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Zwittermicin A biosynthetic cluster

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Abstract

The goal of this study was to identify the biosynthetic cluster for zwittermicin A, a novel, broad spectrum, aminopolyol antibiotic produced by *Bacillus cereus*. The nucleotide sequence of 2.7 kb of DNA flanking the zwittermicin A self-resistance gene, *zmaR*, from *B. cereus* UW85 revealed three open reading frames (ORFs). Of these ORFs, two had sequence similarity to acyl-CoA dehydrogenases and polyketide synthases, respectively. Insertional inactivation demonstrated that *orf2* is necessary for zwittermicin A production and that *zmaR* is necessary for high-level resistance to zwittermicin A but is not required for zwittermicin A production. Expression of ZmaR was temporally associated with zwittermicin A production. The results suggest that *zmaR* is part of a cluster of genes that is involved in zwittermicin A biosynthesis, representing the first biosynthetic pathway for an aminopolyol antibiotic.

Keywords: Acyl-CoA dehydrogenases; Acyltransferases; Polyketide synthases; Self-resistance

1. Introduction

Zwittermicin A is an aminopolyol antibiotic produced by *B. cereus* and *B. thuringiensis* (Stabb et al., 1994; He et al., 1994; Raffel et al., 1996) that has a broad target range (Silo-Suh et al., 1998). Producers of zwittermicin A are ubiquitous in soil. A worldwide survey of *Bacillus* soil isolates revealed that there were 10^4 cfu of zwittermicin A producers in every gram of soil tested (Stabb et al., 1994; Raffel et al., 1996). Zwittermicin A inhibits diverse eukaryotes and prokaryotes, including many plant pathogenic fungi and Oomycetes (Silo-Suh et al., 1998), contributes to the ability of *B. cereus* to suppress certain plant diseases (Silo-Suh et al., 1994), and interacts synergistically with Bt toxin to enhance the insecticidal activity of *B. thuringiensis* (Manker et al., 1994; Broderick et al., 1998). Spontaneous zwittermicin A-resistant mutants of *Escherichia coli* contain lesions in genes encoding subunits of RNA polymerase, although zwittermicin A inhibited neither DNA replication nor RNA transcription, suggesting that zwittermicin A may have an unusual mechanism of action (Stabb and Handelsman, 1998).

Zwittermicin A is the only known aminopolyol antibiotic and has structural features in common with peptide and polyketide antibiotics (Fig. 1). The alternating hydroxyl groups on the carbon backbone are similar to a partially reduced polyketide structure; the nitrogen-rich end of zwittermicin A may be derived from an amino acid, possibly citrulline, similar to a peptide antibiotic. This intriguing hybrid structure begs a more thorough understanding of the mechanism of biosynthesis of zwittermicin A. Several peptide antibiotic biosynthetic pathways have been described from *Bacillus* spp. (Nakano and Zuber, 1990). Although the polyketidic antibiotics difficidin (Zimmerman et al., 1987) and aurantinin (Nakagawa et al., 1988) have been identified from species of *Bacillus*, nothing is known about the genes necessary for their biosynthesis. In fact, the vast majority of genetic studies of polyketide antibiotic biosynthesis have been conducted in *Streptomyces* spp. (Córtes et al., 1990; Katz and Donadio, 1993). Therefore, elucidation of the pathway for biosynthesis of zwittermicin A will increase our understanding of this novel, bioactive molecule and the organisms that produce it, and contribute to fundamental knowledge of the diversity of antibiotic biosynthetic pathways.

Abbreviations: Ap, ampicillin; Cm; chloramphenicol; CM, carboxymethyl; DTT, dithiothreitol; Em, erythromycin; LB, Luria–Bertani; ORF, open reading frame; Sp, spectinomycin; TSB, trypticase soy broth.

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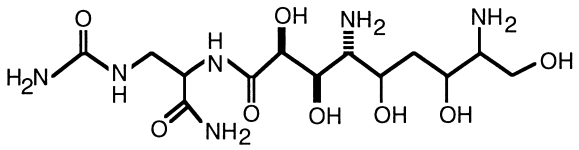

Fig. 1. Structure of zwittermicin A.

We previously identified a zwittermicin A-resistance gene, *zmaR*, from *B. cereus* UW85, which produces zwittermicin A (Milner et al., 1996). Mutants of UW85 that are deficient in zwittermicin A production contain large genomic deletions that span *zmaR* (Milner et al., 1996), and zwittermicin A production among soil isolates of *B. cereus* is highly correlated with the presence of *zmaR* (Raffel et al., 1996). Antibiotic-producing organisms require antibiotic self-resistance genes to protect themselves from the action of their toxic metabolites, and these genes are often found in a cluster with antibiotic biosynthetic genes (Cundliffe, 1989). Therefore, it seemed plausible that *zmaR* would be found in close genetic proximity to the genes required for zwittermicin A biosynthesis. In this paper, we report the identification and analysis of a region necessary for zwittermicin A resistance and biosynthesis in *B. cereus* UW85.

