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Validation of the Intact Zwittermicin A Biosynthetic Gene Cluster and Discovery of a Complementary Resistance Mechanism in *Bacillus thuringiensis*▽†

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Zwittermicin A (ZmA) is a hybrid polyketide-nonribosomal peptide produced by certain *Bacillus cereus* group strains. It displays broad-spectrum antimicrobial activity. Its biosynthetic pathway in *B. cereus* has been proposed through analysis of the nonribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) modules involved in ZmA biosynthesis. In this study, we constructed a bacterial artificial chromosome (BAC) library from *Bacillus thuringiensis* subsp. *kurstaki* strain YBT-1520 genomic DNA. The presence of known genes involved in the biosynthesis of ZmA in this BAC library was investigated by PCR techniques. Nine positive clones were identified, two of which (covering an approximately 60-kb region) could confer ZmA biosynthesis ability upon *B. thuringiensis* BMB171 after simultaneous transfer into this host by two compatible shuttle BAC vectors. Another previously unidentified gene cluster, named *zmaWXY*, was found to improve the yield of ZmA and was experimentally defined to function as a ZmA resistance transporter which expels ZmA from the cells. Putative transposase genes were detected on the flanking regions of the two gene clusters (the ZmA synthetic cluster and *zmaWXY*), which suggests a mobile nature of these two gene clusters. The intact ZmA gene cluster was validated, and a resistance mechanism complementary to that for *zmaR* (the previously identified ZmA self-resistance gene) was revealed. This study also provided a straightforward strategy to isolate and identify a huge gene cluster from *Bacillus*.

Zwittermicin A (ZmA) was first discovered in culture supernatants of *Bacillus cereus* strain UW85, which has the ability to suppress plant disease (11). ZmA has a broad spectrum of antimicrobial activity, inhibiting certain Gram-positive, Gram-negative, and eukaryotic microorganisms (32). It also has the ability to potentiate the insecticidal activity of the protein toxins produced by *Bacillus thuringiensis* (3). ZmA is a linear aminopolyol and represents a new structural class of antibiotic (12). Its unusual structure and diverse biological activities prompted investigation of its biosynthesis.

The ZmA self-resistance gene (*zmaR*) was first isolated from *B. cereus* UW85 by Handelsman's group (23). *zmaR* encodes an acetyltransferase, which acetylates ZmA, rendering it inactive (34). Insertional inactivation of *zmaR* in strain UW85 demonstrated that *zmaR* is necessary for high-level resistance to ZmA but is not required for ZmA production (35).

A 16-kb DNA fragment (*zma16Bc*) from strain UW85, including nine genes and a partial gene (covering *zmaR*), has been isolated. The presence of genes encoding nonribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) homologues in this fragment suggested that ZmA is synthesized by a mixed NRPS/PKS pathway (8). Handelsman's group proposed a model for the ZmA biosynthesis pathway and clearly showed that this 16-kb fragment does not contain all the genes necessary for the biosynthesis of ZmA, as some important genes required for the proposed pathway were missing.

Subsequently, our research group identified three new genes (*zwa6*, *zwa5A*, and *zwa5B*) from another ZmA-producing strain, *B. thuringiensis* subsp. *kurstaki* strain YBT-1520, by sequencing the region downstream of the *zma16Bc* cluster (38, 39). These three genes could fulfill the functions in Handelsman's proposed ZmA biosynthetic pathway, so they were predicted to take part in ZmA biosynthesis.

Recently, Thomas' group revealed a 62.5-kb region consisting of 22 open reading frames (ORFs) related to ZmA biosynthesis by mapping the *zma16Bc* cluster from *B. cereus* UW85. They predicted that ZmA is biosynthesized in an unusual manner that involves processing of both its N and C termini, potentially resulting in the production of two additional metabolites besides ZmA (18). The metabolite (acyl-D-Asn-ZmA) synthesized by the gene cluster is proposed to be cleaved by the secretion transporter ZmaM between the D-Asn and D-Ser, releasing ZmA and fatty acyl-D-Asn (metabolite A), and then they are exported out of the cell by ZmaM (18). One of the two putative additional metabolites (metabolite B) was subsequently detected by liquid chromatography-mass spectrometry (LC-MS) (4). Until now, the proposed mechanism of ZmA biosynthesis has relied mainly on bioinformatics analysis, and no direct experiments have been performed to define whether this gene cluster is sufficient for ZmA biosynthesis.

Heterologous expression in related bacteria has been successfully employed to identify large gene clusters (2, 9, 17, 37). A 12-kb thuringiensin biosynthetic gene cluster from *Bacillus* has been identified in this way by us previously. Thuringiensin

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TABLE 1. Bacterial strains and plasmids used in this study

Species and strain or plasmid	Description[a]	Source or reference
E. coli DH10B	Δ(*mrr hsdRMS mcrBC*) *mcrA recA1*	New England Biolabs
E. herbicola LS005	Indicator strain for ZmA production	31
B. cereus UW85	Produces ZmA	31
B. thuringiensis		
YBT-1520	*B. thuringiensis* subsp. *kurstaki*	38
BMB171	Acrystalliferous mutant of *B. thuringiensis*	13
BMB1212	BMB171 with plasmid pEMB1212, carrying *zmaR*	This study
BMB1236	Mutant of strain YBT-1520 in which the *orf123* gene was knocked out	This study
BMB1237	BMB1236 with plasmid pEMB1237, in which the knocked-out *orf123* gene was complemented	This study
Plasmids		
pBeloBAC11	BAC cloning vector for *E. coli*	21
pHT304	*E. coli* and *B. thuringiensis* shuttle vector	1
pHT304-ts	pHT304 derivative with a temp-sensitive *Bacillus* replicon; Erm^r for *B. thuringiensis*	20
pDG780	Vector with Kan^r for *B. thuringiensis*	BGSC
pEMB0557	*E. coli*-to-*B. thuringiensis* shuttle BAC vector; *ori60*, Erm^r for *B. thuringiensis*	19
pEMB0603	*E. coli*-to-*B. thuringiensis* shuttle BAC vector; *ori44*, Kan^r for *B. thuringiensis*	This study
pEMB1212	1.2-kb PCR fragment containing *zmaR* cloned into pHT304 at SphI/BamHI sites	This study
pEMB1231	2.9-kb PCR fragment containing *zmaWXY* cloned into pHT304 at SphI/BamHI sites	This study
pEMB1232	33.7-kb 4B1 NotI fragment containing *zmaR* cloned into pEMB0603 at NotI site	This study
pEMB1236	Interrupted *orf123* gene cloned into pHT304-ts	This study
pEMB1237	*orf123* gene cloned into pHT304	This study

[a] Kan^r, kanamycin resistance; Erm^r, erythromycin resistance.

production could be observed when this 12-kb region was electroporated into a surrogate *Bacillus* host with the *B. thuringiensis-Escherichia coli* shuttle bacterial artificial chromosome (BAC) vector pEMB0557, which confirms that this 12-kb region is sufficient for the thuringiensin production (20). With the discovery of increasing numbers of large gene clusters, the limitation of this approach is the restriction of the loading capacity of the vector and the transformation efficiency of the large recombinant plasmid in *Bacillus*. We have developed an improved electroporation protocol for *Bacillus* that efficiently transfers a recombinant plasmid with up to 40 kb of DNA inserted in the shuttle BAC vector pEMB0557 (19, 25).

