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Zwittermicin A Resistance Gene from *Bacillus cereus*

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Zwittermicin A is a novel aminopolyol antibiotic produced by *Bacillus cereus* that is active against diverse bacteria and lower eukaryotes (L. A. Silo-Suh, B. J. Lethbridge, S. J. Raffel, H. He, J. Clardy, and J. Handelsman, Appl. Environ. Microbiol. 60:2023–2030, 1994). To identify a determinant for resistance to zwittermicin A, we constructed a genomic library from *B. cereus* UW85, which produces zwittermicin A, and screened transformants of *Escherichia coli* DH5α, which is sensitive to zwittermicin A, for resistance to zwittermicin A. Subcloning and mutagenesis defined a genetic locus, designated *zmaR*, on a 1.2-kb fragment of DNA that conferred zwittermicin A resistance on *E. coli*. A DNA fragment containing *zmaR* hybridized to a corresponding fragment of genomic DNA from *B. cereus* UW85. Corresponding fragments were not detected in mutants of *B. cereus* UW85 that were sensitive to zwittermicin A, and the plasmids carrying *zmaR* restored resistance to the zwittermicin A-sensitive mutants, indicating that *zmaR* was deleted in the zwittermicin A-sensitive mutants and that *zmaR* is functional in *B. cereus*. Sequencing of the 1.2-kb fragment of DNA defined an open reading frame, designated ZmaR. Neither the nucleotide sequence nor the predicted protein sequence had significant similarity to sequences in existing databases. Cell extracts from an *E. coli* strain carrying *zmaR* contained a 43.5-kDa protein whose molecular mass and N-terminal sequence matched those of the protein predicted by the *zmaR* sequence. The results demonstrate that we have isolated a gene, *zmaR*, that encodes a zwittermicin A resistance determinant that is functional in both *B. cereus* and *E. coli*.

Emerging resistance raises intense concern for the continued efficacy of antimicrobial agents in medicine and agriculture. Certain mechanisms of antibiotic resistance that are emerging in target organisms are thought to have their origins in antibiotic-producing organisms (4, 10, 11, 49). Thus, investigations into mechanisms of antibiotic self-resistance may be relevant to understanding mechanisms by which sensitive organisms become antibiotic resistant. Studies of antibiotic self-resistance have been useful in identifying clusters of biosynthetic genes and in defining modes of action of antibiotics. Studies of self-resistance have uncovered fundamental knowledge of molecular processes in bacteria and have allowed the development of molecular systems for the study of a broad range of bacteria. For example, the understanding of sporulation, secondary metabolite production, and molecular biology of the *Streptomyces* spp. has advanced by elucidating mechanisms of self-resistance, regulation of resistance mechanisms, and the relationship of resistance to antibiotic biosynthesis (16–18, 22, 37, 50–52).

Antibiotic-producing bacteria that contain a target that is sensitive to the antibiotic produced by the cell must carry genetic determinants for self-resistance. Mechanisms of self-resistance fall into three general classes (10): (i) inactivation of the antibiotic by chemical modification (22, 33, 35); (ii) rapid efflux or sequestering of the antibiotic from the vicinity of the target site or the interior of the cell (45, 51); and (iii) modification or replacement of the sensitive target site with an insensitive form (16, 23, 34, 46). Genes encoding self-resistance are often clustered with genes encoding other functions required for antibiotic production, namely, biosynthetic enzymes, export pathways, and regulators of production. Certain biosynthetic enzymes or export pathways do double duty as mechanisms of self-resistance (10).

We have identified a novel antibiotic from *Bacillus cereus*, designated zwittermicin A (21, 32, 38, 39, 42). Zwittermicin A is a linear aminopolyol that is active against a variety of gram-positive and gram-negative eubacteria (38). Certain oomycetes (lower eukaryotes) that are pathogenic on plants are highly sensitive to zwittermicin A (38). Zwittermicin A also interacts synergistically with Bt toxin to enhance the insecticidal activity of *Bacillus thuringiensis* (28). The production of antibiotics by *Bacillus* species was previously considered to be limited to peptide antibiotics and to antibiotics containing amino sugars (24, 25, 29). Thus, zwittermicin A is a molecule with a striking range of biological activities which may represent a new class of molecules from *Bacillus* spp.

Elucidation of the mechanisms by which zwittermicin A-producing strains are rendered zwittermicin A resistant will enhance our understanding of the biology of *Bacillus* spp. and of this novel molecule. A study of zwittermicin A self-resistance will advance our understanding of the mode of action of zwittermicin A and may provide an indirect approach to isolating the genes required for zwittermicin A biosynthesis in *B. cereus*. In this study, we exploited the zwittermicin A sensitivity of *Escherichia coli* to isolate and characterize a zwittermicin A self-resistance gene, designated *zmaR*, from *B. cereus* UW85.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture media. The strains and plasmids used in this study are described in Table 1. Mutants of *B. cereus* UW85 were isolated by screening mitomycin C-induced mutants (UW030) or spontaneous mutants (all others) of UW85 for loss of either biological control activity or zwittermicin A production (38, 39). Spontaneous mutants carried a Tn*917* marker on their indigenous plasmid, pBC85. Plasmids constructed in this study are described in Fig. 1.

