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ORIGINAL PAPER

Identification of three Zwittermicin A biosynthesis-related genes from *Bacillus thuringiensis* subsp. *kurstaki* strain YBT-1520

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Abstract Zwittermicin A (ZwA) is a novel, broad-spectrum linear aminopolyol antibiotic produced by some *Bacillus cereus* and *Bacillus thuringiensis*. However, only part of its biosynthesis cluster has been identified and characterized from *B. cereus* UW85. To better understand the biosynthesis cluster of ZwA, a bacterial artificial chromosome (BAC) library of *B. thuringiensis* subsp. *kurstaki* strain YBT-1520, a ZwA-producing strain, was constructed. Two BAC clones, 1F8 and 5E2, were obtained by PCR, which overlap the known ZwA biosynthesis cluster of *B. cereus* UW85. This ZwA biosynthesis cluster is at least 38.6 kb and is located on the chromosome, instead of the plasmid. Partial DNA sequencing revealed both BAC clones carry three new ZwA biosynthesis-related genes, *zwa6*, *zwa5A* and *zwa5B*, which were found at the corresponding location of *B. cereus* UW85. Putative amino acid sequences of these genes shown that ZWA6 is homologous to a typical carbamoyltransferase from *Streptomyces avermitilis*, while ZWA5A and ZWA5B are homologs of cysteine synthetase and ornithine cyclodeaminase which jointly synthesize 2,3-diaminopropionate in the viomycin biosynthesis pathway, respectively. The identification of these three genes further supports the hypothesized ZwA biosynthesis pathway.

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Abbreviations

A	Adenylation domain
ACP	Acyl carrier domain
BGSC	Bacillus Genetic Stock Center
C	Condensation domain
KS	Ketosynthase
KR	Ketoreductase
NRPS	Nonribosomal peptide synthetase
PCP	Peptidyl carrier protein
PKS	Polyketide synthase
PLP	Pyridoxal 5′-phosphate
ZwA	Zwittermicin A

Introduction

Zwittermicin A (ZwA) is a novel aminopolyol antibiotic produced by some *Bacillus cereus* and *Bacillus thuringiensis* (He et al. 1994; Silo-Suh et al. 1994; Stabb et al. 1994; Raffel et al. 1996). It is highly active against *Oomycetes* and their relatives, and has moderate activity against diverse Gram-negative bacteria and certain Gram-positive bacteria, as well as activity against a wide range of plant pathogenic fungi (Silo-Suh 1994, 1998). On the other hand, ZwA also facilitates the insecticidal activity of the protein toxin produced by *B. thuringiensis*, increasing mortality of insects that are typically resistant to this toxin (Broderick et al. 2000, 2003).

Zwittermicin A is different from polyketide and peptide antibiotics in chemical structure with a molecular formula of $C_{13}H_{28}N_6O_8$ (He et al. 1994; Stabb and Handelsman 1998). The hydroxyl groups on the linear

carbon backbone are similar to a partially reduced polyketide structure, but the nitrogen-rich end of ZwA may be derived from an amino acid, like some peptide antibiotics (Stohl et al. 1999a).

A 16-kb DNA fragment (*zwa16Bc*) covering *zmaR* (ZwA resistance gene) was cloned from *B. cereus* strain UW85 and its DNA sequence revealed a potential ZwA biosynthesis cluster, including nine genes and a partial gene (Stohl et al. 1999a; Emmert et al. 2004). Based on the structural and genetic information, Handelsman's group hypothesized that the premature ZwA is synthesized by a nonribosomal peptide synthetase and polyketide synthetase (PKS) hybrid pathway from five kinds of precursors: L-serine, malonyl-CoA, aminomalonyl-ACP, hydroxymalonyl-ACP and 2,3-diaminopropionate (Ansari et al. 2004; Emmert et al. 2004; Fig. 1a). Then, a hypothetic carbamoyltransferase catalyzes the premature ZwA into ZwA (Emmert et al. 2004; Fig. 1b). However, neither the carbamoyltransferase gene nor the 2,3-diaminopropionate synthetase gene has been identified in *zwa16Bc*.