In this study, we present a multiplasmid heterologous expression approach to validate the ZmA biosynthetic gene cluster. We constructed a BAC library with an average insert size of 45 kb from *B. thuringiensis* strain YBT-1520, whose genome sequencing is nearly complete. Nine BAC clones, whose DNA inserts are related to *zma16Bc*, were screened. Two of these clones were individually cloned into two compatible shuttle BAC vectors and transferred simultaneously into a ZmA-nonproducing strain, *B. thuringiensis* BMB171 (13). Several sets with different combinations of the nine BAC clones were also formed. Finally, two strains of *B. thuringiensis* BMB171 containing the recombinant plasmids did produce ZmA, with different yields. We demonstrated that a 60,235-bp region covered by two BAC clones is sufficient for ZmA biosynthesis. Furthermore, we also identified three new ORFs, *zmaWXY*, downstream of this 60-kb cluster, which function as a resistance transporter, forming a different self-resistance mechanism for a ZmA producer strain in addition to *zmaR*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *B. thuringiensis* strain YBT-1520 was isolated from Chinese soil by our group and was shown to produce ZmA. *B. cereus* UW85 is the first strain found to produce ZmA. *B. thuringiensis* strain BMB171 is an acrystalliferous mutant strain and acted as a surrogate host in this study. Two compatible shuttle BAC vectors, pEMB0557 and pEMB0603, were constructed for cloning large fragments in *Bacillus*. The strains and plasmids used in this study are listed in Table 1.

E. coli was grown in Luria-Bertani (LB) medium at 37°C, while *B. thuringiensis* strains were grown at 28°C in 50% (wt/vol) tryptic soy broth (TSB) medium or LB medium. Ampicillin (100 μg/ml for *E. coli*), chloromycetin (12.5 μg/ml for *E. coli*), erythromycin (25 μg/ml for *Bacillus*), and kanamycin (50 μg/ml for *Bacillus*) were used in solid and liquid media for the propagation of plasmids.

DNA manipulation. Established protocols for molecular biology techniques were performed as described by Sambrook et al. (29). Plasmid DNA was obtained from *B. thuringiensis* strains by the modified method of Andrup et al. (1). Oligonucleotides synthesis and DNA sequencing were carried out by Invitrogen Biotechnology Co., Ltd. (Shanghai).

Construction of a BAC library of *B. thuringiensis* strain YBT-1520. It has proved difficult to transfer large recombinant plasmids into *B. thuringiensis* by electroporation (24, 36). Therefore, we developed a transformation procedure to effectively transfer up to 40-kb fragments into *B. thuringiensis* (19). A BAC library from *B. thuringiensis* strain YBT-1520 was constructed as previously described (21), with an average insert size of 45 kb. *B. thuringiensis* strain YBT-1520 was cultured for 5 h at 28°C in LB medium to a density of 3×10^8 cells/ml. Cells were harvested by centrifugation (10 min at 4°C and 12,000 × *g*), and agarose plugs were prepared. Genomic DNA embedded in the agarose plugs was partially digested with HindIII and then separated by pulsed-field gel electrophoresis (PFGE). High-molecular-weight genomic DNA was recovered and ligated into the BAC vector pBeloBAC11, which was previously digested with HindIII. The ligation mix was transformed into *E. coli* strain DH10B by electroporation with a Bio-Rad Gene Pulser. To estimate the insert sizes, the BAC recombinant plasmids were cleaved with NotI and then separated by PFGE with a Bio-Rad CHEF III instrument. The library consisted of 1,200 clones, with an insert size distribution ranging from 25 to 55 kb.

Transformation of large plasmids into *B. thuringiensis*. Transformation of large plasmids into *B. thuringiensis* strain BMB171 was performed by electroporation with the Bio-Rad Gene Pulser set, as previously described (25).

ZmA production assays. ZmA antibacterial activity was determined in an agar diffusion assay with *Erwinia herbicola* LS005 as the indicator on 10% (wt/vol) TSB agar plates (31). Culture supernatants of sporulated *B. thuringiensis* strains and their transformants were filtered with MF-Millipore filters (PES membrane, 0.22 μm) and then used to test their growth inhibition of *E. herbicola* LS005. The plates were incubated for 24 h at 28°C before scoring for the presence or absence

TABLE 2. Primers used in this study

Primer	Sequence[a] (5′ to 3′)	Product size (bp)	Description
F1	CCAGGTCTCAGAAGGAGTAA	671	Probe for screening YBT-1520 BAC library
F2	TGCATCAGAACCAACCTCTG		
F3	ATTGGCAAGAGGTGGGTATGTCACTT	1005	Probe for screening YBT-1520 BAC library
F4	TAGCATATCGAACATGGTGCGGTTCT		
F5	ATTGCATGCATTTGCCATACCATCCACTAAG	2878	For amplifying the *zmaWXY* genes from YBT-1520
F6	ATTGGATCCTTCTCCGATCCCAATTTTCC		
F7	ATTGCATGCACTTGTTCTCAAAAGGGAGG	1241	For amplifying the *zmaR* gene from YBT-1520
F8	ATTGGATCCGAATAATGGGATCCTACGCC		
F9	GGAAAGCTTGGTATTCAGCGTGCTCATTC	800	For amplifying the upstream of gene *orf123* from YBT-1520
F10	GGAGTCGACAATGATTAGTCGTGTTTACCG		
F11	GGAGGATCCGTATCTGAGCTTAGTGATGG	791	For amplifying the downstream of gene *orf123* from YBT-1520
F12	GGAGGTACCCATCCGATAACAAACCTCTC		
F13	GGAGTCGACATTCGATATCAAGCTTATCG	1512	For amplifying the kanamycin resistance cassette from pDG780
F14	GGAGGATCCCGGTATCGATACAAATTCCT		
F15	TAAGAATTCTTGGGAAGAAGTCTGTCGTG	1396	For amplifying the gene *orf123* from strain YBT-1520
F16	TCCGAATTCGCTTATGTAATCTCCTAATTC		

[a] Restriction sites within the PCR primers are underlined.

of an inhibition zone. ZmA production was indicated by the presence of an inhibition zone. The diameter of the growth inhibition zone was measured, and the relative concentrations of ZmA in the supernatants were deduced by comparing the inhibition zone sizes of the test strains with the zone size of a ZmA producer strain.