Unless otherwise indicated, *B. cereus* strains were grown in Trypticase soy broth (TSB) or on Trypticase soy agar (TSA) prepared to half the strength directed by the manufacturer (Difco Laboratories, Detroit, Mich.), with the following antibiotics added at the indicated concentrations: spectinomycin, 200 mg/liter; erythromycin, 10 mg/liter. *E. coli* strains were grown on Luria-Bertani

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference or source
B. cereus		
UW85	Wild type	20
UW030	UW85 *zma-030*; lacks pBC85	39
UW101	UW85 pBC85-101::Tn*917*	39
UW101C	UW101 cured of pBC85-101::Tn*917*	38
UW167	UW85 *zma-167*; pBC85-167::Tn*917*	39
UW167C	UW167 cured of pBC85-167::Tn*917*	This study
UW226	UW85 *zma-226*; pBC85-226::Tn*917*	39
UW226C	UW226 cured of pBC85-226::Tn*917*	This study
UW325	UW85 *zma-325*; pBC85-325::Tn*917*	39
UW325C	UW325 cured of pBC85-325::Tn*917*	This study
UW419	UW85 *zma-419*; pBC85-419::Tn*917*	39
UW419C	UW419 cured of pBC85-419::Tn*917*	38
UW452	UW85 *zma-452*; pBC85-452::Tn*917*	39
UW452C	UW452 cured of pBC85-452::Tn*917*	38
UW457	UW85 *zma-457*; pBC85-457::Tn*917*	39
UW457C	UW47 cured of pBC85-457::Tn*917*	38
SNY14	Zwittermicin A nonproducing soil isolate	43
E. coli		
C2110	*polA* Nal^r	43
DH5α	ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR supE44 hsdR17 recA1 endA1 gyrA95 thi-1 relA1*	19
DH5αF′IQ	DH5α F′ *proAB*$^+$ *lac*^{q}ZΔM15 *zzf*::Tn*5* (Km^r)	Gibco-BRL
HB101	F^- *hsdS20* (r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rspL20* (Sm^r) *xyl-5 mtl-1 supE44* λ^-	36
Plasmids		
pDL278	Broad-host-range shuttle vector; Spc^r	12
pHT304	*E. coli-Bacillus* shuttle vector; Amp^r Erm^r; multiple cloning site of pUC18	3
pGEM-3Zf(+)	Cloning and sequencing vector; Amp^r	Promega Corp.
pLA2917	Broad-host-range cosmid vector; Km^r Tc^r	1
pZMR6	pLA2917 with 18-kb insert; *zmaR*	This study
pZMR7	pLA2917 with 19-kb insert; *zmaR*	This study
pZMR7-3	pZMR7 *zmaR*::Tn*3*	This study
pHoKmGus	Tn*3*-*gus* vector; *tnpA* Amp^r Km^r *tnpA*$^+$ Cm^r	5
pSShe		42
pRK2013	Helper plasmid; Km^r	13

[a] *zma* designations such as *zma-030* indicate defects in zwittermicin A production. *zmaR* designates the zwittermicin A resistance gene. Cm^r, chloramphenicol resistant.

(LB) broth or agar (36) with the following antibiotics added at the indicated concentrations: tetracycline, 20 mg/liter; kanamycin, 10 or 50 mg/liter; ampicillin, 100 mg/liter; spectinomycin, 100 mg/liter; chloramphenicol, 25 mg/liter; nalidixic acid, 10 mg/liter. Isopropyl-β-D-thiogalactopyranoside (IPTG), supplied at 1 mM, and 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc), supplied at 50 μM, were obtained from Research Organics (Cleveland, Ohio).

DNA manipulations. Plasmid DNA was isolated from *E. coli* by the alkaline lysis protocol (36) or with Qiagen (Chatsworth, Calif.) plasmid kits. Total genomic DNA was isolated from *B. cereus* strains as described previously (39). *B. cereus* strains were transformed by the electroporation method described previously (39). *E. coli* strains were transformed by electroporation as directed in the operating manual for the Gene Pulser transfection apparatus (Bio-Rad Laboratories, Hercules, Calif.). Plasmid profiles of *B. cereus* strains were visualized on Eckhardt gels (15, 48), or plasmids were recovered by the alkaline lysis protocol. Agarose gel electrophoresis, restriction digests, and ligation reactions (T4 DNA ligase) were performed by standard procedures and as described in the manufacturers' instructions. DNA fragments were recovered from agarose gels with the Qiaex gel extraction kit (Qiagen).

For Southern blot analysis, DNA was transferred to Magna nylon membrane as described in the manufacturer's specifications (Micro Separations, Inc., Westboro, Mass.). Digoxigenin-dUTP labeling of probes, hybridization, and blot development were performed with a labeling and colorimetric development kit as described in the manufacturer's specifications (Boehringer Mannheim Corp., Indianapolis, Ind.). We routinely used the high-stringency conditions defined in the instructions. However, for Southern blots in which *B. cereus* genomic DNA was the target, we observed that probes failed to hybridize to the target under high-stringency conditions, presumably because *B. cereus* DNA is AT rich (36% GC) (9). For those blots, we used reduced-stringency hybridization conditions, the hybridization temperature was reduced from 42 to 40°C and excess probe was washed from blots with 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C instead of 0.1× SSC at 68°C.

Elimination of Tn*917*-marked pBC85 from *B. cereus* strains. *B. cereus* UW85 carries a cryptic plasmid, designated pBC85 (38, 39). pBC85 was cured from mutants of UW85 in which the plasmid was marked with Tn*917* (erythromycin resistant [Erm^r]) by the following procedure (38). Mutants were transformed with pDL278, which carries spectinomycin resistance (12), and transformants were selected on TSA-spectinomycin plates. A spectinomycin-resistant (Spc^r) transformant was grown for 4 days in TSB with spectinomycin, the culture was plated for single colonies on TSA plates containing spectinomycin, and single colonies were patched on TSA-erythromycin plates. Erm^s colonies arose at a high frequency, 10^{-2}, which suggested that pBC85::Tn*917* was incompatible with pDL278 (38, 39). An Erm^s Spc^r colony was inoculated into TSB without antibiotics, grown for 4 days, and plated on TSA plates without antibiotics. Colonies were patched on TSA containing spectinomycin and TSA containing erythromycin to identify Erm^s Spc^s colonies, which would lack both pDL278 and the Tn*917*-marked pBC85. These putative cured isolates were tested for the loss of

