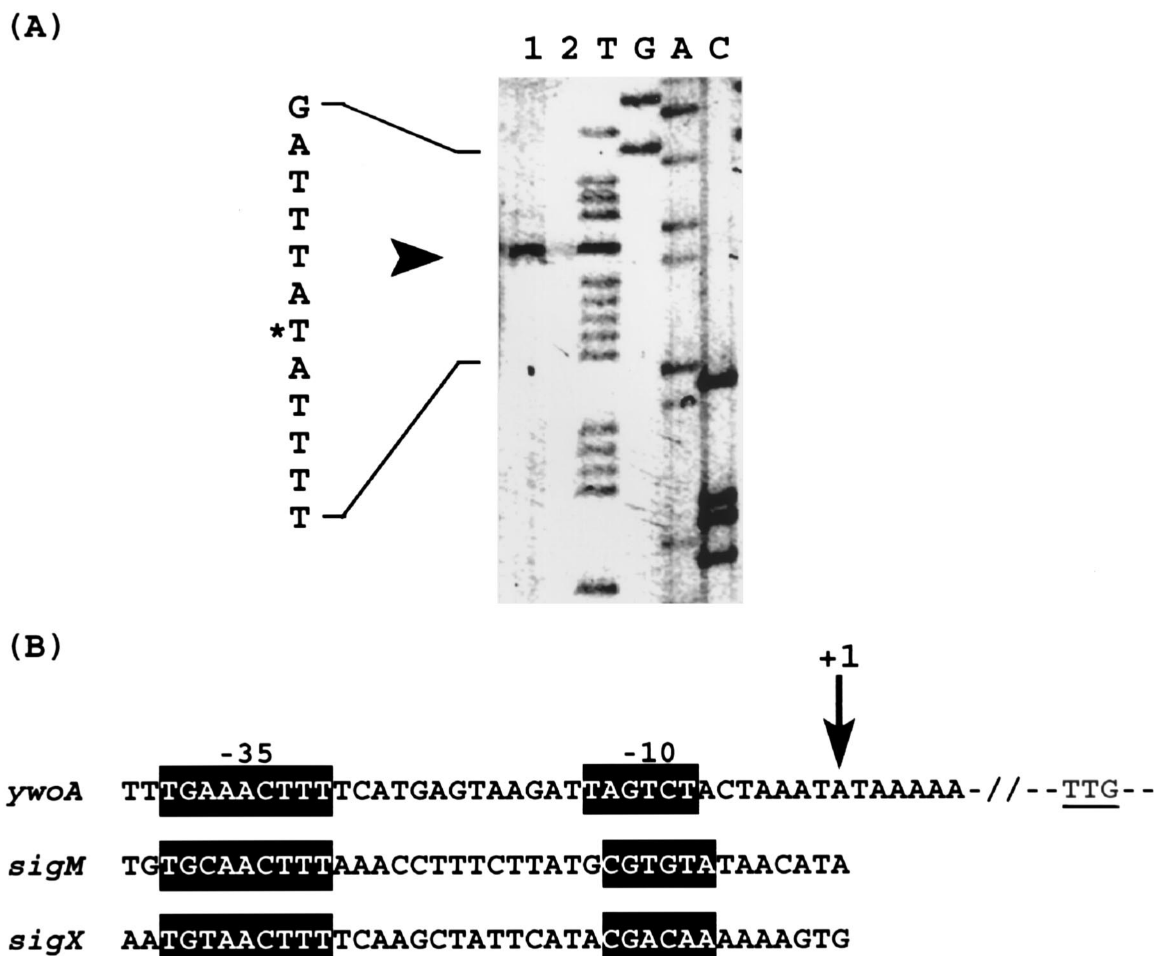

FIG. 4. Mapping of the 5′ end of the *ywoA* mRNA by primer extension analysis. (A) Total RNA was isolated from early log phase culture of wild-type strain 168 and SIGMd mutant grown in L medium. Fifteen micrograms of total RNA was used for primer extension analysis. The potential transcription start site is marked with arrows. Lanes: 1, 168; 2, SIGMd; T, G, A, and C, dideoxy sequencing ladder obtained with the same primer used for primer extension. (B) Comparison of *ywoA* promoter sequence with promoters recognized by the ECF σ factors, σ^M and σ^X, of *B. subtilis*.