2. Materials and methods

2.1. Bacterial strains, plasmids, and culture conditions

The strains and plasmids used in this study are described in Table 1. Unless otherwise indicated, *E. coli* strains were grown at 37°C on Luria–Bertani (LB) broth or agar. Unless otherwise indicated, *Bacillus* strains were grown at 28°C on 50% trypticase soy broth (TSB) or agar (TSA) (Difco Laboratories, Detroit, MI). 100MH8.1 was prepared by amending Mueller–Hinton medium (Difco) with 40 mM 3-(morpholino)propanesulfonic acid (MOPS) and 40 mM tris(hydroxymethyl)-aminomethane (Tris) and adjusting the pH to 8.1 with NaOH. Antibiotics were added at the following levels for *E. coli*: ampicillin, 50 mg/l; spectinomycin, 100 mg/l; chloramphenicol, 12 mg/l, and at the following levels for *Bacillus*: spectinomycin, 150 or 200 mg/l; erythromycin, 10 mg/l; chloramphenicol 5 mg/l.

2.2. DNA manipulations and analysis

Plasmid DNA was isolated from strains of *E. coli* using Wizard minipreps (Promega Corp., Madison, WI) or Qiagen (Chatsworth, CA) plasmid kits. Plasmid DNA was isolated from strains of *Bacillus* using a modified alkaline lysis extraction described elsewhere (Milner et al., 1996). Total genomic DNA was isolated from *B. cereus* using the Easy-DNA kit (Invitrogen Corp., Carlsbad, CA). Restriction and modification enzymes were used according to the suppliers' directions (Promega Corp.; New England Biolabs, Beverly, MA; Gibco BRL, Richmond, CA). To change buffer conditions between different enzymatic reactions and to isolate DNA fragments from agarose gels, DNA was purified using QIAquick kits (Promega Corp.). Southern blot analysis was carried out with the Genius Kit (Boehringer Mannheim Biochemicals, Indianapolis, IN) as described previously (Milner et al., 1996). Plasmids were introduced into *E. coli* and *Bacillus* by electroporation as described previously (Milner et al., 1996). To increase the transformation efficiency of *B. cereus*, we found it necessary to first introduce and purify plasmid DNA from *B. thuringiensis* EG10368 (Table 1), a highly transformable strain, prior to electroporation of *B. cereus* (E.A.B. Emmert and J. Handelsman, unpublished data).

2.3. DNA sequencing and analysis

B. cereus DNA fragments cloned into pLA2917 or pHT304 (Table 1) were isolated from *E. coli* and used as templates for sequencing. Both strands of a 2.7 kb region were sequenced by primer walking and with M13/pUC forward and reverse primers. Primers 677 (5′-TAAAGCTCGTCCCTCTTCAG-3′) (Milner et al., 1996) (nucleotide sequence 2580–2561 in GenBank Accession No. AF155831) and 1721 (5′-GCACTA-GATCTAGGATGG-3′) (Milner et al., 1996) (nucleotide sequence 2491–2508 in GenBank Accession No. AF155831) were used to determine the orientation of a Sp^r cassette in plasmids used for insertional inactivation of *zmaR* and *orf2*. Sequencing reactions were carried out using the PRIZM cycle sequencing kit (Applied Biosystems, Inc., Foster City, CA). Primer synthesis and separation of DNA sequencing products were performed by the University of Wisconsin-Madison Biotechnology Center (Madison, WI).

The partial DNA sequences were aligned and compiled, open reading frames were identified, and codon usage was analyzed with SeqMan and EditSeq software (DNAStar, Inc.), and Codon Use 3.3 (Conrad Halling, University of Chicago). DNA and protein comparisons were carried out using the BLAST algorithms (Altschul et al., 1990) via the NCBI BLAST E-mail server and the GCG Wisconsin Sequence Analysis Software Package (Genetics Computer Group, Inc.).

2.4. Construction of plasmids for insertional inactivation *of* zmaR *and* orf2

pGEMdSH was constructed by digesting pGEM-3Zf(+) (Promega Corp.) with *Sma*I and *Hin*dIII, creating blunt ends with Klenow enzyme, and self-ligating the DNA. A 5.5 kb *Eco*RI fragment from