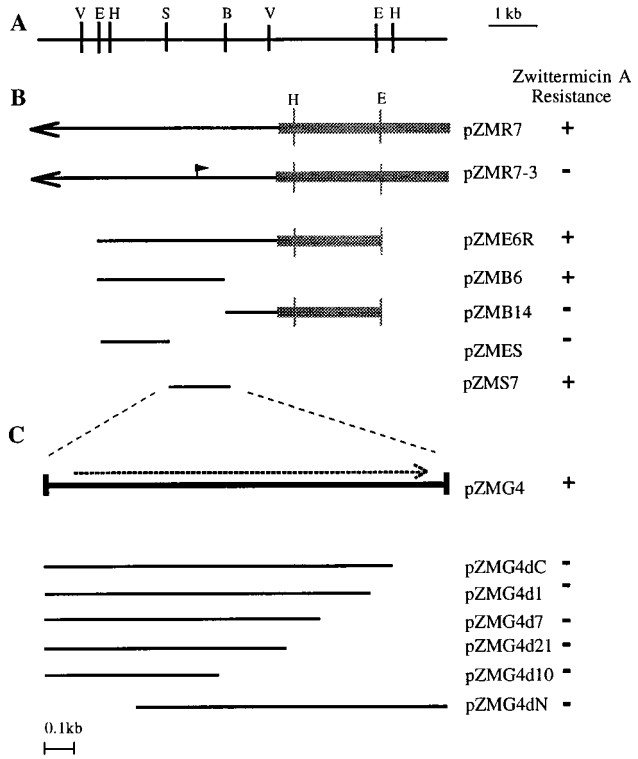

FIG. 1. Cloning and subcloning of the zwittermicin A resistance determinant. The columns on the right side indicate plasmid names and the zwittermicin A resistance phenotype of DH5α carrying the plasmid (100 μg of zwittermicin A). Symbols: +, no zone of inhibition; −, an 8- to 11-mm zone of inhibition as measured for DH5α or DH5α containing the relevant vector in same experiment. (A) Partial restriction map of UW101C DNA. Vertical bars on the top line represent restriction sites, which are abbreviated as follows: E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; S, *Sph*I; V, *Eco*RV. (B) Extent of inserts in pLA2917 and pHT304. Thin horizontal lines represent UW101C-derived DNA. Thick horizontal lines represent pLA2917-derived DNA. The flag (pZMR7-3) represents the site and direction of the Tn*3*. (C) The heavy line bounded by vertical bars represents the 1.2-kb insert in pZMG4. For deletion constructs, the undeleted portion of the insert is indicated by horizontal lines. The precise limits of the deletions are (based on the numbering from Fig. 3) as follows: pZMG4d1, nucleotides 873 to 1227 deleted; pZMG4d7, nucleotides 775 to 1227 deleted; pZMG4d10, nucleotides 474 to 1227 deleted; pZMG4dN, nucleotides 1 to 301 deleted; pZMG4dC, nucleotides 1063 to 1227 deleted. The horizontal dashed line represents the extent of ZmaR (Fig. 3).

pBC85 by examining their plasmid profiles in Eckhardt gels. Erm^s Spc^s isolates for which the marked pBC85 was not detectable on an Eckhardt gel were considered cured of the Tn*917*-marked pBC85 and are so designated by the addition of the letter C to the strain designation (Table 1).

Construction of cosmid libary of UW101C. Strain UW101C is derived from UW85 (38). UW101C produces zwittermicin A, is zwittermicin A resistant, and has been cured of pBC85. UW101C was used as the source of insert DNA in the construction of the cosmid library (Table 1) so that pBC85 sequences would not be overrepresented in the library. Total genomic DNA from UW101C was prepared and partially digested with the restriction enzyme *Sau*3AI, and restriction fragments ranging in size from 10 to 30 kb were recovered from an agarose gel. The insert DNA was ligated into the *Bgl*II site of cosmid vector pLA2917, which carries tetracycline and kanamycin resistance markers (1), thereby disrupting the kanamycin resistance marker on the cosmid vector. The ligation mixture was packaged with a lambda DNA packaging kit (Boehringer Mannheim) and transfected into *E. coli* DH5α. A total of 343 tetracycline-resistant (Tc^r) kanamycin-sensitive (Km^s) transfectants were obtained. One batch of 244 transfectants and a second batch of 99 transfectants were pooled, and the pools were stored at −70°C in 10% dimethyl sulfoxide. The average insert size, as determined by analyzing the products of restriction digestion on agarose gels, was 19 ± 2 kb (n = 10). Assuming a genome size of 5.5 Mb (6), the probability that the entire genome was represented in the cosmid bank was 0.69 (8, 36).

Assays for zwittermicin A resistance. Assays for zwittermicin A resistance were conducted on Mueller-Hinton agar (Difco) adjusted to pH 8.0 with 50 mM Tris and MOPS (morpholinepropanesulfonic acid) buffers (41). Zwittermicin A-resistant transformants of the sensitive *E. coli* strain DH5α were identified by spreading cells (10^2 to 10^5 CFU per plate) on Mueller-Hinton agar plates, applying zwittermicin A (100 μg) to three evenly spaced wells cut in the agar plate, and incubating the plates at 28°C for 24 h. Zwittermicin A-resistant colonies were identified as those that grew within the zone of growth inhibition around the wells. Zwittermicin A-resistant isolates from the amplified library were detected at a frequency of 6×10^{-3}. This frequency suggested that two cosmids able to confer zwittermicin A resistance would be present in the library on the basis of the prediction that the frequency of zwittermicin A-resistant transformants would be 3×10^{-3} (1 in 343) if one cosmid was present in the library that conferred zwittermicin A resistance on DH5α. When cosmid DNA was prepared from zwittermicin A-resistant transformants and retransformed into DH5α cells, zwittermicin A resistance was transferred with tetracycline resistance.

The resistance phenotype of individual isolates was determined in a radial streak assay. Zwittermicin A (50 to 100 μg for *E. coli* strains; 300 μg for *B. cereus* strains) was applied to a sterile filter disk placed in the center of a Mueller-Hinton agar plate, and a sterile wooden stick dipped in the test culture was streaked radially from the disk outward to the edge of the plate. After incubation at 28°C for 24 h, the radius of growth inhibition was measured. Two phenotypes of DH5α transformants were detected in radial streak assays. Zwittermicin A resistance was indicated by no zone of inhibition. Sensitivity was indicated by zones of inhibition sized between 8 and 11 mm.