indicated that the *ywoA* gene is transcribed as a monocistronic mRNA from a promoter located upstream of the *ywoA* coding region and terminated at the ρ-independent transcriptional terminator signal found downstream of the *ywoA* gene. The amount of *ywoA* mRNA was higher in early-log-phase than in late-log-phase cells grown in L medium as well as in DSM medium (Fig. 2A). In order to investigate the expression pattern of the *ywoA* gene during various growth phases, the expression of the reporter *lacZ* gene was monitored in YWOAd which contained the transcriptional fusion of the *ywoA* promoter region to the *lacZ* gene. The expression of *lacZ* fusion was maximal during early exponential phase and was followed by an immediate decrease as cells reached the stationary phase (Fig. 3A). This corresponds to the results obtained in Northern hybridization.

A putative promoter sequence was found in the upstream region of the *ywoA* gene. A −35 consensus sequence is similar to those typical of ECF σ factor-dependent promoters (Fig. 4B) (7, 8). In order to determine what σ factor is involved in the transcription of the *ywoA* gene, we measured the bacitracin resistance of the mutants in which one of the ECF σ factor genes, *sigM*, *sigX*, *sigV*, or *sigI*, was disrupted. SIGMd and SIGXd showed a significant decrease in resistance both in the presence or absence of $ZnSO_4$, whereas SIGVd and SIGId showed a small decrease in the absence of $ZnSO_4$ (Table 2, experiment 1). As the decrease in resistance was maximal in SIGMd, the dependency of *ywoA* transcription on σ^M was also examined in the expression of *ywoA-lacZ* fusion. As shown in Fig. 3B, the expression of the *ywoA-lacZ* fusion decreased to a lower level during all growth phases in YWOAd/*sigMd*. When YWOAd transformed with pDG148-*sigM* was grown in the presence of 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG), the expression of *ywoA-lacZ* fusion was increased severalfold (Fig. 3C and D). Northern hybridization experiments showed that the amount of *ywoA* mRNA decreased to almost 50% of that in the wild-type strain in both L and DSM mediums (Fig. 2A). The growth phase dependency of *ywoA* mRNA level in SIGMd was similar to that in the wild-type strain. All of the results indicate that transcription of *ywoA* gene was dependent on σ^M.

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TABLE 2. Bacitracin resistance of various sigma factor-deficient strains

Expt	Strain	Relative resistance in medium	
		L	L+$ZnSO_4$
1	168	1	1
	SIGMd	0.32	0.51
	SIGXd	0.63	0.68
	SIGVd	0.84	1.1
	SIGId	0.78	1.0
2	168	1	1
	SIGMd/*LacZ*::*tet*	0.40	0.37
	SIGXd/*sigM*d	0.056	0.027
	SIGVd/*sigM*d	0.22	0.28
	YWOAd	0.034	0.063
3	168/pDG148 − IPTG	1	1
	+ IPTG (0.2mM)	0.98	1.0
	168/pDG148-*sigM* − IPTG	1.2	1.1
	+ IPTG (0.2mM)	0.92	1.0

About 30 to 40% of the decrease in relative resistance in SIGXd showed that the expression of *ywoA* was partially dependent on σ^X. The resistance to bacitracin in SIGMd was still higher than that of the *ywoA* disruptant. To determine whether σ^X is involved in the residual expression of the *ywoA* gene in the absence of σ^M, we constructed strains in which two ECF σ factors were inactivated. In a SIGXd/*sigMd* double mutant, the resistance decreased to a low level similar to that of the *ywoA* disruptant, whereas the SIGVd/*sigMd* double mutant showed a level of resistance similar to that of SIGMd (Table 2, experiment 2). These results indicate that in the absence of σ^M, most of the transcription of the *ywoA* gene was dependent on σ^X.