In this study, a bacterial artificial chromosome (BAC) library was constructed with the total DNA of *B. thuringiensis* subsp. *kurstaki* strain YBT-1520 (briefly, *B. thuringiensis* YBT-1520), a ZwA-producing bacterium with high toxicity against *Helicoverpa arrnigera* larvae (Sun et al. 2000). From this library, nine clones overlapping *zwa16Bc* were identified and two of them, 1F8 and 5E2, were found carry the carbamoyltransferase gene and the 2,3-diaminopropionate synthetase gene.

Materials and methods

Bacterial strains and primers

The bacterial strains and primers used in this study are listed in Tables 1 and 2, respectively.

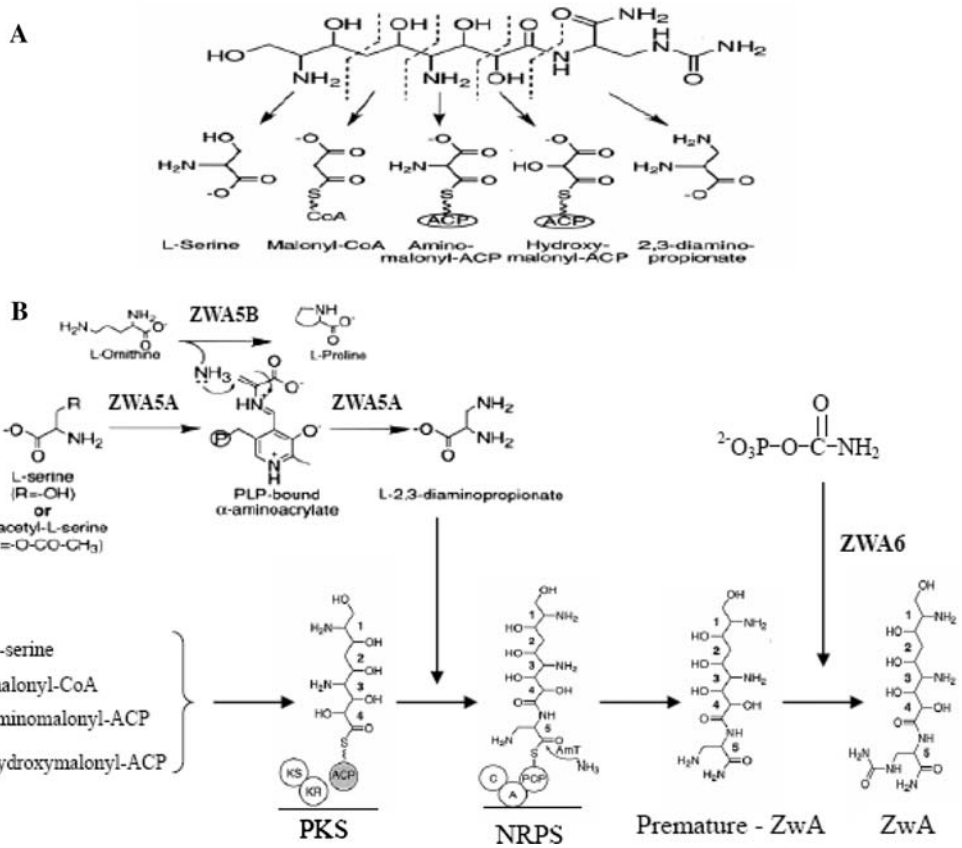

Fig. 1 Chemical structure and proposed biosynthesis pathway of ZwA (modified from Thomas et al. 2003; Emmert et al. 2004). **a** Structure of ZwA and proposed biosynthesis precursors. **b** Hypothesized function of ZWA5A, ZWA5B and ZWA6 in the biosynthesis of ZwA. The premature ZwA is synthesized by a NRPS and PKS hybrid pathway from five kinds of precursors: L-serine, malonyl-CoA, aminomalonyl-ACP, hydroxymalonyl-ACP and 2,3-diaminopropionate. ZWA5A and ZWA5B synthesize 2,3-diaminopropionate by their concerted actions. Then ZWA6, a typical carbamoyltransferase, catalyzes the premature ZwA into ZwA. *A* adenylation domain, *ACP* acyl carrier domain, *C* condensation domain, *KR* ketoreductase, *KS* ketosynthase, *NRPS* nonribosomal peptide synthetase, *PCP* peptidyl carrier protein, *PKS* polyketide synthase, *PLP* pyridoxal 5′-phosphate, *ZwA* zwittermicin A

Table 1 Bacterial strains used in this study

Strain	Description	Source or reference
Escherichia coli		
E. coli DH10B	Δ(*mrr_hsdRMS_mcrBC*)*mcrA recA*1	New England Biolabs
Erwinia herbicola		