Table 1
Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
Escherichia coli		
DH5α	Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*) *U169deoR supE44 hsdR17 recA1 endA1 gyrA95 thi-1 relA1*	Hanahan (1983)
DH5αF′IQ	DH5αF′ *proAB*$^+$ *lac*^{q}ZΔM15 *zzf*::Tn*5* (Km^r)	Gibco-BRL
Bacillus		
B. cereus UW85	Wild type	Handelsman et al. (1990)
B. cereus UW030	UW85 *zma-030*; lacks *zmaR*	Silo-Suh et al. (1994); Milner et al. (1996)
B. cereus UW85Δ*zmaR*	UW85 derivative with *zmaR* deleted Sp^r	This study
B. cereus UW85Δ*orf2*	UW85 derivative with *orf2* deleted Sp^r	This study
B. thuringiensis EG10368	Highly transformable strain	Ecogen Corp.
Plasmids		
pLA2917	Broad-host-range cosmid vector; Km^r Tc^r	Allen and Hanson (1985)
pZMR6	pLA2917 with *zmaR*-containing insert; used for DNA sequencing; Km^r Tc^r	Milner et al. (1996)
pHT304	*E. coli–Bacillus* shuttle vector; Ap^r Em^r	Arantes and Lereclus (1991)
pZME6R	pHT304 with 6 kb *Eco*RI fragment containing *zmaR*, *orf1*, *orf2*, and additional vector sequence from pLA2917; Ap^r Em^r	Milner et al. (1996)
pZMES17/pZMES18	pHT304 with 1.2 kb *Eco*RI–*Sph*I fragment from pZME6R, used for DNA sequencing; Ap^r Em^r	Milner et al. (1996)
pZMB14/pZMB20	pHT304 with 3.1 kb *Bam*HI fragment from pZME6R, used for DNA sequencing; Ap^r Em^r	Milner et al. (1996)
pZMS7	pHT304 with 1.2 kb *Sph*I–*Bam*HI subclone of *zmaR*; Ap^r Em^r	Milner et al. (1996)
pORF2	pHT304 with 1.7 kb subclone of *orf2*;Ap^r Em^r	This study
pGEM-3Zf(+)	Cloning and sequencing vector; Ap^r	Promega Corp.
pGEMdSH	pGEM-3Zf(+) with *Sma*I–*Hin*dIII of MCS deleted; Ap^r	This study
pBeloBAC	*E. coli* cloning vector based on F-factor; Cm^r	Shizuya et al. (1992)
HM11	pBeloBAC with 14 kb insert containing *zmaR*; Cm^r	M. Rondon, R. M. Goodman, J. Handelsman, unpublished data
pZME5.5	pGEMdSH with 5.5 kb *Eco*RI fragment from HM11 containing *zmaR*, *orf1*, and *orf2*; Ap^r	This study
pZMG6	pGEM with 1.2 kb *Sph*I–*Bam*HI subclone of *zmaR*; Ap^r	Milner et al. (1996)
pDG1726	Sp^r cassette from *Enterococcus faecalis* in plasmid pSB119; Ap^r Sp^r	Guérout-Fleury et al. (1995)
pZMG6S+	pZMG6 with deletion of a 754 bp *Bgl*II–*Blp*I fragment of *zmaR* and Sp^r cassette inserted; Ap^r Sp^r	This study
pZME5.5Δ*zmaR*	pZME5.5 with deletion of a 754 bp *Bgl*II–*Blp*I fragment of *zmaR* and Sp^r cassette inserted; Ap^r Sp^r	This study
pZME5.5Δ*orf2*	pZME5.5 with deletion of a 881 bp *Bam*HI–*Eco*RV fragment of *orf2* and Sp^r cassette inserted; Ap^r Sp^r	This study
pKM10	*Bacillus–E. coli* vector with temperature-sensitive origin of replication for marker exchange mutagenesis; Ap^r Cm^r	A. Klimowicz, D. Beecher, A. Wong, J. Handelsman, unpublished data
pΔ*zmaR*	pKM10 with *Eco*RI fragment from pZME5.5Δ*zmaR*; Ap^r Cm^r Sp^r	This study
pΔ*orf2*	pKM10 with *Eco*RI fragment from pZME5.5Δ*orf2*; Ap^r Cm^r Sp^r	This study

[a] A *zma* designation indicates a defect in zwittermicin A production. Km^r, Cm^r, Ap^r, Em^r, and Sp^r indicate resistance to kanamycin, chloramphenicol, ampicillin, erythromycin, and spectinomycin, respectively.

HM11 containing *orf1*, *zmaR*, and *orf2* was subcloned into pGEMdSH, yielding pZME5.5. The strategy for creating a plasmid construct for insertional inactivation of *zmaR* was to delete 754 bp of DNA internal to *zmaR* and to insert a 1.2 kb spectinomycin resistance (Sp^r) cassette from plasmid pDG1726 (Table 1) in the same transcriptional orientation as *zmaR* at the site of the deletion. pZMG6, which is vector pGEM-3Zf(+) carrying *zmaR* on a 1.2 kb *Sph*I–*Bam*HI fragment (Table 1), was digested with *Bgl*II and *Blp*I, blunt-ended with Klenow, and ligated to a 1.2 kb, blunt-ended *Pst*I fragment containing the Sp^r cassette, yielding pZMG6S+. By sequencing with primer 677, we determined that the Sp^r cassette was in the same transcriptional orientation as *zmaR*. The 1.7 kb *Sph*I–*Bam*HI fragment from pZMG6S+ was cloned into pZME5.5, which had been digested with *Sph*I and *Bam*HI, treated with CIP, and gel-purified away from the *zmaR*-containing 1.2 kb *Sph*I–*Bam*HI fragment, yielding pZME5.5Δ*zmaR*. The *Eco*RI fragment from pZME5.5Δ*zmaR* was cloned into pKM10, yielding pΔ*zmaR* (Fig. 2). pKM10 (Table 1) is an *E. coli–B. cereus* shuttle vector, which has a temperature-sensitive origin of replication, and is used for marker exchange mutagenesis in *B. cereus* (A. Klimowicz, D. Beecher, A. Wong, J. Handelsman, unpublished data).