When wild-type strain 168 transformed with pDG148-*sigM* was grown in L medium containing 0.2 mM IPTG, no increase in resistance was observed (Table 2, experiment 3). From the results obtained using the *ywoA-LacZ* fusion gene, overexpression of *sigM* by the addition of IPTG resulted in increased transcription of the *ywoA* gene. These results indicated that the cellular level of *ywoA* mRNA was not a limiting factor for determining resistance in wild-type strain.

The transcriptional start site was determined by primer extension of RNA extracted from both wild type and SIGMd. As shown in Fig. 4, a single band was detected, corresponding to a start site located 23 bp upstream of the *ywoA* translational start site in the wild-type strain. A weak band at the same site was detected in SIGMd. These results indicate that in the absence of the σ^M protein, transcription of the *ywoA* gene dependent on σ^X started at the same initiation site.

Increased resistance induced by preincubation with low concentrations of bacitracin. Wild-type cells were grown overnight in L medium containing bacitracin at 10 or 20 U/ml and were grown to early log phase after inoculation into the same medium. When this culture was used for the bacitracin resistance assay, an approximately twofold increase in relative resistance was observed comparing the cells grown in L medium without bacitracin (Table 3). The induction of resistance was also observed in BcrR as well as the SIGMd mutant (Table 3). In order to determine whether the increase in resistance was due to the increase of *ywoA* gene expression at the transcriptional level, the amount of *ywoA* mRNA prepared from wild-type cells grown in L or DSM medium with or without addition of bacitracin was determined. Northern hybridization analysis revealed that preincubation with bacitracin did not cause an increase in the amount of *ywoA* mRNA (Fig. 2B).

Growth inhibition by purified bacitracin A. Commercially available bacitracin (Sigma) is a mixture of similar peptides (3, 17, 30, 32). Bacitracin A and B make up about 40 to 60% of commercial bacitracin and are major components responsible for antibiotic activity (3). It was also reported that commercial bacitracin is contaminated with a subtilisin-type protease (23). In order to confirm that the results obtained with commercial bacitracin in this study are dependent on purified bacitracin rather than other impurities, we fractionated commercial bacitracin by reverse-phase HPLC. The chromatographic profile of commercial bacitracin using gradient elution as described in the Materials and Methods is shown in Fig. 5A. The elution pattern obtained is similar to that reported previously (3, 32). The amount of bacitracin A was calculated to be 42%. We scaled up to preparative HPLC to obtain sufficient amounts of purified bacitracin A. The HPLC pattern of the purified bacitracin A preparation is shown in Fig. 5B, and the purity was calculated as 92%. The mass spectrum of this fraction is shown in Fig. 5C. Purified bacitracin A exhibited a protonated molecular ion $[M+H]^+$ at *m/z* 1423.5 and the sodium adduct ion $[M+Na]^+$ at *m/z* 1445.5 (molecular weight of bacitracin A: $C_{66}H_{103}N_{17}O_{16}S$, 1,422.7 g/mol). We used this purified preparation of bacitracin A in the experiments of growth inhibition and induction of bacitracin resistance and found that similar results were obtained with purified bacitracin A as with crude bacitracin, indicating that contaminating substances were not responsible for the results reported in this paper. The specific activity of purified bacitracin A to inhibit growth per unit of OD_{254} increased (Table 4).

Disruption of the *ywoA* gene had no effect on resistance to other drugs. We tested the resistance of the *ywoA* disrupted mutant to surfactin, iturin A, vancomycin, tunicamycin, gramicidin D, valinomycin, ethidium bromide, deoxycholate, daunomycin, daunorubicin, and rhodamine 6G. No significant difference in sensitivity to these drugs was observed between the wild-type strain and YWOAd, indicating that YWOA is specifically involved in resistance to bacitracin.

TABLE 3. Increase in resistance by preincubation with bacitracin

Strain	Preincubation medium	Relative IC_{50}[a]
168	L	1
	L+bacitracin (10 U/ml)	2.0
	L+bacitracin (20 U/ml)	2.0
BcrR-1	L	1
	L+$ZnSO_4$ + bacitracin (20 U/ml)	4.5
SIGMd	L	1
	L+bacitracin (2.5 U/ml)	2.2
	L+bacitracin (5 U/ml)	2.2

[a] Relative IC_{50} was determined by dividing the IC_{50} obtained with preincubation by the IC_{50} without preincubation.