Construction and analysis of plasmids. For plasmid constructions, ligation mixtures were transformed into *E. coli* DH5α by electroporation. For pHT304- and pGEM-based plasmids, ampicillin-resistant (Amp^r) transformants that were white on LB agar containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were streak purified. Several individual colonies were screened for zwittermicin A resistance, and plasmid DNA was prepared from the isolates to determine the presence and orientation of inserts by restriction analysis. Plasmid DNA was retransformed into DH5α cells, and the transformants were subjected to the same analysis to ensure that the phenotypes detected in the primary transformation were plasmid conferred. Transconjugants carrying representative plasmids were stored at −70°C in 10% dimethyl sulfoxide.

pZME6 and pZME6R were constructed by ligating a 6-kb *Eco*RI fragment from pZMR7 into the unique *Eco*RI site of shuttle vector pHT304. The strategy for subcloning smaller fragments from the 6-kb *Eco*RI is summarized in Fig. 1. The 1.2-kb *Sph*I-*Bam*HI fragment was inserted in both orientations in the shuttle vector pHT304 (plasmids pZMS6 and pZMS7) and in the sequencing vector pGEM-3Zf(+) (pZMG4 and pZMG6) by isolating the 1.2-kb *Sph*I fragment from pZMB6, created by the insert-derived *Sph*I site and the *Sph*I site derived from the multiple cloning site, and ligating it into the unique *Sph*I site within the multiple cloning site of each vector.

pZMG4dC was constructed by digesting pZMG4 with *Bgl*II and *Bam*HI and religating the DNA. This deleted a 165-nucleotide fragment from the 3′ end of the insert in pZMG4. pZMG4dN was constructed by digesting pZMG4 with *Blp*I and *Hin*dIII, filling in the ends with Klenow fragment, and religating the DNA. This deleted a 301-nucleotide fragment from the 5′ end of the insert in pZMG4.

Tn*3* mutagenesis. Mutagenesis of cosmid clones by Tn*3*, which carries a kanamycin resistance marker, was conducted as described previously (5).

DNA sequencing and sequence analysis. Nested deletions into the 1.2-kb *Sph*I-*Bam*HI fragment were constructed from pZMG4 (Fig. 1; Table 1) with the Erase-a-Base deletion-generating system (Promega Corp.) by taking advantage of the *Kpn*I and *Bam*HI restriction sites in the multiple cloning site of pGEM-3Zf(+). Sequencing reactions were performed with the PRISM Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit (Perkin-Elmer Corp., Foster City, Calif.). Plasmids pZMG4, pZMG6, pZMG4d1, pZMG4d7, and pZMG4d10 were used as template DNA. Primer walking, using the forward and reverse univeral M13pUC primers and the following primers, numbered according to the DNA sequence in Fig. 3, was used to complete the sequencing of both strands: primer 677 (5′-TAAAGCTCGTCCCTCTTCAG-3′), nucleotide sequence 1142 to 1123; primer 678 (5′-ATGTGCACTTGTATGGGCAG-3′), nucleotide sequence 191 to 210; primer 1641 (5′-TTTGGGAGTCAACTCAGG-3′), nucleotide sequence 550 to 567; primer 1720 (5′-ACTAGCGGGAAAAGTTGG-3′), nucleotide sequence 803 to 820; primer 1721 (5′-GCACTAGATCTAGGATG G-3′), nucleotide sequence 1053 to 1070; primer 1737 (5′-ACAAGTGCACATC TAGGG-3′), nucleotide sequence 202 to 185. Primer 677 and 678 (44) were also used to PCR amplify a DNA fragment internal to *zmaR* that was labeled and used as a probe for *zmaR*.

The partial DNA sequences were aligned and compiled, open reading frames were identified, and codon usage was analyzed with SeqMan and SeqEdit software (DNASTAR Inc.) and Codon Use 3.3 (Conrad Halling, Universty of Chicago). The nucleotide sequence and deduced primary amino acid sequence were compared with sequences in the database by use of the BLASTN, BLASTX, and BLASTP algorithms (2) via the NCBI BLAST electronic mail server and with the GCG Wisconsin Sequence Analysis Package software (Genetics Computer Group, Inc.), which was also used to predict the secondary structure of deduced primary amino acid sequences.

Protein visualization, purification, and N-terminal sequencing. *E. coli* DH5αF′IQ (Table 1) carrying different plasmids was grown to an optical density (at 600 nm) of 0.5 to 0.6 in LB broth with ampicillin and kanamycin. IPTG (1 mM) was added to induce gene expression from the *lac* promoter, and 1-ml samples were taken at 1-h intervals. Cells were collected by centrifugation (16,000 × *g*, 5 min) and resuspended in 100 μl of water. An equal amount of 2× sodium dodecyl sulfate (SDS) sample buffer was added, and the samples were stored at −20°C. The samples were heated to 95°C in preparation for loading on a gel. The protein concentration of the cell extracts was quantified by the bicinchoninic acid assay (Pierce Chemicals, Rockford, Ill.) (40). Polyacrylamide gels (12%) were prepared by standard methods (27) and run at 15 mA through the stacking gel and 20 mA through the separating gel. Prestained protein markers and broad-range protein markers (Bio-Rad Laboratories) were included on all gels. Protein bands were visualized by staining with Coomassie blue.