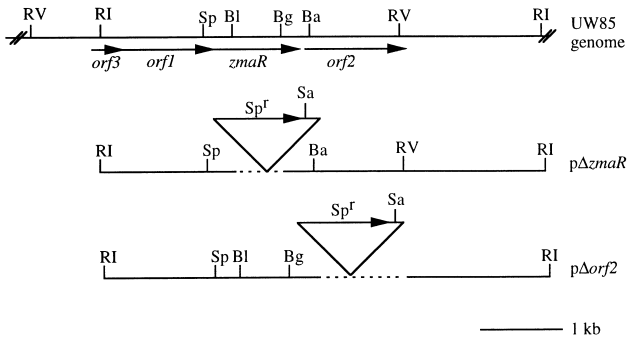

Fig. 2. Insertional inactivation of *zmaR* and *orf2*. Restriction maps are shown for the UW85 genomic region containing *orf3*, *orf1*, *zmaR*, and *orf2*, and the constructs pΔ*zmaR* and pΔ*orf2*, used to disrupt each gene in the UW85 genome. Arrows indicate the direction of transcription of the genes. Restriction sites are abbreviated as follows: RI, *Eco*RI; Sp, *Sph*I; Ba, *Bam*HI; Bl, *Blp*I; Bg, *Bgl*II; RV, *Eco*RV; Sa, *Sal*I. Dotted lines indicate deleted DNA.

The strategy for creating a plasmid construct for insertional inactivation of *orf2* was to delete 881 bp of DNA internal to *orf2* and to insert a Sp^r cassette in the same transcriptional orientation as *orf2* at the site of the deletion. pZME5.5 was digested with *Bam*HI and *Eco*RV and ligated to the Sp^r cassette prepared as described above, yielding pZME5.5Δ*orf2*. By sequencing with primer 1721, we determined that the Sp^r cassette was in the same transcriptional orientation as *orf2*. The *Eco*RI fragment from pZME5.5Δ*orf2* was cloned into pKM10, yielding pΔ*orf2* (Fig. 2). To construct pORF2, pZME6R was digested with *Bgl*II and *Hin*dIII, restriction sites present directly upstream of *orf2* and in the vector sequence of pZME6R downstream of *orf2*, respectively. The 1.7 kb fragment containing *orf2* was gel-purified and ligated to *Bam*HI/*Hin*dIII-digested and CIP-treated vector pHT304 (Table 1).

2.5. *Insertional inactivation of* zmaR *and* orf2

Plasmid pΔ*zmaR* or pΔ*orf2* was transformed into *B. cereus* UW85. Cultures were grown without antibiotic selection at 42°C, a non-permissive growth temperature for pKM10-derived plasmids. Putative single integrants were identified by plating at 42°C on media containing chloramphenicol and spectinomycin, and integration was confirmed by Southern blot analysis. Single integrants were grown without antibiotic selection for 48 h at 42°C with repeated sub-culturing to promote excision and loss of the plasmid, and plated on non-selective media. Colonies were subsequently screened for spectinomycin resistance and chloramphenicol sensitivity on TSA at 28°C. One-fourth to one-third of spectinomycin-resistant colonies were sensitive to chloramphenicol, indicating that they had lost the vector and wild-type allele, but maintained the mutant allele.

Southern blot analysis of genomic DNA from the Cm^s, Sp^r clones was performed to establish the genomic structure of the region (Fig. 2). When *Sph*I/*Bam*HI-digested genomic DNA from UW85 and UW85Δ*zmaR* was probed with a PCR fragment (Milner et al., 1996) internal to the *zmaR*-coding region (bp 1628–2580 in GenBank Accession No. AF155831), a 1.2 kb band was detected from UW85, and a 1.7 kb band was detected from UW85Δ*zmaR*, confirming the presence of a disrupted copy of the *zmaR* gene. When *Sal*I/*Eco*RV-digested DNA from UW85 and UW85Δ*orf2* was probed with the same PCR fragment, a band of approximately 4.5 kb was detected in UW85, whereas a slightly larger band was detected in UW85Δ*orf2*. The increase in size of this fragment in UW85Δ*orf2* was predicted from the relative locations of the *Sal*I site in the Sp^r cassette and the *Eco*RV site upstream of *orf3* in the UW85 genome (Fig. 2), confirming the presence of a disrupted copy of the *orf2* gene.

2.6. *Recombinant ZmaR protein visualization, purification, and concentration*

A 500 ml culture of *E. coli* DH5ΔF′IQ carrying pZMG4 was grown to an optical density (at 600 nm) of 0.5–0.6 in LB with ampicillin and kanamycin. IPTG (1 mM) was added, and cells were allowed to grow for 3 h. Cells were collected by centrifugation and resuspended in 50 ml of 50 mM Tris (pH 8.0), 2 mM EDTA buffer. 0.6 mg of lysozyme and 2.5 ml of 1% Triton X-100 were added to the sample, and the sample was incubated at 30°C for 15 min. Cells were disrupted by sonication, and inclusion bodies were purified initially as described previously (Milner et al., 1996). Inclusion bodies were further purified by repeated washing with increasing concentrations of urea and subsequently solubilized in 5 ml of 8 M urea, 1% SDS, 20 mM Tris-acetate, pH 8.0. After addition of 5× SDS sample buffer, the sample was stored at −20°C. Inclusion bodies were further purified on preparative 12% polyacrylamide gels, prepared and run by standard methods (Laemmli, 1970). Protein was visualized by staining the gel with cupric chloride (Deutscher, 1990), and the 43.5 kDa protein band was excised from the gel and stored at 4°C. Protein was electroeluted from the gel using a unidirectional electroelutor as directed by the operating manual (IBI, New Haven, CT). Protein concentration and buffer exchange to 20 mM Tris (pH 8.0) were achieved through ultrafiltration using Centriplus MWCO10 and Centricon 10 columns (Amicon, Inc., Beverly, MA). Protein concentration was determined by the bicinchoninic acid assay (Pierce Chemicals, Rockford, IL) (Smith et al., 1985).