For further identification of ZmA production, culture filtrates were further filtered with Biomax filters (Millipore; nominal molecular mass limit, 3 kDa). High-performance liquid chromatography (HPLC) combined with ion trap/time-of-flight mass spectrometry (LC-MS–IT-TOF) (Shimadzu) was performed to detect ZmA by its molecular weight (*m*/*z*, 397 [MH^+]). The separation was performed on a C_{18} column, using a gradient elution consisting of mobile phase A (0.1% [wt/vol] formic acid) and mobile phase B (acetonitrile-water [20:80, vol/vol]).

Insertional inactivation of gene *orf123*. An 800-bp fragment and a 791-bp fragment, corresponding to the DNA regions upstream and downstream of the open reading frame of the *orf123* gene in strain YBT-1520, respectively, were generated by PCR using the primer pairs F9/F10 and F11/F12 (Table 2) and digested with HindIII-SalI and BamHI-KpnI, respectively. A kanamycin resistance cassette (1,512 bp) was amplified with primer pairs F13/F14 (Table 2) from plasmid pDG780 and digested with SalI-BamHI. These three fragments were cloned into the temperature-sensitive plasmid pHT304-ts at HindIII-KpnI sites. The resulting plasmid, pEMB1236, was transformed into strain YBT-1520 by electroporation. The transformant was cultivated in LB medium with kanamycin added for 8 h. The transformant was then cultivated at 45°C for 4 days to eliminate the unintegrated temperature-sensitive plasmid pEMB1236. Kanamycin-resistant but erythromycin-sensitive colonies were harvested. The colonies with allelic double exchange were confirmed by PCR, and the correct mutant strain was named BMB1236.

Gene *orf123* was amplified by primer pair F15/F16 (Table 2). The PCR product was digested with EcoRI and then inserted into the EcoRI site of shuttle vector pHT304 to create recombinant plasmid pEMB1237. pEMB1237 was then introduced into strain BMB1236, generating BMB1237, to complement the *orf123* mutation. The vector pHT304 was introduced into strain BMB1236 as a negative control.

ZmA sensitivity test. The ZmA sensitivities of *B. thuringiensis* strain BMB171 and other stains were determined in agar diffusion tests. One hundred microliters of stationary-phase culture was inoculated into 5 ml of TSB medium and grown to an optical density at 600 nm (OD_{600}) of 0.8. Two hundred microliters of a 10^{-2} dilution was spread on a 10% (wt/vol) TSB agar plate. Various amounts of ZmA which were HPLC purified (in a volume of 100 μl) were added to Oxford cups (8-mm diameter) on the test plate. The plate was kept for 2 h at 4°C for diffusion and then incubated overnight at 28°C. The diameter of the growth inhibition zone was measured. ZmA sensitivity was indicated by the size of the inhibition zone.

LC-MS–IT-TOF assay for acetylated ZmA in intracellular contents. The test stains were grown in 100 ml LB medium at 28°C until the cell density reached an OD_{600} of 0.3. ZmA was then added to the cultures at final concentrations of 30 μg/ml or 150 μg/ml. The cultures were then incubated at 28°C until the OD_{600} reached 0.8. The cells were harvested and washed three times with 50 mM Tris-HCl, pH 8.0. The cells were then quickly frozen in liquid nitrogen and adequately triturated. After centrifugation (10 min at 2°C and 12,000 × *g*), the supernatants were collected and LC-MS–IT-TOF was used to detect the molecular weight (*m*/*z* 439.2152 [MH^+]) corresponding to the acetylated ZmA molecule $C_{15}H_{30}N_6O_9$ (34).

Database research. Transmembrane regions were predicted with the DAS-Transmembrane Prediction server (7) (http://www.sbc.su.se/~miklos/DAS/).

Nucleotide sequence accession number. The nucleotide sequence of the ZmA resistance genes of *B. thuringiensis* strain YBT-1520, *zmaWXY*, has been assigned GenBank accession number HQ846969.

RESULTS

Construction of a ZmA biosynthesis-related gene cluster linkage group. To isolate the ZmA biosynthetic genes, we probed the BAC library constructed from the genomic DNA of *B. thuringiensis* strain YBT-1520. Based on the sequences of the 5′ and 3′ termini of *zma16Bc*, the 16-kb region from *B. cereus* UW85 related to ZmA biosynthesis (8), two primer pairs were designed as the probes (Table 2). The relative positions of the targets amplified by PCR are shown in Fig. 1. Using these two primer pairs, PCR amplification from the YBT-1520 BAC library showed that there were nine BAC clones overlapping *zwa16Bc*. Both ends of the target BAC clones were sequenced. Comparing the end sequences with the genome sequence of strain YBT-1520 and the restriction maps of the BAC clones (data not shown), the nine BAC clones were all located to the region containing the ZmA biosynthesis-related gene cluster, as indicated in Fig. 1. The isolated BAC