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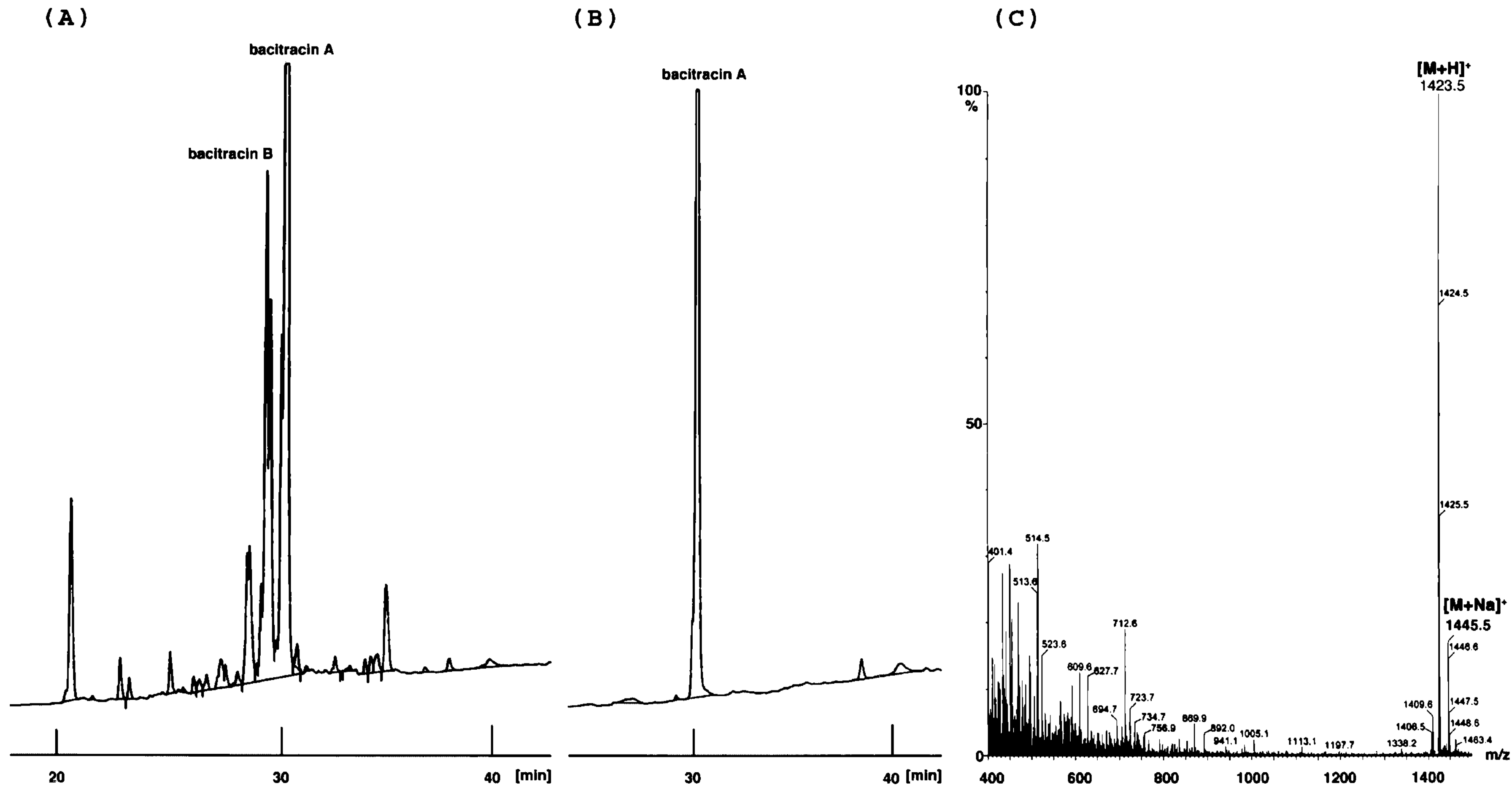