For N-terminal sequencing, *E. coli* DH5αF′IQ(pZMG4) was grown overnight in LB broth containing IPTG, ampicillin, and kanamycin. Cells were collected from 1 ml of culture by centrifugation (16,000 × *g*, 5 min). The pellet was resuspended in 50 mM Tris (pH 8.0)–2 mM EDTA buffer. Lysozyme (10 μg) and 1% Triton X-100 (10 μl) were added to the samples, and the samples were incubated at 30°C for 15 min. Cells were disrupted by three 10-s pulses of a Vibra-Cell probe sonicator at output setting 3 (Sonics & Materials, Inc., Danbury, Conn.). Insoluble proteins, membranes, and undisrupted cells were recovered by centrifugation at 16,000 × *g* for 15 min at 4°C. The pellet was treated further (36), and proteins were separated on a 12% polyacrylamide gel. Gels were blotted to a 0.22-μm-pore-size polyvinylidene difluoride membrane (Bio-Rad Laboratories) with a Bio-Rad transfer cell at 60 V for 5 h at 4°C. The membrane was stained, and the prominent 43.5-kDa protein band was excised for N-terminal sequencing directly on the polyvinylidene difluoride membrane by Edman degradation (30) (E. D. Eccleston, Jr., MicroChemical Facility, Institute of Human Genetics, University of Minnesota).

Purification and quantification of zwittermicin A. Zwittermicin A was purified by cation-exchange chromatography and high-voltage paper electrophoresis (39). To quantify zwittermicin A in culture supernatants, zwittermicin A was partially purified by the Sep-Pak (Millipore Corp.) method and quantified in an end-point dilution assay (32, 39).

Nucleotide sequence accession number. The sequence for *zmaR* (see Fig. 3) was assigned the GenBank accession number U57065.

RESULTS

Identification of a cosmid conferring zwittermicin A resistance on *E. coli* strain DH5α. To identify a zwittermicin A resistance determinant, we cloned DNA from *B. cereus* UW85, which produces zwittermicin A and is resistant to it, into cosmid pLA2917. *E coli* DH5α, which is sensitive to zwittermicin A, was used to identify cosmids that would confer zwittermicin A resistance. Restriction and Southern hybridization analyses of DNA from 10 of 118 zwittermicin A-resistant transformants of DH5α identified two distinct cosmids that carried overlapping insert DNA. The cosmids, designated pZMR6 and pZMR7, were estimated to have inserts of 18 and 19 kb, respectively. We designated the determinant of zwittermicin A resistance *zmaR*.

Localization of *zmaR* within the cosmid insert. To localize the region required for zwittermicin A resistance within the cosmid insert, we mutagenized pZMR7 with Tn*3* (5). The one

TABLE 2. Phenotype conferred by *zmaR* on *B. cereus* strains

Strain	Zwittermicin A sensitivity[a] (zone of growth inhibition [mm])					Zwittermicin A production[b] (μg/ml)		
	No plasmid	pHT304	pZME6R	pZMS6	pZMS7	No plasmid	pHT304	pZME6R
UW85	0	0	0	NT[c]	NT	16.1	12.5	15.0
UW101C[d]	0	0	0	NT	NT	13.1	10.2	9.7
UW030 (Δ*zmaR*)	3	3	0	0	0	<0.5	<0.5	<0.5
UW167C (Δ*zmaR*)	3	3	0	0	0	<0.5	<0.5	<0.5
UW226C	0	0	0	NT	NT	<0.5	<0.5	<0.5
UW325C (Δ*zmaR*)	3	3	0	0	NT	<0.5	<0.5	<0.5
UW457C	0	0	0	NT	NT	4.4	3.0	3.0
SNY14	8	8	0	NT	NT	NT	NT	NT

[a] Zwittermicin A resistance to 300 μg of zwittermicin A was determined by the radial streak assay on at least three independent cultures. Zones of inhibition are reported for a single representative determination.
[b] Zwittermicin A production was assayed by the end-point dilution method described in the text. The mean of three independent cultures is reported.
[c] NT, not tested.
[d] Mutants cured of the Tn*917*-marked pBC85 (designated with "C") were used for these assays because the erythromycin resistance marker from Tn*917* interfered with the erythromycin selection for transformants carrying pHT304 and pHT304-based plasmids.

pZMR7::Tn*3* derivative of the 160 screened that did not confer zwittermicin A resistance on *E. coli* DH5α was designated pZMR7-3. We mapped the Tn*3* to a site corresponding to a 6-kb *Eco*RI fragment on pZMR7 (Fig. 1). A portion of this fragment was derived from the cosmid vector pLA2917.

The 6-kb *Eco*RI fragment from pZMR7 was ligated into pHT304. Representative plasmids carrying the insert in each orientation, designated pZME6 and pZME6R, were isolated from zwittermicin A-resistant transformants (Fig. 1; Table 1). By subcloning, we identified a 1.2-kb *Sph*I-*Bam*HI fragment, which corresponded to the site of the Tn*3* mutation in pZMR7-3, as the smallest fragment to confer zwittermicin A resistance on DH5α (Fig. 1). This fragment conferred zwittermicin A resistance on *E. coli* DH5α in both orientations in pHT304 (pZMS6, pZMS7) or pGEM-3Zf(+) (pZMG4, pZMG6).

Mutants of *B. cereus* UW85 contain deletions of *zmaR*. In previous studies (38, 39), we isolated mutants of *B. cereus* UW85 defective in zwittermicin A production (Table 1). Mutants UW030, UW167, UW167C, UW325, and UW325C were zwittermicin A sensitive (Table 2), whereas mutants UW226, UW226C, UW419, UW419C, UW452, UW452C, UW457, and UW457C were resistant. We predicted that a comparative Southern hybridization analysis of the *zmaR* region in UW85 and its mutants could be informative about the nature of the mutations, which arose spontaneously (38).

In the Southern analysis, we used target DNA from *B. cereus* UW85 mutants or the mutants cured of their Tn*917*-marked cryptic plasmid, designated by the suffix "C" (Table 1), to determine whether the presence of the plasmid and the process of curing influenced the results. The genomic DNA was digested with *Eco*RV, *Eco*RI, or *Hin*dIII and hybridized to a PCR-amplified fragment internal to *zmaR* or the 1.2-kb *Sph*I-*Bam*HI fragment containing *zmaR* (Fig. 2 and data not shown).