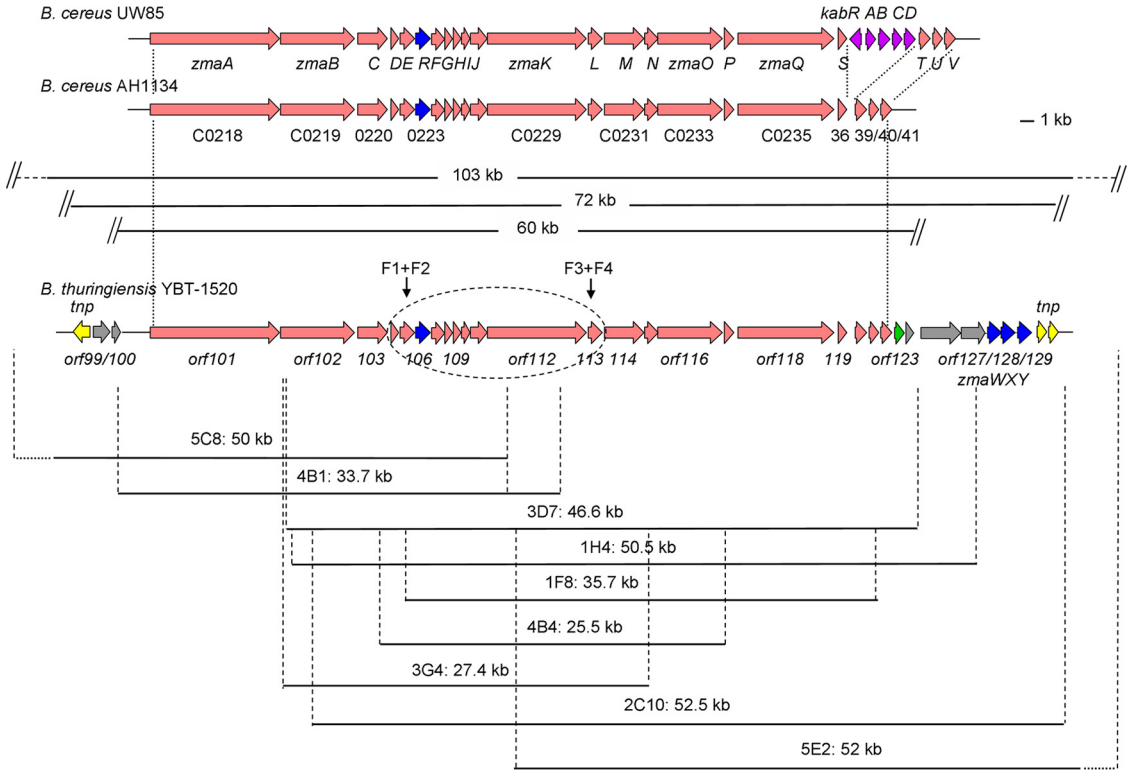

FIG. 1. Construction of ZmA biosynthesis-related gene cluster BAC clone linkage group and comparison of ZmA gene clusters from three strains. The ZmA biosynthesis genes from *B. cereus* UW85 and AH1134 that were discovered by Thomas' group are shown at the top (quoted as described in reference 18). The region of *zma16Bc* is indicated by an ellipse frame on the ZmA gene cluster of strain YBT-1520. The fragments cloned from strain YBT-1520 in nine BAC clones are indicated by black lines under the gene cluster. The names of the clones and the sizes of the DNA fragments are described above the line. Red arrows indicate genes potentially involved in ZmA biosynthesis, blue arrows indicate genes potentially involved in ZmA self-resistance, the green arrow indicates a gene potentially involved in regulation, yellow arrows indicate transposase genes (*tnp*), gray arrows indicate ORFs without clear functions, and purple arrows denote genes involved in kanosamine biosynthesis in UW85 (*kabRABCD*). The target position of the two primer pairs F1/F2 and F3/F4 are indicated by two black arrows.

clones encompassed ~103 kb (from the 5′ terminus of clone 5C8 to the 3′ terminus of clone 5E2) of the YBT-1520 genome.

DNA sequencing reveals the potentially intact ZmA biosynthetic gene cluster. An intact antibiotic gene cluster always comprises three parts: biosynthesis, resistance, and regulation. Sequence analysis of this 103-kb DNA region indicated a 72-kb region which harbors *zma16Bc* and has putative transposase genes on both ends (Fig. 1, yellow arrows). At the 5′ terminus, there is a putative transposase gene (1,449 bp) showing 99% identity with a transposase gene from a *B. thuringiensis* strain (accession no. AY566174). At the 3′ terminus, there are two putative transposase genes (encoding proteins of 138 and 290 amino acids [aa]), both showing 96% identity to transposases from *B. cereus* E33L (accession no. YP_245810 and YP_245811, respectively). The organization and location of these transposase genes suggest that this 72-kb region is a mobile element, and we predicted that this 72-kb mobile element should encompass the intact ZmA gene cluster.

In this 72-kb region, there are 31 ORFs (Fig. 1, *orf99* to *orf129*). Twenty-two of them (*orf101 to 122*) show high similarities (approximately 98% identity) with the predicted ZmA biosynthetic genes (*zmaA* to *-V*) reported by Thomas' group (18) (Fig. 1, red arrows). Adjacent to *orf122* (equivalent to *zmaV*), there are two ORFs: *orf123* (774 bp) and *orf124* (213 bp). They both show 97% identity to conserved hypothetical proteins (BCAH1134_C0242 and BCAH1134_C0244, respectively) from *B. cereus* AH1134. In this ZmA producer strain, *B. cereus* AH1134 (18), these two genes are also located just downstream of the *zmaV* (gene BCAH1134_C0241). ORF123 possesses a ParBc superfamily domain (aa 12 to 90) and a helix-turn-helix DNA-binding motif (27). Given that this gene is connected to the predicted ZmA biosynthetic genes, it is probable that ORF123 is involved in the regulation of ZmA biosynthesis (Fig. 1, green arrow).

There are three ORFs (*orf127* to *-129*) at the 3′ terminus of this region (Fig. 1, blue arrows). *orf127* encodes a 302-aa protein possessing an ATP-binding domain (aa 40 to 190) usually appearing in ATP-binding cassette (ABC) transporters. The next ORFs, *orf128* and *orf129*, whose start codons are GTG, encode 241-aa and 232-aa proteins, respectively. These two ORFs show less than 30% identities to known proteins (data not shown). Both have highly conserved transmembrane domains (TMDs), comprising six trans-membrane α-helices (33). A complete transport complex usually consists of four domains: two ATP-binding domains and two hydrophobic TMDs (15). Based on the above-described similarity, the organization of these three proteins could compose a transport complex. This kind of transport complex has been proven to be involved

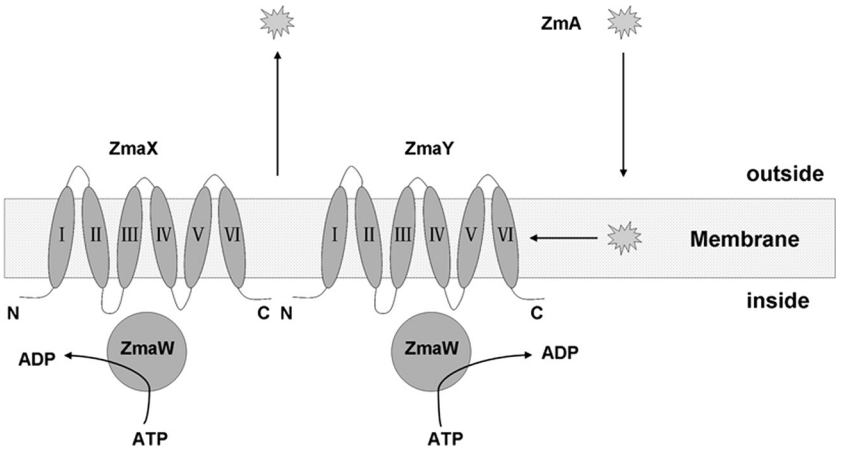

FIG. 2. Prediction of the function of *zmaWXY* in *B. thuringiensis* strain YBT-1520. A schematic representation of the ABC transporter resistance mechanism encompassing two membrane proteins, ZmaX (ORF128) and ZmaY (ORF129) (each with six TMDs), two ZmaW (ORF127) hydrophilic nucleotide-binding proteins, and the proposed location and function of ZmaWXY. TMDs are predicted by the DAS-Transmembrane Prediction server (33). The six *trans*-membrane α-helices are indicated by Roman numerals. The ZmA molecule is indicated by a blast dot.