E. herbicola LS005	Standard bacteriological test strain	Silo-Suh et al. (1994)
Bacillus		
B. cereus UW85	Wild type, ZwA producing	Silo-Suh et al. (1994)
B. cereus UW56	ZwA improved mutant from *B. cereus* UW85	Silo-Suh et al. (1994)
B. thuringiensis HD1	Wild type, *zmaR*$^+$	BGSC
B. thuringiensis YBT-1520	Wild type, *zmaR*$^+$	Sun et al. (2000)

zmaR ZwA resistance gene, *Cm*^{r} chloramphenicol resistance gene, *ZwA* zwittermicin A, *BGSC* Bacillus Genetic Stock Center (The Ohio State University, USA)

Table 2 Primers used in this study

Primer	Sequence
Up-F	CCAGGTCTCAGAAGGAGTAA
Up-R	TGCATCAGAACCAACCTCTG
Down-F	ATTGGCAAGAGGTGGGTATGTCACTT
Down-R	TAGCATATCGAACATGGTGCGGTTCT
6U	AGCTGTTTAGTAGTTTGTATTTACATACAT
6D	CTCTAATTTCTTGAGCATAATTTGCTTCCT
5AU	AATGAGCACGCTGAATACCT
5AD	CCGAAAAGTATTGTATTTAC
5BU	CGCGGATCCAAAAGTCTTGATTTAGATGA
5BD	GGGATGTAAGAGATCTAAGGCCGAAATAAT
PKS-U	TATGTTGGAGGATCACCAAA
PKS-D	AAGCTGTATCAGATAGTGAT
A	ACTTTAGCACCAGATAATCG
B	TAGTTTGTATTTACATACAT
C	TTCCAGGTACGGAGTTATAT
D	GTAACTTTGCGGTTGCTCTT
E	AGGATGTTCAAAATACACCT
F	TCGAGAGTTTGCCAAGTTAA

All the primers were synthesized by TAKARA Biotechnology (Dalian) Co., Ltd

Purification and identification of ZwA produced by *Bacillus thuringiensis* YBT-1520

Zwittermicin A was initially purified from *B. thuringiensis* YBT-1520 with amberlite IRC-50 cation exchange resin (SIGMA) according to the purification method described previously (Silo-Suh et al. 1994, 1998). The eluants were concentrated in a rotary evaporator. Resuspended sample was analyzed by high-performance liquid chromatography-mass spectrometry (HPLC-MS) with a Waters Alliance 2695 instrument. Fractions were collected and assayed for inhibition of *Erwinia herbicola* LS005 by the method reported earlier (Silo-Suh et al. 1994; Stabb et al. 1994).