2.7. *Generation of anti-ZmaR polyclonal antiserum*

Purified ZmaR protein (350 μg) in 20 mM Tris, pH 8.0 (0.5 ml), was mixed with Freund's complete

adjuvant and injected intradermally into a rabbit. The rabbit was boosted with 350 μg of purified protein monthly, and was bled from the marginal ear vein 2 weeks after each boost. Serum was spun in a centrifuge ($9800 \times g$, 10 min) at 4°C to remove blood cells, and frozen in 1 ml aliquots at −20°C (Harlow and Lane, 1988). Immune serum was pre-adsorbed to acetone powders prepared from cell extracts of *E. coli* DH5ΔF′IQ (Stratagene, La Jolla, CA) carrying pGEM-3Zf(+) and cell extracts of *B. cereus* UW030 prepared by standard methods (Harlow and Lane, 1988).

2.8. *Isolation of total protein from* B. cereus

Three milliliters of culture were pelleted by centrifugation ($16\,000 \times g$, 5 min), and the supernatant was removed. Pellets were resuspended in 200 μl of protein extraction buffer [10 mM Tris–Cl (pH 8.0), 100 mM EDTA, 100 mM DTT, 1% SDS], 50 μl of 0.1 mm silica beads were added, and samples were vortexed for 4 min at room temperature. Samples were centrifuged ($14\,000 \times g$, 5 min) at 4°C, 5× SDS protein sample buffer was added to 100 μl of sample supernatant, and the sample was stored at −20°C. A 10 μl sample of the supernatant was used for determining the protein concentration using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA).

2.9. *Western blot analysis*

Overnight cultures of *B. cereus* UW85 and UW85Δ*zmaR* were inoculated 1/100 into fresh media and grown at 28°C with shaking. Six, 12, 24, 48, and 72 h after inoculation, 3.0 ml of culture were removed and pelleted. Total protein was isolated, and the protein concentration determined as described above. Twenty micrograms of total *B. cereus* protein was loaded on each lane of a 12% protein gel prepared and run by standard techniques (Laemmli, 1970). Prestained markers (Bio-Rad Laboratories) were included on all gels. Before transfer, gels were allowed to equilibrate for 10 min in transfer buffer (0.025 M Tris, 0.192 M glycine, 10% methanol) with 0.1% SDS added. Gels were blotted to a 0.45 μm polyvinylidene difluoride (PVDF) membrane (Millipore, Millford, MA) using a Bio-Rad transfer cell (Richmond, CA) at 30 V overnight at 4°C. Western blots were developed according to the ECL Western blotting protocol (Amersham Life Technologies, Arlington Heights, IL) with the following modifications. Blots were blocked for 1 h at 34°C in 25 ml of a 5% solution of Carnation powdered milk (Nestlé Food Company, Glendale, CA) dissolved in blocking solution [Tris-buffered saline (pH 7.6), 0.1% Tween-20]. Primary anti-ZmaR antibodies and secondary anti-rabbit IgG antibodies conjugated to horseradish peroxidase (Sigma, St. Louis, MO) were diluted 1:5000 in blocking solution and incubated at 34°C for 1 h. All washes were performed at room temperature.

2.10. *Assays for production of zwittermicin A and zwittermicin A resistance*

Zwittermicin A was identified in culture supernatants of sporulated cultures of *B. cereus* UW85 and UW85-derived mutant constructs by cation exchange chromatography using CM SEP-PAK cartridges (Millipore) followed by high-voltage paper electrophoresis (HVPE) as described elsewhere (Silo-Suh et al., 1994; Milner et al., 1995). Zwittermicin A production was also tested in a plate bioassay for inhibition of *Erwinia herbicola* LS005 (Silo-Suh et al., 1994) with the modifications that 0.1% TSA plates were used, and plates were incubated for 24 h at 28°C before scoring for the presence or absence of a zone of inhibition. The zwittermicin A-resistance phenotype of strains of *E. coli* and *B. cereus* was determined by radial streak assay on 100MH8.1 agar using 100 and 300 μg of zwittermicin A for each organism, respectively, as described elsewhere (Milner et al., 1996).

2.11. *Nucleotide sequence Accession No.*

The sequence for *orf1*, *orf2*, and *orf3* was deposited in GenBank under Accession No. AF155831.