in resistance to many lantibiotics and is proposed to mediate resistance by active extrusion of lantibiotic molecules from the cytoplasmic membrane (26, 28, 33). Accordingly, we predicted that *orf127*, *orf128*, and *orf129* are involved in ZmA self-resistance in strain YBT-1520. Their contribution to ZmA resistance is the expulsion of ZmA molecules, most likely from the cytoplasmic membrane into the extracellular medium (Fig. 2). We named *orf127*, *orf128*, and *orf129* as *zmaW*, *zmaX*, and *zmaY*, respectively.

From the above analysis, this 72-kb region would consist of a 60-kb biosynthetic cluster, one regulation gene, and a resistance cluster, as well as the transposase genes at both ends.

Validation of the ZmA biosynthetic gene cluster by heterologous expression. To confirm the involvement of the isolated DNA fragments in ZmA biosynthesis, we transferred them into a surrogate host, *B. thuringiensis* strain BMB171, which does not produce ZmA and whose complete genome sequence showed that it lacked the predicted ZmA biosynthesis-related genes (13). Two *B. thuringiensis-E. coli* shuttle BAC vectors, pEMB0557 and pEMB0603, derived from the typical BAC vector pBeloBAC11 by addition of the plasmid replication origins of *B. thuringiensis* large plasmids (90-kb and 60-kb native plasmids of strain YBT-1520, respectively), were constructed for the purpose of heterologous expression with large loading capacity. These two shuttle BAC vectors are compatible with each other in *Bacillus* (unpublished).

According to the relative positions shown in Fig. 1, we first chose BAC clones 4B1 and 2C10, which together encompass nearly the whole predicted ZmA biosynthetic gene cluster. We transferred their inserts into shuttle BAC vectors pEMB0557 and pEMB0603 at the NotI site, respectively, and then cotransferred them into strain BMB171. The ZmA production of the transformant was checked by detecting inhibition of *E. herbicola* strain LS005 by the culture filtrate and by using mass spectrometry for the molecular weight of the ZmA molecule. It was found that the DNA inserts of 4B1 and 2C10 could confer ZmA biosynthesis on host strain BMB171 (Fig. 3). To minimize this region, a transformant with the DNA inserts of 4B1 and 3D7 was constructed. This transformant could still produce ZmA, but the growth inhibition zone was slightly smaller than those of 4B1 and 2C10 (Fig. 3). Other transformants constructed to further minimize this region were unable to produce ZmA (Table 3).

These heterologous expressions demonstrated that the DNA inserts of 4B1 and 3D7 encompass the ZmA biosynthetic gene cluster and that the insert of 3D7 only (or 4B1 and 1F8), which lacks *orf101* and *orf102* (or *orf121* to *-124*) (Fig. 1), is not sufficient for ZmA biosynthesis. The region comprising 4B1 and 3D7 is approximately 60 kb and contains 24 ORFs (Fig. 1), including all 22 ORFs predicted by Thomas' group to be involved in ZmA biosynthesis. The absence of the *zmaWXY* genes in the transformant with inserts of 4B1 and 3D7, which can still produce ZmA, suggests that the *zmaWXY* genes are not essential for ZmA biosynthesis.

The function of the *orf123* (which is included in this 60-kb region) was confirmed by a knockout experiment. ZmA production assays for the *orf123* mutant BMB1236, carrying an interrupted *orf123* gene, demonstrated that this mutant could still produce ZmA, and the area of the growth inhibition zone of 100-μl culture filtrates increased 3.4-fold (the mean of three independent assays) compared to that of strain YBT-1520. Genetic complementation experiments showed that strain BMB1237 which recovered gene *orf123* in BMB1236, could generate the same inhibition zone size as strain YBT-1520. These data demonstrated that *orf123* is also not essential for ZmA biosynthesis, and the *orf123* mutant BMB1236 could yield more ZmA than the wild strain YBT-1520.

***zmaWXY* confer resistance against ZmA to *B. thuringiensis* strain BMB171.** To determine whether the products of *zmaWXY* act as a ZmA resistance transporter and contribute to ZmA production, we assayed its function in strain BMB171, which is highly sensitive to ZmA (13). *zmaWXY* and the known ZmA resistance gene *zmaR* were introduced separately into strain BMB171 using the routine shuttle vector pHT304, resulting in recombinant BMB1231 ($ZmaWXY^+$) and BMB1212 ($ZmaR^+$). Agar diffusion tests showed that *zmaWXY* conferred