The PCR-amplified probe hybridized to a 6-kb *Hin*dIII fragment, a 6-kb *Eco*RI fragment, or a 4.5-kb *Eco*RV fragment from *B. cereus* UW85, UW101C, UW226, UW419, UW452, and UW457, but it did not detect corresponding fragments in mutants UW030, UW167, and UW325 (Fig. 2 and data not shown). The 1.2-kb *Sph*I-*Bam*HI fragment generated the same result. Both probes produced these results against target DNA from the mutants carrying the Tn*917*-marked pBC85 (Fig. 2) or the corresponding cured mutants (data not shown). Thus, the zwittermicin A-sensitive mutants, UW030, UW167, UW167C, UW325, and UW325C, each carry a genomic deletion of at least 8 kb that encompasses *zmaR*.

A second weak band was detected in strain UW85 and all of its mutants when their DNA was hybridized to the PCR-amplified probe. For the *Eco*RV digests, this band was about 8 kb in size (Fig. 2). The 1.2-kb *Sph*I-*Bam*HI fragment detected the same band. Thus, UW85 and its mutants may carry a second genetic element that has sequence similarity with *zmaR*.

Phenotype of *zmaR* in *B. cereus* UW85 and its derivatives. The discovery that the zwittermicin A-sensitive mutants had a deletion of *zmaR* led us to test zwittermicin A resistance with *zmaR* supplied in *trans*. Plasmids pZME6R, pZMS6, and pZMS7 restored full zwittermicin A resistance to mutants UW030, UW167C, and UW325C (Table 2). However, pZME6R did not alter the zwittermicin A production phenotype of *B. cereus* UW85 or its mutants (Table 2). pZME6R also conferred zwittermicin A resistance on a wild *B. cereus* isolate, SNY14, that does not produce zwittermicin A and is the most zwittermicin A-sensitive *B. cereus* isolate we have tested (44). These data demonstrate that *zmaR* is functional as a determinant of zwittermicin A resistance in *B. cereus* as well as in *E. coli*.

Sequence analysis of *zmaR*. The 1,227-nucleotide sequence of the *Sph*I-*Bam*HI fragment is shown in Fig. 3. In database

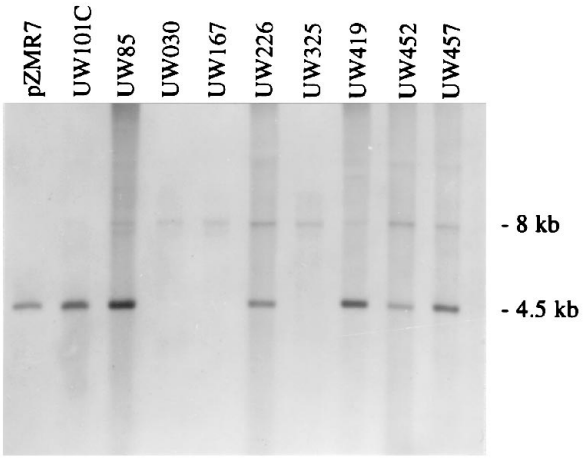

FIG. 2. Southern analysis of UW85 and derivative strains. Genomic DNAs of *B. cereus* UW85 and its mutants digested with *Eco*RV were hybridized to a PCR-amplified probe internal to *zmaR*. Lanes are labeled with the source of the genomic DNA.

```
      SphI
1     GCATGCACTTGCACTTGTTCTCAAAAGGGAGGACTTTTAAGAATATGATTTATGAATTG
                                                  M  I  Y  E  L     5
60    GTAAAAGAAAAGGATGCATTCAAACAAATATCTCCACTTCTCGTTGATTATAAGAACCAT
      V  K  E  K  D  A  F  K  Q  I  S  P  L  L  V  D  Y  K  N  H    25
120   CCAATACTGAACGGTATAGCTCATGGTTACAATAAGGGACATATTTATGTGGATAATCCT
      P  I  L  N  G  I  A  H  G  Y  N  K  G  H  I  Y  V  D  N  P    45
180   AATAACCCTAGATGTGCACTTGTATGGGCAGAACAAGAAATTTTTTATTTATTAGGTGAT
      N  N  P  R  C  A  L  V  W  A  E  Q  E  I  F  Y  L  L  G  D    65
240   CCTACAAGTAATTTTGTTTCAAGTCTACCAACTTTTATTAAAGAAGTAATTGCACCTGAG
      P  T  S  N  F  V  S  S  L  P  T  F  I  K  E  V  I  A  P  E    85
300   GCTGAGCAGATAGGGGATGACTTTTTTCAGGTTGAATTATTACCTGAATCAAAATGGAAA
      A  E  Q  I  G  D  D  F  F  Q  V  E  L  L  P  E  S  K  W  K   105
360   CAGGTTGTCGAGGAACAATTGCAAATGTTTATTCCTAAACCTTACACTCGAGTAACATTT
      Q  V  V  E  E  Q  L  Q  M  F  I  P  K  P  Y  T  R  V  T  F   125
420   ACTTTTGATCCGGAGTCTTATAATAAGTTATCTAAACCTAATATGCCTGAAGAGGTAACG
      T  F  D  P  E  S  Y  N  K  L  S  K  P  N  M  P  E  E  V  T   145
480   GTGGAACGTATTACAATCGACATGCTATCAGATAAAAAGTTCGCAATGGTACGGGATGAT
      V  E  R  I  T  I  D  M  L  S  D  K  K  F  A  M  V  R  D  D   165
540   ATTGTTGATTTTTGGGAGTCAACTCAGGATTTCATCCAAAATGGATTTGGTTATGTAGTA
      I  V  D  F  W  E  S  T  Q  D  F  I  Q  N  G  F  G  Y  V  V   185
600   ATGGTACATGAACAAGTTGTAACTAGTTGCTTATCGGTTTTTGCAACAGATACTGATGTG
      M  V  H  E  Q  V  V  T  S  C  L  S  V  F  A  T  D  T  D  V   205
660   GAAATTGGAATTAATACTTATGATTTATTCCAGCGAGGTAAAGGGTATGCTTGGTTGGCT
      E  I  G  I  N  T  Y  D  L  F  Q  R  G  K  G  Y  A  W  L  A   225
720   GCAAGAGCATTCTTGGATGACTGTTTAAAACAAGGAAGAACACCACATTGGAAGACGGAG
      A  R  A  F  L  D  D  C  L  K  Q  G  R  T  P  H  W  K  T  E   245
780   GATTTTCGTATTCCATCTATTAAACTAGCGGGAAAAGTTGGATTCACAAATCTTCAAACC
      D  F  R  I  P  S  I  K  L  A  G  K  V  G  F  T  N  L  Q  T   265
840   TATACAGCTTATGTGTTTCCTTATAACGAATTAGATAATTTCATTTTTACCGCGTACCAT
      Y  T  A  Y  V  F  P  Y  N  E  L  D  N  F  I  F  T  A  Y  H   285
900   CAATTAAGATACTATTCAAATTTTAATAAAGCTAGTGAGTTTGTACAAAAAGCTCGTACT
      Q  L  R  Y  Y  S  N  F  N  K  A  S  E  F  V  Q  K  A  R  T   305
960   ATAGGAGATTTAAATGCATGGCATCACTTTTTACTTTCATGTGGTTATTCATTAATTGAT
      I  G  D  L  N  A  W  H  H  F  L  L  S  C  G  Y  S  L  I  D   325
1020  AGAATCGATCTTTCGTTAAAGCATATGAATCTTGCACTAGATCTAGGATGGAATGATGTA
      R  I  D  L  S  L  K  H  M  N  L  A  L  D  L  G  W  N  D  V   345
1080  TCAGATATTCGCTATGTAGTTGATTTAGTGAATTTAAGAAAAACTGAAGAGGGACGAGCT
      S  D  I  R  Y  V  V  D  L  V  N  L  R  K  T  E  E  G  R  A   365
1140  TTATTAGATAGAGTTGAAGATAGCTTAAGATGAAAATAAGGGAGATAAAAAGATGAATAT
      L  L  D  R  V  E  D  S  L  R  .                              375
                              BamHI
1200  TGTATTTATGTTTCCTGGCGTAGGATCC
```