3. Results

3.1. *Sequence analysis of* zmaR-*flanking region*

We previously reported the isolation and sequence of a zwittermicin A self-resistance gene, *zmaR*, from a zwittermicin A-producing organism, *B. cereus* UW85 (Milner et al., 1996). To determine whether *zmaR* is linked to the zwittermicin A biosynthetic cluster, we began sequencing the DNA surrounding *zmaR*. The sequence of the region (GenBank Accession No. AF155831) contains 3933 nucleotides (nt), including the previously published nucleotide sequence of a 1.2 kb *Sph*I–*Bam*HI fragment (bases 1338–2665). We also identified an additional base (C, nucleotide position 1448) that was not reported in the previously published sequence (Milner et al., 1996). Analysis of the sequence, applying codon usage typical of *Bacillus* spp., identified three open reading frames (ORFs) having the same orientation of transcription as *zmaR*. Two ORFs were identified upstream of *zmaR*, and one ORF was identified downstream. ORF1 (bases 338–1486), ORF2 (bases 2630–3847), and ORF3, the smallest ORF (bases 78–341) all have putative ribosome binding sites 7–9 bp upstream of the potential ATG translational start sites.

All three ORFs, in addition to *zmaR*, terminate with a TGA stop codon. The TGA stop codon of ORF3 overlaps the ATG start codon of ORF1, and is shifted one reading frame in relation to ORF1. A similar overlapping structure is apparent for the TGA stop codon of ORF1 and ATG start codon of *zmaR*. The ATG start codon of ORF2 is 20 bases downstream of the TGA stop codon of *zmaR*. The lack of a promoter sequence, the overlapping structure of the start and stop codons, and the shared transcriptional orientation suggest that the ORFs and *zmaR* are organized in an operon.

3.2. Deduced functions of ORFs 1, 2, and 3

Comparison of the deduced amino acid sequences of the three ORFs using the BLAST algorithm (Altschul et al., 1990) revealed that each of the ORFs has homology to proteins with known function. ORF1 encodes a predicted protein of 42.1 kDa with an isoelectric point of 6.7 that has a high degree of sequence similarity to acyl-CoA dehydrogenase enzymes from diverse organisms, for example *B. subtilis* (Gen Prot Accession No. Z49782; 54% amino acid similarity, 34% amino acid identity) and *Rattus norvegicus* (SwissProt Accession No. P15651; 53% amino acid similarity, 31% amino acid identity) over the full length of the 382 amino acid ORF. Acyl-CoA dehydrogenases are flavoproteins that catalyze the initial step in the oxidative breakdown of fatty acids. Four distinct acyl-CoA dehydrogenases have been identified from mammals, and they are classified by their substrate specificity for long-, medium-, short-, and very-long-chain fatty acids. The relative location of a glutamic acid residue determines the substrate specificity of the various acyl-CoA dehydrogenases (Thorpe and Kim, 1995). ORF1 contains the catalytic residue identified for medium-chain acyl-CoA dehydrogenase, Glu-376 (Kim et al., 1993), located at aa residue 355 in ORF1 (GenBank Accession No. AF155831), which is believed to extract the α-proton from the substrate.

ORF2 is predicted to encode a 45.6 kDa protein with an isoelectric point of 5.3 that has sequence similarity to both polyketide and fatty acid synthase enzymes over parts of the predicted protein. ORF2 has 36–60% identity and 51–75% similarity to polyketide synthase enzymes (Schwecke et al., 1995), and 37–48% identity and 55–74% similarity to fatty acid synthase enzymes, specifically the acyltransferase portion of polyketide synthases (for example, *Streptomyces hygroscopicus*, GenProt Accession No. X86780) and the transacylase of fatty acid synthase (for example, *Bacillus subtilis* GenProt Accession No. L42526) enzymes. The region of homology includes active site sequences, (aa 85–98), identified for acyltransferases (Córtes et al., 1990). The acyltransferase motif P′xGHSxG′A(x = any amino acid, S being the serine involved in the formation of the acyl-enzyme intermediate) is conserved in ORF2 apart from a His→Tyr substitution within this sequence motif. Although both His and Tyr are positively charged amino acids, we do not know what effect this substitution would have. ORF2 also contains additional conserved residues found in acyltransferases, including an Arg residue (aa 117 in ORF2) spaced 25 residues C-terminal of the active site Ser residue. However, a second His residue located approximately 100 aa C-terminal to the active site and a Gln residue found near the C-terminus of acyltransferases are not present in ORF2 (Aparicio et al., 1996).

ORF3 is predicted to encode a 10.2 kDa protein with an isoelectric point of 4.3. ORF3 only has small regions of weak sequence similarity to peptide synthetase enzymes, including gramicidin S synthetase (Turgay et al., 1992) and surfactin synthetase (Cosmina et al., 1993). Furthermore, the known active sites of these enzymes are not included in the region of homology, and peptide synthetases are typically very large proteins. It is therefore difficult to predict the function of ORF3 based on homology searches.

The data suggest that all of these ORFs are genes that encode proteins. Therefore, we designated the ORFs as *orf1*, *orf2*, and *orf3* and the deduced proteins encoded by them as Orf1, Orf2, and Orf3, respectively. They will be referred to by these designations for the remainder of this paper.