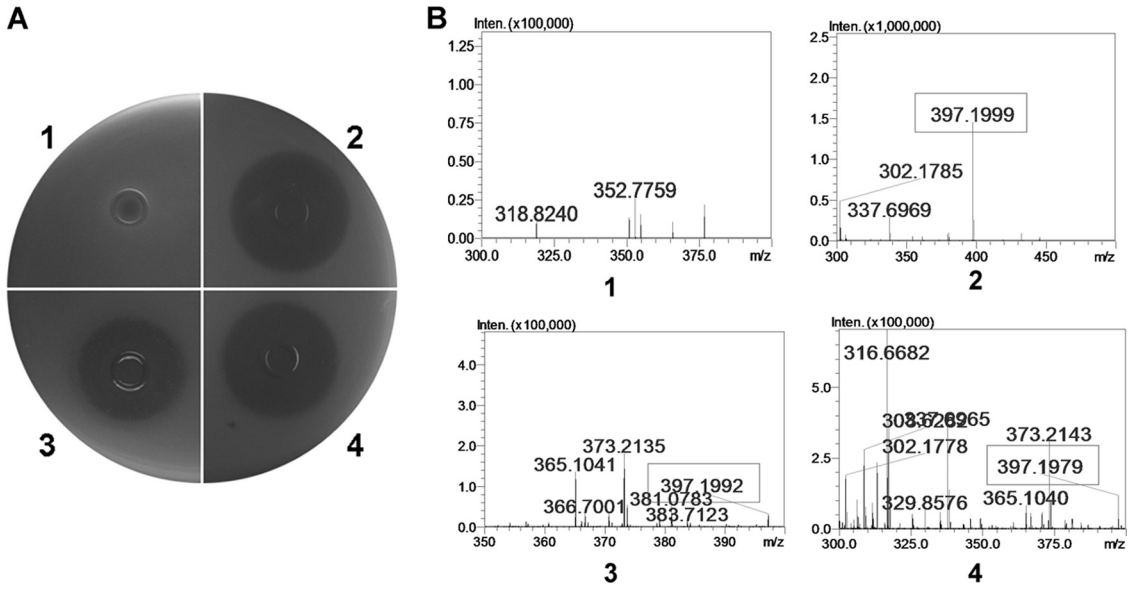

FIG. 3. Expression of the ZmA biosynthesis-related gene cluster in a surrogate host, *B. thuringiensis* strain BMB171. (A) Detection of the ZmA product by agar diffusion tests. A 150-μl volume of culture supernatant was added to each well. (B) LC-MS–IT-TOF detection of a molecular weight corresponding to ZmA in the culture supernatants. The molecular weight corresponding to ZmA is indicated by a rectangular frame. 1, strain BMB171 with vectors pEMB0557 and pEMB0603; 2, strain YBT-1520 (ZmA producer); 3, strain BMB171 with the inserts of BAC clone 4B1 and 3D7; 4, strain BMB171 with the inserts of BAC clone 4B1 and 2C10.

improved resistance against ZmA on strain BMB171 compared to strain BMB171 with vector pHT304 only. However, BMB1231 (ZmaWXY$^+$) had less resistance than BMB1212 with *zmaR* and less than strain YBT-1520, which has both *zmaWXY* and *zmaR* (Fig. 4). These data show that ZmA resistance can be conferred on the ZmA-sensitive strain BMB171 by *zmaWXY*. The data also demonstrated that the acetyltransferase ZmaR and the resistance transporter ZmaWXY could act independently and that ZmaWXY show less tolerance to ZmA than ZmaR. The highest tolerable quantity of ZmA can be calculated from the point of interception with the abscissa, as shown in Fig. 4B. For BMB1212 (ZmaR$^+$), the highest tolerable concentration of ZmA was approximately 200 μg/ml, while for BMB1231 (ZmaWXY$^+$), the value was approximately 80 μg/ml (the point of interception with the abscissa represents approximately 20 and 8 μg ZmA, respectively, and the constant volume of each ZmA sample was 0.1 ml).

TABLE 3. ZmA production by recombinants with various BAC recombinant plasmids

DNA insert(s) of BAC clones (clone vector) in BMB171	Inhibition activity of culture filtrate[a]	Mass spectrometry for mol wt of ZmA[a]
4B1 (pEMB0603) + 2C10 (pEMB0557)	+	+
4B1 (pEMB0603) + 3D7 (pEMB0557)	+	+
4B1 (pEMB0603) + 1F8 (pEMB0557)	−	−
4B1 (pEMB0603) + 4B4 (pEMB0557)	−	−
4B1 (pEMB0603) + 3G4 (pEMB0557)	−	−
3D7 (pEMB0557)	−	−
4B1 (pEMB0603)	−	−

[a] +, inhibition activity was observed or molecular weight of ZmA could be detected; −, inhibition activity was not observed or molecular weight of ZmA could not be detected.

We demonstrated that a transformant with the DNA inserts of 4B1 and 2C10 yields more ZmA than one harboring 4B1 and 3D7, which do not cover *zmaWXY* (Fig. 3). To further investigate the effect of *zmaWXY* on ZmA production, growth profiles were carried out for the two recombinants and ZmA quantification was performed from 48-h cultures, conditions under which ZmA yields were maximal. For the transformant with inserts of 4B1 and 3D7, the area of the growth inhibition zone of 150-μl culture supernatants was approximately 420 mm^2 (see Fig. S1A2 in the supplemental material), whereas the inhibition area increased 1.3-fold to approximately 550 mm^2 for BMB171 with the inserts of 4B1 and 2C10, which included *zmaWXY* (see Fig. S1A4 in the supplemental material). These data suggested that the transformant with *zmaWXY* yielded more ZmA than the transformant without *zmaWXY*.

Furthermore, *zmaWXY* and *zmaR* were transferred into *B. cereus* strain UW85, using pHT304, to detect their contributions to ZmA production in a ZmA native producer strain. For strain UW85 with the vector only, the area of the growth inhibition zone of 100-μl culture supernatants was approximately 320 mm^2 (see Fig. S1B3 in the supplemental material). It increased 1.5-fold to approximately 470 mm^2 after coexpression of *zmaWXY* (see Fig. S1B2 in the supplemental material) and increased 2.1-fold to approximately 690 mm^2 after coexpression of *zmaR* (see Fig. S1B4 in the supplemental material). These data showed that the ZmA yield could be enhanced by additional copies of *zmaWXY* as well as *zmaR*, which act as the ZmA resistance system.

ZmaWXY expel ZmA from *B. thuringiensis* strain BMB171. To clarify the mechanism of action of the ZmA resistance transporter ZmaWXY, a number of recombinants were constructed. *zmaWXY* were transferred into strain BMB171 via shuttle vector pHT304, resulting in BMB1231 (ZmaWXY$^+$).