FIG. 3. Sequence of the 1.2-kb *Sph*I-*Bam*HI fragment. The terminal restriction sites are underlined and labeled. The deduced amino acid sequence of the 1,125-nucleotide open reading frame is labeled below the nucleotide sequence. The consensus ribosome binding site is indicated by underlining, and the inverted repeats that flank the GGAGG sequence are overlined. The nucleotide position number is labeled on the left, and the amino acid position number is labeled on the right.

searches (BLASTX), we detected no DNA sequences with identity or significant similarity to the *zmaR* sequence. Translation of the nucleotide sequence in all six reading frames revealed a 1,125-nucleotide open reading frame whose codon usage was typical of *B. cereus* (bases 45 to 1170) (Fig. 3). This predicted translation product was designated ZmaR. A consensus *Bacillus* ribosome binding site (GGAGG) was located 12 bases upstream of the putative ATG initiation codon, and it was flanked by repeats that could form a canonical stem-loop structure associated with ribosome binding (47). The genetic evidence was consistent with the assignment of ZmaR as the *zmaR* gene product because plasmids derived from pZMG4 that had deletions in the insert DNA (Fig. 1) were unable to confer zwittermicin A resistance on *E. coli* DH5α. Thus, most or all of the 1.2-kb insert DNA was essential for zwittermicin A resistance.

The sequence analysis predicted that ZmaR would have 375 amino acids (187 polar, acidic, or basic residues and 139 hydrophobic residues), a molecular mass of 43.5 kDa, a pI of 5.0, and a net charge of −13.7 at pH 7.0. Computer modeling of the secondary structure (7, 14) predicted that ZmaR has characteristics typical of a globular, soluble protein. Hydropathy analysis (26) predicted that ZmaR has no hydrophobic secondary structure sufficient to represent a membrane-spanning domain. No primary amino acid sequences with significant similarity to ZmaR were identified in a BLASTP or BLASTN search: the highest-scoring matches gave scores of only 62, and there were no probabilities of less than 0.99 that the matches occurred by random chance. The MOTIFS and PROFILESCAN programs (Genetics Computer Group) did not identify any matches in ZmaR to structural or sequence motifs defined in the PROCITE catalog (Genetics Computer Group). Thus, comparison of the *zmaR* sequence with the database entries did not assist us in predicting the mechanism by which ZmaR confers zwittermicin A resistance.

Identification of ZmaR. In DH5αF′IQ, gene expression can be controlled by relieving repression of a plasmid-encoded *lac* promoter by supplying the synthetic inducer IPTG. We predicted that *zmaR* would be under *lac* promoter control in pZMG4 but not in pZMG6 and that ZmaR would be detectable in cell extracts of DH5αF′IQ(pZMG4) grown in the presence of IPTG. We observed overexpression of a *zmaR*-dependent protein band at approximately 43.5 kDa as early as 1 h after addition of IPTG (Fig. 4). Additional evidence that the 43.5-kDa protein was a product of *zmaR* was gained from N-terminal sequencing. The 10 N-terminal amino acids were identical to those predicted from the *zmaR* sequence, i.e., Met-Ile-Tyr-Glu-Leu-Val-Lys-Glu-Lys-Asp. The genetic, molecular, and biochemical evidence collectively demonstrate that *zmaR* is expressed as a 43.5-kDa protein, ZmaR.

DISCUSSION

By using genetic and molecular approaches, we have identified a zwittermicin A resistance gene, *zmaR*, from the zwittermicin A-producing bacterium *B. cereus* UW85, that is functional in *B. cereus* and *E. coli*. The product of *zmaR*, designated ZmaR, was a 43.5-kDa protein.