3.3. Effect of insertional inactivation of zmaR and orf2 in B. cereus UW85

To investigate further the roles of *orf2* and *zmaR* in zwittermicin A biosynthesis and zwittermicin A resistance, each wild-type gene was replaced with a mutant allele carrying a deletion and an antibiotic resistance gene through homologous recombination of plasmid DNA (Fig. 2) into the UW85 genome. Southern blot analysis confirmed the genomic structure of the Δ*zmaR* and Δ*orf2* mutants (data not shown), and the mutants appeared to grow and sporulate normally (data not shown). UW85Δ*zmaR* was sensitive to zwittermicin A; UW85Δ*orf2* was resistant to zwittermicin A, as determined by radial streak assay. UW85Δ*orf2* did not produce any detectable zwittermicin A, as shown by a plate assay for inhibition of *Erwinia herbicola* and direct isolation of zwittermicin A from culture supernatants, whereas UW85Δ*zmaR* did produce zwittermicin A (Table 2).

When *zmaR* was introduced in trans on plasmid pZMS7, it restored zwittermicin A resistance to UW85Δ*zmaR* (Table 2). pHT304, the plasmid vector, does not confer zwittermicin A resistance on sensitive strains of *B. cereus* (Milner et al., 1996). When *orf2* was introduced in trans on plasmid pORF2, it restored zwittermicin A production to UW85Δ*orf2*. pHT304 did

Table 2
Phenotypes of UW85 and mutants

Strain	Zwittermicin A resistance[a]	Zwittermicin A production[b]
UW85	R	+
UW85Δ*zmaR*	S	+
UW85Δ*zmaR*+pZMS7[c]	R	+
UW85Δ*orf2*	R	−
UW85Δ*orf2*+pHT304	R	−
UW85Δ*orf2*+pORF2[d]	R	+

[a] Zwittermicin A resistance to 300 μg of zwittermicin A was measured by a radial streak assay on at least three independent cultures. R (resistant) indicates no zone of inhibition; S (sensitive) indicates a 3 mm zone of inhibition.

[b] Zwittermicin A production was measured by bioassay against a lawn of *Erwinia herbicola* and by biochemical purification of zwittermicin A using Sep-Pak cation exchange columns and subsequent high-voltage paper electrophoresis. The results reported are representative of at least three independent cultures.

[c] pZMS7 contains *zmaR* in plasmid pHT304.

[d] pORF2 contains *orf2* in plasmid pHT304.

not restore zwittermicin A production to UW85Δ*orf2* (Table 2). Together, the data indicate that *zmaR* is necessary for zwittermicin A resistance and that *orf2* is necessary for zwittermicin A production.

3.4. Expression of ZmaR in B. cereus UW85 and UW85Δ zmaR

To determine the pattern of expression of ZmaR in *B. cereus* UW85 and UW85Δ*zmaR*, we performed a Western blot analysis. From the amino acid sequence of *zmaR* and the expression of ZmaR in *E. coli* (Milner et al., 1996), we predicted that ZmaR would migrate as a 43.5 kDa protein in *B. cereus*. We detected a 43.5 kDa band in cell extracts of UW85, but not in UW85Δ*zmaR*, the mutant in which *zmaR* had been deleted. We also detected no band in cell extracts of UW030 (Table 1), a mutant containing a large genomic deletion that spans *zmaR* (data not shown). Therefore, we conclude that the 43.5 kDa band is ZmaR. We observed expression of ZmaR in UW85 after 12 h, at the end of the logarithmic growth phase. The level of ZmaR protein increased over time, showing maximal expression after 48–72 h (Fig. 3). Interestingly, although addition of phosphate and iron to cultures of UW85 suppressed and increased zwittermicin A production, respectively (Milner et al., 1995), they did not appear to affect the expression of ZmaR (E.A. Stohl and J. Handelsman, unpublished data).

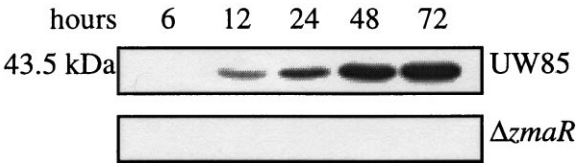

Fig. 3. Expression of ZmaR in *B. cereus* UW85 and UW85Δ*zmaR*. Cultures of UW85 and UW85Δ*zmaR* were grown in 50% TSB. Time points consisting of 3.0 ml of culture were taken at 6, 12, 24, 48, and 72 h. SDS–PAGE gels containing 20 μg of total protein per lane were run, and protein was transferred to PVDF membrane, with subsequent Western blot analysis using the ECL kit. Production of zwittermicin A is first detected after 48 h. The limit of detection for zwittermicin A is 0.33 μg/ml (Milner et al., 1995).

4. Discussion

We identified a genetic region required for zwittermicin A synthesis. DNA sequence analysis identified open reading frames with sequence similarity to acyl-CoA dehydrogenases and the acyltransferase domain of polyketide synthases. Gene inactivation studies in *B. cereus* UW85 identified genes necessary for zwittermicin A production and resistance. Expression of ZmaR, the resistance protein, was temporally associated with zwittermicin A production. This work constitutes the only investigation of an aminopolyol biosynthetic pathway.