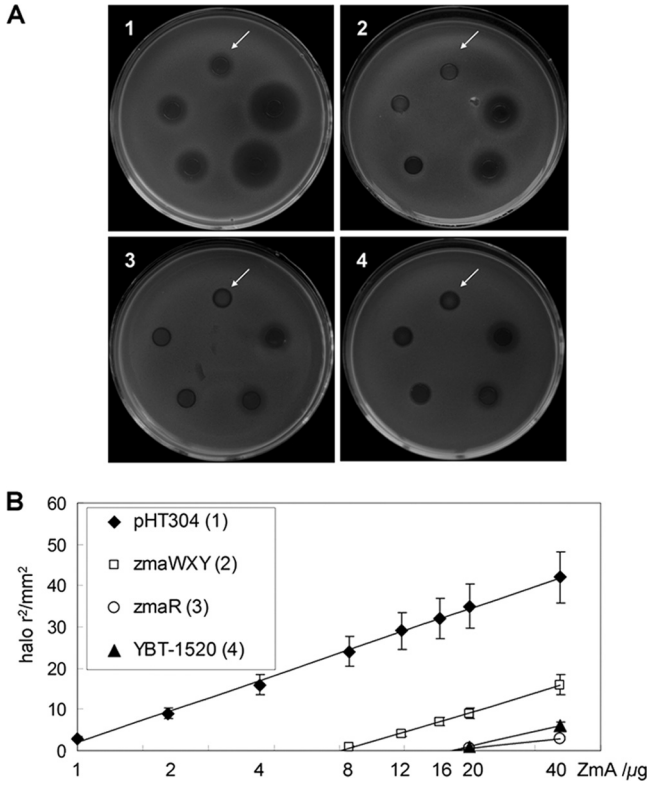

FIG. 4. Functional analysis of ZmA resistance in strain BMB171. The ZmA sensitivities of different strains were detected by agar diffusion tests. (A) Strain BMB171 was transformed with vector pHT304 (plate 1), *zmaWXY* (plate 2), and *zmaR* (plate 3); plate 4 contained *B. thuringiensis* strain YBT-1520. The quantities of ZmA applied to the plates (counterclockwise starting from the arrow) were 1, 2, 4, 20, and 40 μg. ZmA amounts of 8, 12, and 16 μg were also applied in this study but are not shown in these plates. (B) Quantitative representation of the diffusion resistance assay. According to the second law of diffusion (also referred to as Fick's law), the square of the diffusion distance of a given solute in a liquid is directly proportional to the natural logarithm of its initial concentration (33). Thus, with constant volumes (100 μl), linear dependencies between the square of the halos and the natural logarithm of the applied ZmA amounts were obtained. Standard errors were less than 15% for all values (means from three independent assays).

zmaR was transferred into strain BMB171 via shuttle BAC vector pEMB0603, resulting in BMB1232 (ZmaR$^+$). *zmaWXY* and *zmaR* were simultaneously transferred into strain BMB171 via compatible vectors pHT304 and pEMB0603, respectively, resulting in BMB1233 (ZmaR$^+$ and ZmaWXY$^+$). BMB1231 (ZmaWXY$^+$), BMB1232 (ZmaR$^+$), and BMB1233 (ZmaR$^+$ and ZmaWXY$^+$) were incubated in LB medium with 30 μg/ml or 150 μg/ml ZmA overnight. The intracellular contents of all strains were then analyzed by LC-MS–IT-TOF. BMB1231 (ZmaWXY$^+$) could not grow in LB medium with 150 μg/ml ZmA. The molecular weight corresponding to acetylated ZmA, which is the inactive form of ZmA catalyzed by ZmaR, could not be detected in the intracellular fraction of BMB1233 (ZmaR$^+$ and ZmaWXY$^+$) but could be detected in BMB1232 (ZmaR$^+$) with 30 μg/ml ZmA added to the LB medium. The molecular weight corresponding to ZmA also could not be detected in the intracellular fraction of BMB1231 (ZmaWXY$^+$) with 30 μg/ml ZmA added to the LB medium (see Fig. S2A and Table S1 in the supplemental material). These data indicate that ZmA at this concentration (30 μg/ml) cannot migrate into BMB1231 and BMB1233, which harbored *zmaWXY* or both *zmaWXY* and *zmaR*, but can migrate into BMB1232, which harbored only *zmaR*. When the final concentration of ZmA in medium was increased to 150 μg/ml, the molecular weight corresponding to acetylated ZmA could be detected in the intracellular fraction of BMB1233 (ZmaR$^+$ and ZmaWXY$^+$) (see Fig. S2B and Table S1 in the supplemental material). These results suggest that ZmaWXY can prevent ZmA from diffusing into the cells at lower concentrations, such as 30 μg/ml, but when the final concentration of ZmA in the medium increases to 150 μg/ml or exceeds a concentration limit, ZmA can diffuse into the cells and be inactivated (acetylation) by ZmaR.

Meanwhile, the intracellular contents of a *B. thuringiensis* strain YBT-1520 (ZmaR$^+$ and ZmaWXY$^+$) culture, whose yield of ZmA is approximately 20 μg/ml, was also subjected to LC-MS–IT-TOF detection. No molecular weight corresponding to acetylated ZmA was found (data not shown). This result confirms that ZmaWXY can prevent ZmA molecules from diffusing into the cells when the concentration of ZmA surrounding the cells corresponds to the level of ZmA production.

DISCUSSION

Secondary metabolites, such as antibiotics, are generally encoded by large and complicated gene clusters, which are difficult to identify. In this study, we developed a straightforward strategy to address this problem. We set out to circumvent the bias when identifying a whole gene cluster by exhaustive bioinformatics analysis of the DNA sequence and by mutation or deletion analysis for the predicted ORFs. The strategy identified a large functional gene cluster from *Bacillus* by heterologous expression using a series of combinatorial DNA fragments related to the gene cluster in a surrogate host. Compatible shuttle BAC vectors (pEMB0557 and pEMB0603) make it possible to clone large DNA fragments without the capacity limit of the insert size in the BAC library and reduce the pressure of transformation of large recombinant plasmids into *Bacillus*. By screening the target function of the transformants, a series of combinations of different BAC clone inserts expressed in the surrogate host directly indicate which DNA inserts are obligatory or sufficient for the target function and which are not.

In this study, we present the intact ZmA gene cluster, which is proposed to comprise three parts: biosynthesis, resistance, and regulation. In addition, we validated this gene cluster by heterologous expression. A 60-kb fragment, containing 24 ORFs, was demonstrated to be sufficient for ZmA biosynthesis in a surrogate host, which gives experimental support to the hypothetical ZmA biosynthetic pathway proposed by Thomas' group. No BAC clone that harbored the region covering the 22 ORFs but without *orf123* and *orf124* (Fig. 1), both of which were not discovered by Thomas' group, was isolated in this work. Therefore, we could not define whether *orf123* and *orf124* are essential for ZmA biosynthesis by heterologous expression directly. However, further gene knockout experiments showed that the gene *orf123* is not essential for ZmA biosyn-