Construction of BAC library

Bacillus thuringiensis YBT-1520 was cultured for 5 h at 28°C in Luria Bertani medium to a density of 3×10^8 cells/ml. Cells were harvested by centrifugation and agarose plugs were prepared as described (Rondon et al. 1999). Genomic DNA embedded in the agarose plugs was partially digested with *Hin*dIII (TAKARA) and then separated by pulse field gel electrophoresis (PFGE). High molecular weight genomic DNA was recovered and ligated into cloning-ready BAC vector pBeloBAC11 (GenBank accession no. U51113) digested with *Hin*dIII. The ligation mix was transformed into *Escherichia coli* strain DH10B by electroporation with a Bio-Rad GenePulser instrument. To estimate the insert sizes of BAC clones, BAC clones were digested with *Not*I and *Hin*dIII, and then separated by PFGE with a Bio-Rad CHEF III instrument.

PCR screening and DNA sequencing

In order to identify those BAC clones related to ZwA synthesis by PCR, two primer pairs were designed according to the sequence of the 5′- and 3′-terminals of *zwa16Bc* (Emmert et al. 2004). Primer pair up-F and up-R, were designed to amplify the 5′-terminal, while primer pair down-F and down-R for the 3′-terminal.

Both ends of target BAC clones were sequenced with T7 and SP6 universal primers, respectively. A 1.2-kb DNA sequence located on the 3′-terminal of BAC clone 1F8 was found to encode a putative protein ZWA6. The 2-kb DNA sequence following it was tested by means of primer walking with BAC clone 5E2 as a template. Another two potential proteins ZWA5A and ZWA5B were found. All of these three genes, *zwa6*, *zwa5A* and *zwa5B* were deposited to GenBank under accession no. DQ120781.

Southern hybridization

Southern hybridization was performed to determine the location of the ZwA biosynthesis cluster. A 1,058-bp DNA fragment encoding 2,3-diaminopropionate synthetase was amplified from strain YBT-1520 with primer 5BU and primer 5BD and labeled by (α-^{32}P)-dGTP with hexanucleotide random primer amplification. Intact total DNA from bacteria was separated by

PFGE and then transferred to a nylon membrane. Southern hybridization was performed according to a standard protocol (Sambrook and Russell 2001).

Results and discussion

Identification of ZwA produced by *Bacillus thuringiensis* YBT-1520

The initial purified ZwA from strain YBT-1520 was analyzed by HPLC. There is a main peak distinguish from the others obviously (Fig. 2a). The fraction eluted from 5.5 to 6.0 min was collected to give pure ZwA. This purified sample showed activity to inhibit *Erwinia herbicola* LS005 (data not shown), which is sensitive to ZwA. The mass spectrogram (Fig. 2b) showed that the molecular weight of this sample is 397 Da, the molecular weight of ZwA determined by Handelsman's group (Silo-Suh et al. 1994). Previous studies also showed that many *B. thuringiensis* strains could produce ZwA, especially *B. thuringiensis* subsp. *kurstaki* (Nair et al. 2004). In addition, ZwA-resistant gene *zmaR*, which is necessary for ZwA production (Milner et al. 1996; Stohl et al. 1999b), was detected from YBT-1520 by PCR and sequence analysis (unpublished data).

BAC library of *Bacillus thuringiensis* YBT-1520

To isolate the ZwA biosynthesis cluster, a BAC library of *B. thuringiensis* YBT-1520 was constructed. This library consists of 960 clones, with an average insert size of 50 kb and a distribution of insert size ranging from 24.5 to 72.5 kb. Assuming the size of the YBT-1520 genome is 5 Mb, the library would have a 9.6-fold coverage of the YBT-1520 genome and the probability