Three zwittermicin A nonproducing mutants of *B. cereus* UW85, i.e., UW030, UW167, and UW325, and their plasmid-cured derivatives, were zwittermicin A sensitive. Those mutants carried a deletion spanning *zmaR*, and *zmaR* supplied in *trans* restored zwittermicin A resistance (Table 2; Fig. 2). Four other zwittermicin A-nonproducing mutants were resistant to

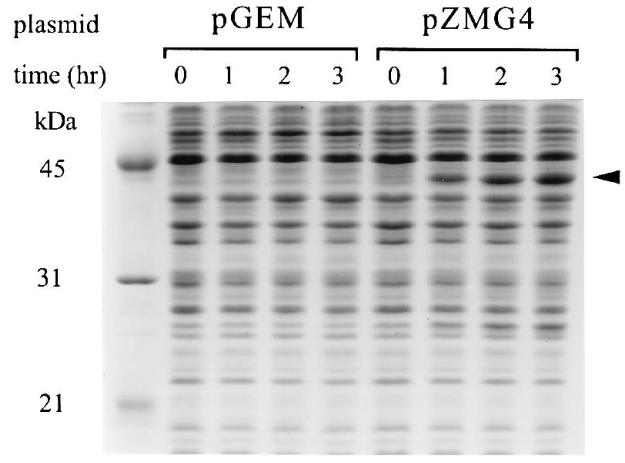

FIG. 4. Coomassie blue-stained polyacrylamide gel of DH5αF′IQ carrying pGEM or pZMG4. Cultures were grown to an optical density at 600 nm of 0.5 to 0.6 in LB broth with ampicillin (50 μg/ml) and kanamycin (10 μg/ml). IPTG (1 mM) was added, and samples were withdrawn immediately prior to IPTG addition (time 0) and at 1, 2, and 3 h following the addition of IPTG. All lanes contain 40 μg of total protein. The arrowhead indicates the position of the *zmaR*-dependent protein.

zwittermicin A and did not have a deletion of *zmaR*. The similarity of the deletions in UW030, UW167, and UW325 leads us to speculate that a reproducible genetic event accounts for their occurrence. If the presence of *zmaR* is correlated with zwittermicin A production in wild isolates of *B. cereus*, then screening isolates for *zmaR* may be a reliable strategy to identify zwittermicin A-producing strains. Results with a PCR-based screen to amplify *zmaR* support that hypothesis (44).

It is intriguing to us that a deletion of *zmaR* was associated with mutations affecting zwittermicin A production in three of seven mutants. One explanation for this high frequency of linked phenotypes is that the genomic deletions span genes, *zmaR* or others, that are required for zwittermicin A biosynthesis and production. As a consequence, deletions of this region would be causal to loss of both resistance and production. The 6-kb fragment containing *zmaR* did not restore zwittermicin A production to the nonproducing mutants of UW85 (Table 2), but because the UW101C-derived portion of the fragment was not sufficient to cover the entire deletion, it may not have been sufficient to restore that phenotype. Also, cosmid pZMR7, which contained approximately 19 kb of UW101C-derived DNA including *zmaR*, did not confer zwittermicin A production on *E. coli* DH5α (31). A second explanation for the associated loss of both resistance to and production of zwittermicin A in the mutants is that a deletion of *zmaR* in a producing strain may be lethal, rendering the cells sensitive to zwittermicin A, and thereby may provide a powerful selection for mutations that impair zwittermicin A production. This second scenario does not make any predictions about the relative genomic positions of *zmaR* and other genes required for zwittermicin A production. Additional studies to define the extent of the genomic deletions and to identify other genes associated with zwittermicin A production will contribute to the resolution of this question.

In database searches, we did not detect sequences with significant similarity to the *zmaR* DNA sequence of the ZmaR primary sequence. Thus, the DNA sequence does not suggest testable hypotheses for the mechanism of resistance. This result may reflect the paucity of database entries for organisms with AT-rich DNA, specifically for *B. cereus* and the closely related species *B. thuringiensis*, *Bacillus anthracis*, and *Bacillus mycoides*. It also indicates that the mechanism of resistance mediated by ZmaR may have features that have not been described previously. In either case, elucidation of the mechanism of ZmaR-mediated resistance to zwittermicin A will represent a contribution to understanding the biology of this novel antibiotic and the *B. cereus* group of bacteria.

Studies on antibiotic production, mostly in the actinomycetes, indicate that self-resistance to antibiotics is achieved by excluding the antibiotic from the environment of the sensitive target, by modifying or degrading the antibiotic (often by an enzyme in the biosynthetic pathway), or by modifying or replacing the target so that it is no longer antibiotic sensitive (10, 16, 22, 23, 34, 35, 45, 46, 49–52). Does the mechanism for ZmaR-based resistance fit one of these profiles? On the basis of secondary structure analysis of the predicted protein sequence, ZmaR is predicted to be a soluble protein rather than a membrane protein. This prediction leads us to disfavor the hypothesis that ZmaR is a zwittermicin A exporter. Preliminary experiments suggest that ZmaR does not directly modify or degrade zwittermicin A because zwittermicin A can be recovered quantitatively from cultures of DH5α(pZMR7) grown in LB broth containing zwittermicin A (31). Our Southern hybridization analysis suggests that *B. cereus* UW85 carries *zmaR* and a *zmaR* homolog. This result is consistent with the hypothesis that ZmaR is a zwittermicin A-resistant target that replaces a sensitive homolog. Future studies will be directed to systematically testing these hypotheses and to elucidating the mechanism by which ZmaR confers resistance.

The overexpression system for ZmaR developed in this study will form the basis for purification of ZmaR to be used in future studies directed to establishing the mechanism by which ZmaR confers resistance. We plan to discern whether the mechanisms of resistance are the same in *B. cereus* and *E. coli*. Because zwittermicin A is a novel antibiotic and *zmaR* encodes a protein unlike others described to date, our studies will contribute to the fundamental understanding of resistance mechanisms. Since zwittermicin A-producing strains of *B. cereus* are ubiquitous in soil (42), this knowledge will enhance our understanding of bacteria and antibiotic resistance in the environment and may suggest strategies for managing resistance in medicine and agriculture.

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