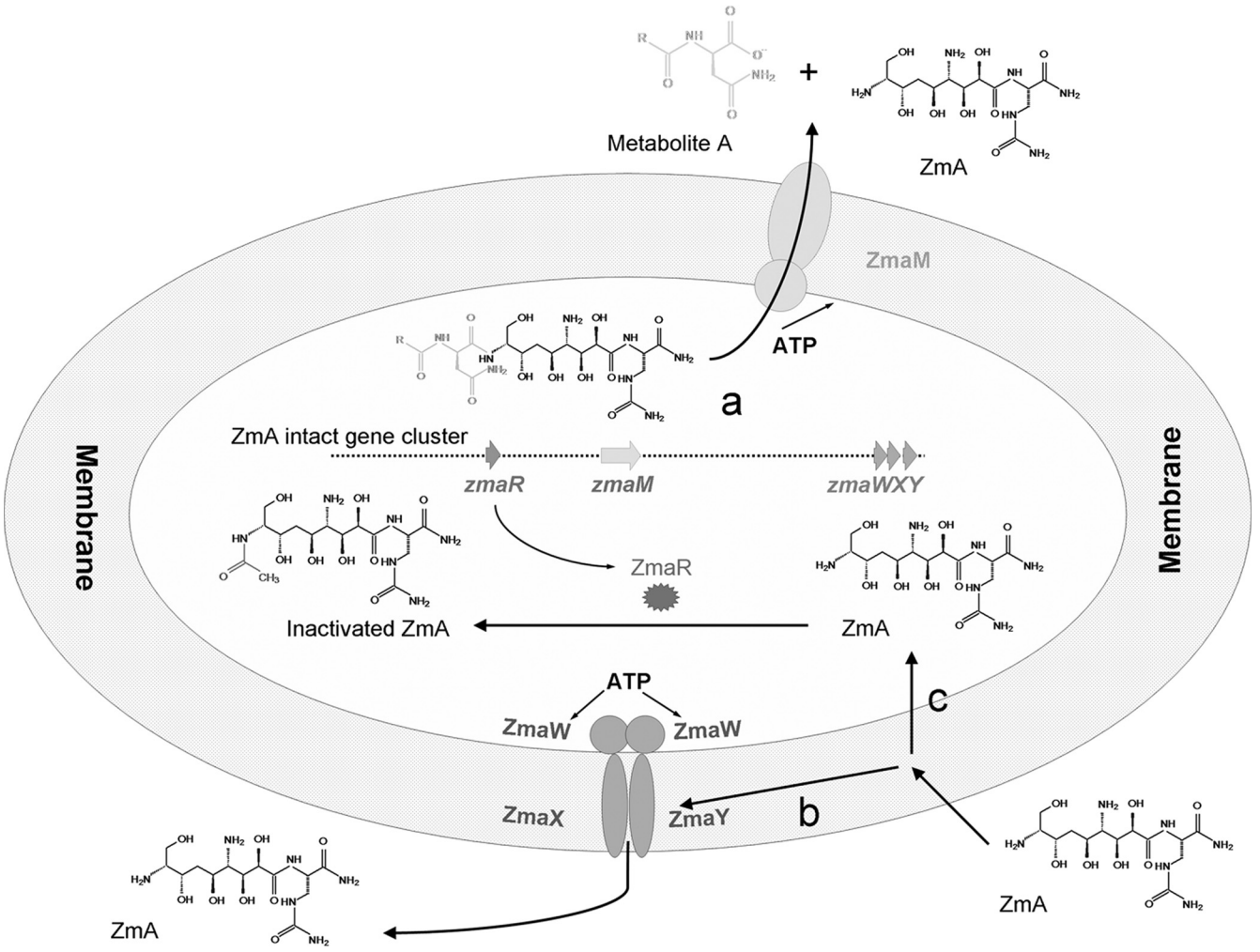

FIG. 5. Schematic model for the transport and resistance of ZmA. ZmA is synthesized by the gene cluster indicated by a dotted line. Resistance genes *zmaR* and *zmaWXY*, and secretion transporter gene *zmaM* are shown on the dotted line. ZmaM and ZmaWXY are located on the membrane, while ZmaR is located in the intracellular space. Black arrows indicate the pathway of ZmA secretion and self-resistance in the ZmA producer strain. See the text for a detailed description of the proposed model.

thesis, and the *orf123* mutant BMB1236 could yield more ZmA than the wild strain YBT-1520. Given that ORF123 is predicted to be involved in the regulation of ZmA biosynthesis, it is implied that ORF123 may act as a negative regulator of ZmA biosynthesis.

In this study, transposase genes were found at both ends of the 72-kb DNA region which encompasses the intact ZmA gene cluster. Some investigators have insisted that *B. thuringiensis* acquired insecticidal factors in the course of coevolution with insects through a host-parasite relationship (22, 30). Therefore, it could be predicted that the ZmA gene cluster was acquired in the *B. cereus* group by horizontal gene transfer. Furthermore, the presence of a sequence with similarities to a *B. cereus* transposon suggests the possibility that gene transfer between *B. cereus* and *B. thuringiensis* occurred. The overall GC content of this 72-kb mobile element, 32.5%, is slightly lower than that of the genomes of many *B. thuringiensis* and *B. cereus* strains, such as *B. thuringiensis* serovar konkukian strain 97-27, *B. thuringiensis* strain Al Hakam, *B. cereus* ATCC 14579, *B. cereus* E33L, and *B. thuringiensis* strain BMB171, at 35% (5, 10, 13, 16).

Handelsman's group found that the *zmaR* mutant UW85Δ*zmaR* was sensitive to high levels of ZmA (300 μg) but could still produce ZmA at near-wild-type levels (12.5 μg/ml of culture) without compromising growth compared to the parent strain, suggesting that *zmaR* is not essential to ZmA biosynthesis and that another mechanism of self-resistance must be present (8, 35). This additional mechanism of self-resistance has remained undiscovered until now. In this study, three genes, *zmaW*, *zmaX*, and *zmaY* (*zmaWXY*), were found at the end of the intact ZmA gene cluster and were shown to function as another mechanism of self-resistance against ZmA. Furthermore, *zmaWXY*, as well as *zmaR*, were found to contribute to the increased yield of ZmA, which agrees with reports that the introduction of additional antibiotic resistance genes into antibiotic-producing bacteria results in increased antibiotic production (6, 14). It is probable that the presence of additional copies of *zmaWXY* and *zmaR* in a ZmA producer strain could

reduce the growth pressure of ZmA on the mother cells and stimulate the yield of ZmA.

Taken together, the whole mechanism for ZmA self-resistance of the producer strain can be predicted. First, the metabolite (acyl-D-Asn-ZmA) synthesized by the gene cluster is cleaved by the secretion transporter ZmaM between D-Asn and D-Ser, releasing ZmA and fatty acyl-D-Asn (metabolite A). The secretion transporter ZmaM then exports them out of the cell (18) (Fig. 5a). ZmA in the culture supernatants could easily interact with the cytoplasmic membrane. The resistance transporter ZmaWXY will recognize it and expel it from the cytoplasmic membrane, thus keeping ZmA molecules out of the cell (Fig. 5b). This process requires ATP to provide energy. If the concentration of ZmA in the culture supernatants is higher than the concentration that ZmaWXY can cope with, ZmA molecules could diffuse into the cell. Once the ZmA molecules get into the cell, ZmaR acetylates ZmA, thereby inactivating it and rendering the cell resistant to ZmA (Fig. 5c). This hypothesis provides a reasonable explanation for why the *B. cereus* mutant UW85Δ*zmaR* is sensitive to ZmA at a high concentration (300 μg per well) but at a low concentration of ZmA can still produce near-wild-type levels of ZmA (12.5 μg/ml of culture) (8).

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