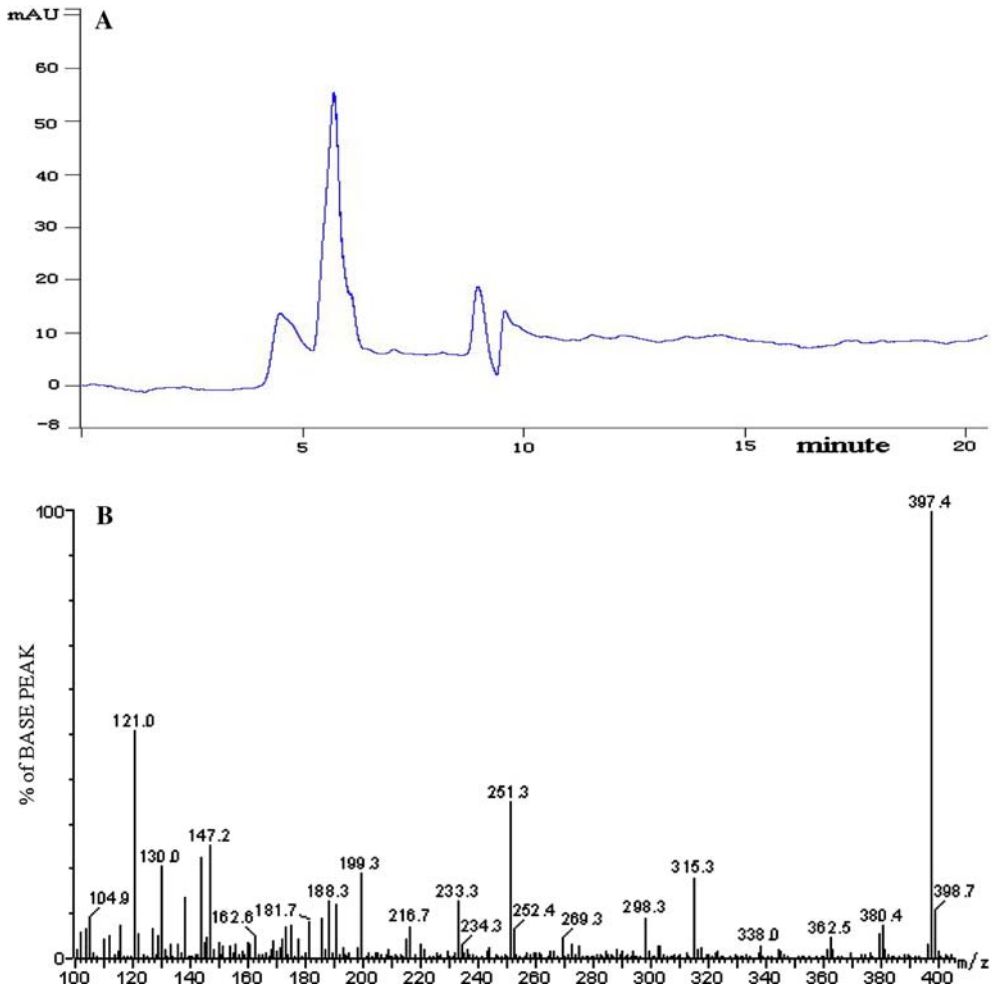

Fig. 2 HPLC-MS analysis of ZwA produced by *B. thuringiensis*. **a** The chromatogram (C18 column was used, 40% methanol–water used as mobile phase, monitored at 220 nm wavelength) of initial purified antibiotic. **b** The mass spectrogram (electron ionization, 70 eV were used) of the fraction collected from 5.5 to 6.0 min in HPLC analysis

of the library covering any particular 1 kb gene is 99.999%.

Identification of ZwA biosynthesis-related BAC clones

PCR amplification showed that there were nine BAC clones overlapping *zwa16Bc*. Among them, clone 1G7, 4B1 and 5C8 overlap 671 bp region of the 5′-terminal (nt54–nt724), clone 1F8, 4C5 and 5E2 overlap 1,005 bp region of the 3′-terminal (nt14847–nt15851), while clone 1H4, 3D7 and 4B4 overlap both.