FIG. 5. Reverse-phase HPLC chromatograms of commercially available bacitracin and purified bacitracin A. Commercially available bacitracin (Sigma) (A) and purified bacitracin A (B) were analyzed by HPLC using a UV detector at 254 nm. Reversed-phase C_{18} column (particle size, 5 μm; column dimensions, 3.0 mm by 15 cm; Shiseido) was used with a 30-min linear gradient of acetonitrile-water-TFA from 0:100:0.1 to 50:50:0.1 (by volume) at a flow rate of 0.7 ml/min. Contents of bacitracin A in commercially available bacitracin was calculated as 40%. Purity of bacitracin A preparation was calculated as 92%. (C) Mass spectrum of purified bacitracin A. Mass spectrometry analysis of bacitracin A was carried out as described in Materials and Methods.

TABLE 4. Growth inhibition by purified bacitracin A

Strain	Antibiotic	Relative IC_{50} medium	
		L	L+$ZnSO_4$
168	Bacitracin[a]	1	1
	Bacitracin A	0.28	0.26
YWOAd	Bacitracin[a]	1	1
	Bacitracin A	0.38	0.49

[a] Commercially available bacitracin (Sigma).

DISCUSSION

In *B. licheniformis*, a bacitracin producer, BcrABC proteins belonging to the ABC transporter family are involved in bacitracin resistance. We found that a disruptant of the *ywoA* gene which encodes a homologue of BcrC showed hypersensitivity toward bacitracin in *B. subtilis*. Although there is no direct evidence of the efflux of bacitracin by YwoA as well as BcrC, the results indicate that growth inhibition of *ywoA* disrupted mutant is hypothesized to be caused by the lack of bacitracin efflux.

Transcription of the *ywoA* gene is dependent on ECF σ factors σ^M and σ^X. In the absence of both σ factors, no expression of the *ywoA* gene was observed, at least under the conditions in this study. One of the most well-characterized σ^M-dependent operons in *B. subtilis* is the σ^M operon itself, which contains *sigM*, *yhdL*, and *yhdK* genes (7). The expression of the σ^M operon and that of the *ywoA* gene have the same growth phase dependency, maximal in early log phase and minimal in late log phase. Many σ^X-dependent promoters have been reported, some of which are also recognized by σ^W (8). Our result show that both σ^M and σ^X dependent transcription of *ywoA* initiate from the same position indicating that there is also some overlap in promoter selectivity between σ^M and σ^X.

Neumüller et al. reported that in *B. licheniformis*, preincubation with bacitracin induced an increase in resistance to bacitracin along with an increase the cellular level of BcrA (14). They reported that two component regulatory system, BacRS was involved in the regulation. While searching for a homologue of BacRS in *B. subtilis*, we found that YcbLM showed a relatively high homology to BacRS. However, disruption of *ycbL* or *ycbM* had no effect on resistance to bacitracin or on the induction of resistance by preincubation.

In the vicinity of the *ywoA* gene, no gene encoding nucleotide-binding domain (NBD) was found. In order to find an NBD partner of *ywoA*, we examined the bacitracin resistance in disruptants of the *bcrA* homologue, YCBNd, YHCHd, YFILd, and YXIPd. No decrease in resistance was observed in these disruptants. Quentin et al. reported that the *B. subtilis* genome encodes for at least 78 ABC transporters that can be divided into 38 importers and 40 extruders (21). Among 40 extruders, 7 orphan NBD genes are not associated with membrane-spanning domain partners, whereas 33 NBD genes are associated with membrane-spanning domain subunit genes in the same operon or in their neighborhood. If YwoA has a NBD partner, it is most probable that it belongs to the former group. We examined bacitracin resistance in disruptants of six genes, *expZ*, *ydiF*, *yfmM*, *yfmR*, *ykpA*, and *ycbN*, except *yurY* which has been shown indispensable for growth (T. Tanaka, personal communication). No decrease in bacitracin resistance was observed in these disruptants. Therefore, it has not yet been determined whether YwoA has an NBD partner, or NBD which belongs to another ABC transporter contributes to the energy supply for the bacitracin transporter.

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