The discovery of a region necessary for the biosynthesis of zwittermicin A, a previously undescribed class of aminopolyol antibiotic, may provide a new type of pathway for antibiotic biosynthesis in *Bacillus*. Construction of an *orf2* mutant resulted in the loss of zwittermicin A production, demonstrating the involvement of Orf2 in zwittermicin A production. Orf2 has sequence similarity to the acyltransferase domain of polyketide synthase enzymes, including the active-site sequence (Córtes et al., 1990). Furthermore, since the alternating hydroxyl groups on the carbon backbone of zwittermicin A (Fig. 1) are similar to a partially reduced polyketide, it is possible that zwittermicin A could be synthesized via a mechanism similar to polyketides. However, polyketide synthases utilize multiple enzymatic activities, including an acyltransferase activity, to catalyze the condensation of short-chain carboxylic acids into long carbon chains (Katz and Donadio, 1993). Since *orf2* is the first and only locus thus far identified as being necessary for zwittermicin A production, identification of other enzymatic activities required for zwittermicin A biosynthesis, as well as a thorough understanding of the biochemistry involved in its synthesis, is vital to establish a more convincing link with polyketide synthases. Although preliminary sequence analysis of the DNA further downstream of *orf2* revealed DNA with sequence similarity to the β-ketoacyl ACP synthase domain of polyketide synthases (E.A. Stohl and J. Handelsman, unpublished data), further characterization of the protein potentially encoded by this sequence is necessary. Alternatively, the role of Orf2 in zwittermicin A biosynthesis may be unrelated to polyketide synthases. An acyltransferase was identified as being important for biosynthesis of penicillin, a

β-lactam antibiotic, in *Aspergillus nidulans* (Montenegro et al., 1990); however, this acyltransferase does not contain the acyltransferase active site sequences identified in polyketide synthases or Orf2.

The potential roles of Orf1 and Orf3 in zwittermicin A biosynthesis are not easily predicted. Because of its overall sequence similarity and conserved catalytic residue, Orf1 is likely to function as an acyl-CoA dehydrogenase. A dehydrogenase is involved in the synthesis of sterigmatocystin (Brown et al., 1996), a polyketide, so it is possible that Orf1 is involved in zwittermicin A biosynthesis. Because of the weak sequence similarity of Orf3 with peptide synthetase enzymes, it is difficult to infer the role of Orf3 in zwittermicin A biosynthesis. However, there are many examples of peptide antibiotics produced by *Bacillus* spp. (Nakano and Zuber, 1990), and the structure of zwittermicin suggests that it may be partially derived from an amino acid. Further analysis of the region upstream of *orf3* will establish the nature of *orf3* and its role, if any, in zwittermicin A biosynthesis.

It is intriguing that a *zmaR* mutant could be constructed in a zwittermicin A-producing background. *zmaR* encodes an acetyltransferase that inactivates zwittermicin A (E.A. Stohl, S.F. Brady, J. Clardy, J. Handelsman). Self-resistance proteins that covalently modify the produced antibiotic sometimes perform a dual role as a biosynthetic enzyme (Kumada et al., 1988; Cundliffe, 1989). However, the *zmaR* mutant produced near-wild-type levels of zwittermicin A, as judged by both bioassay and direct isolation of the antibiotic, demonstrating that *zmaR* is not required for zwittermicin A production. We cannot rule out the possibility that the *zmaR* mutant produces an intermediate of zwittermicin A that retains biological activity and possesses biochemical properties sufficiently similar to authentic zwittermicin A to be indistinguishable by our biochemical analyses, although this seems unlikely. Furthermore, despite the loss of a resistance gene, growth of the *zmaR* mutant was not compromised, although the *zmaR* mutant was sensitive to exogenous zwittermicin A. These results suggest the presence of a second mechanism of antibiotic self-resistance in UW85. It is, in fact, common for antibiotic-producing organisms to contain multiple self-resistance mechanisms (August et al., 1994; Calcutt and Schmidt, 1994; Lomovskaya et al., 1996).

Antibiotic self-resistance is often found clustered with, and under similar regulation as, antibiotic biosynthetic genes (Cundliffe, 1989; Matsuo et al., 1997). *zmaR* appears to be arranged in an operon with genes involved in antibiotic biosynthesis, suggesting that these genes may be co-regulated. We were first able to detect zwittermicin A production after 48 h, and production reached maximal levels after 72–96 h (Milner et al., 1995). Expression of ZmaR was first detected after 12 h, and the level of ZmaR increased concomitant with zwittermicin A production, reaching maximal levels after 72 h. Thus, it is possible that *zmaR* and the genes necessary for zwittermicin A production are co-regulated. However, the addition of phosphate and iron to cultures of UW85 did not affect expression of ZmaR, but did affect zwittermicin A production (Milner et al., 1995), suggesting that there may be multiple levels of regulation that differ between the zwittermicin A biosynthetic genes and *zmaR*, the self-resistance gene.

Further analysis of this region will establish the full suite of functions necessary for zwittermicin A resistance and biosynthesis, its regulation and export. Elucidating the biosynthetic pathway of this unique aminopolyol antibiotic will increase our understanding of the diverse mechanisms of antibiotic biosynthesis, and may provide clues about how to engineer the pathways for desired practical outcomes.

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