As for clone 1F8, its 5′-terminal DNA analysis showed 676-bp DNA sequence shares 98% nucleotide identities with *zmaR* in *zwa16Bc* (nt621–nt1296). Another pair of primers named PKS-U and PKS-D was designed to check whether clone 1F8 contains the *pks* gene, which is present at the 3′-terminal of *zwa16Bc*. A 1,069-bp DNA fragment was amplified from clone 1F8 with this primer pair. DNA sequence of PCR product has a 97% nucleotide identity to the corresponding region in *pks* gene (nt11201–nt12269). This implied that clone 1F8 covers a counterpart of *zwa16Bc* (Fig. 3).

The 3′-terminal DNA sequence of clone 1F8 was determined and PCR primers were then designed according to the determined sequence. PCR amplification found that clone 5E2 overlaps the 3′-terminal of clone 1F8. Furthermore, PCR amplification (with primer pair down-F/R and primer pair PKS-U/D) and DNA analysis showed that clone 5E2 overlaps the 3′-terminal and *pks* gene of *zwa16Bc* as well as 1F8 (Fig. 3).

These results indicated that the gene organization of ZwA biosynthesis cluster of *B. thuringiensis* YBT-1520 is most likely similar to that of *B. cereus* UW85.

Identification and characterization of *zwa6*, *zwa5A* and *zwa5B* genes

In the proposed ZwA biosynthesis pathway, neither the carbamoyltransferase gene nor the 2,3-diaminopropionate synthetase gene has been included in *zwa16Bc* (Emmert et al. 2004).

Interestingly, end DNA sequencing found that the 5′-terminal of clone 1F8 shares high similarity to the carbamoyltransferase gene. An intact open reading frame (ORF) *zwa6* was identified from clone 1F8 by walking sequencing with primer A. Based on the sequence of *zwa6*, three ORFs (*zwa6–zwa5A–zwa5B*) were identified from clone 5E2 by primer walking with five sequencing primers (primer B, C, D, E and F) step by step. At the upstream of *zwa5B*, no obvious promoter could be recognized. Therefore, most likely these genes are in the same operon with *zwa5A* and share a common promoter.

Gene *zwa6* encodes a putative protein sharing 56% amino acid identity with ornithine carbamoyltransferase from *Streptomyces avermitilis* (Omura et al. 2001). According to the proposed ZwA biosynthesis hypothesis (Emmert et al. 2004), ZWA6 would be the candidate enzyme catalyzing the premature ZwA into ZwA in the last step of ZwA biosynthesis (Fig. 1b). Further work is in progress to test this hypothesis.

Meanwhile, ZWA5A and ZWA5B share 39% identity and 33% identity to VioB and VioK, which are homologs of cysteine synthetase and ornithine cyclodeaminase and involved in viomycin biosynthesis by concerted actions to synthesize the precursor—2,3-diaminopropionate (Thomas et al. 2003), respectively. Therefore, it is reasonable to draw a conclusion that ZW A5A and ZWA5B synthesize 2,3-diaminopropionate

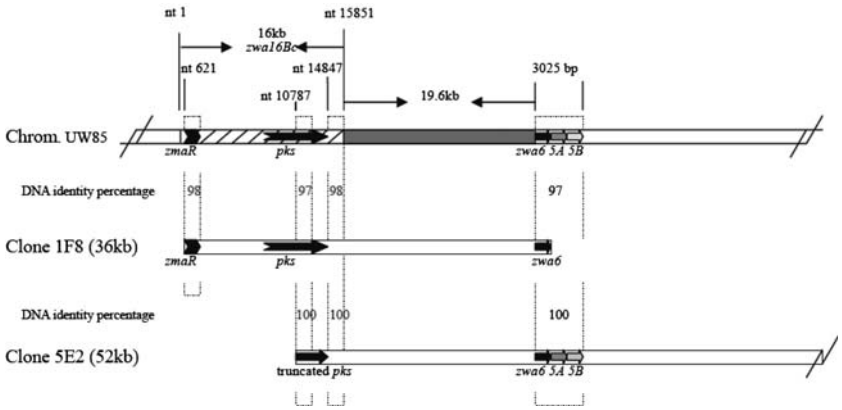

Fig. 3 Schematic show of the putative alignment of BAC clones 1F8 and 5E2 relative to the ZwA biosynthesis cluster of *B. cereus* strain UW85. The *diagonal frame* represents part of ZwA biosynthesis cluster, *zwa16Bc*, identified from *B. cereus* UW85. All of the DNA fragments in the *dashed frames* were sequenced and analyzed, DNA identity percentages of corresponding sequence are as shown in the *middle* of the frames

by their concerted actions in ZwA biosynthesis (Fig. 1b) and that 2,3-diaminopropionate is actually one of the precursors of ZwA. PCR amplification showed *zwa6*, *zwa5A* and *zwa5B* could be amplified from strain UW85 with primer pairs 6U/6D, 5AU/5AD and 5BU/5BD, respectively. PCR product shares 97% DNA identity with *zwa6–zwa5A–zwa5B* from *B. thuringiensis* YBT-1520. All these three genes cloned from *B. cereus* are registered in GenBank as DQ336618.

Furthermore, according to the insert size in BAC clone 1F8 (36 kb) and its alignment with *zwa16Bc* (Fig. 3), the minimal size of ZwA biosynthesis cluster should be 38.6 kb (36 kb plus the 2 kb fragment of *zwa5A* and *zwa5B*, and 0.6 kb of *zmaR* upstream). However, it is necessary to determine whether the 38.6-kb DNA fragment is sufficient for the biosynthesis of ZwA. Long-range PCR with down-F and 5BD as primers showed that the distance between 3′-terminal of *zwa16Bc* and *zwa6* gene is 19.6 kb (subtracting 1 kb of *zwa16Bc* 3′-terminal and 3 kb of *zwa6–zwa5A–zwa5B* from the total 23.6 kb PCR product) in clone 5E2, *B. cereus* UW85 and *B. thuringiensis* YBT-1520 (Fig. 4). The 23.6-kb DNA fragment amplified from *B. thuringiensis* YBT-1520 and *B. cereus* UW85 showed similar restriction fragment length polymorphisms except that *B. thuringiensis* YBT-1520 has two more *Hin*dIII sites, while *B. cereus* UW85 has one more *Eco*RI site. It indicates that ZwA biosynthesis clusters from YBT-1520 and UW85 have a similar organization.

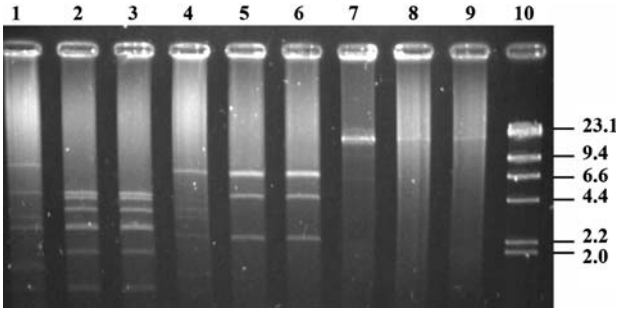

Fig. 4 Restriction fragment length polymorphisms of the 23.6-kb DNA fragment amplified from *B. thuringiensis* YBT-1520, *B. cereus* UW85 and BAC clone 5E2. *Lane 1* UW85/*Hin*dIII, *lane 2* YBT-1520/*Hin*dIII, *lane 3* 5E2/*Hin*dIII, *lane 4* UW85/*Eco*RI, *lane 5* YBT-1520/*Eco*RI, *lane 6* 5E2/*Eco*RI, *lane 7* UW85, *lane 8* YBT-1520, *lane 9* 5E2, *lane 10* lambda DNA/*Hin*dIII (size in kb)

Localization of the ZwA biosynthesis cluster

As far as we know, several antibiotic biosynthesis gene clusters have been found on the chromosome of the host, even though in many *Streptomyces* and other bacteria, gene(s)/gene cluster responsible for antibiotics biosynthesis are located on plasmids, instead of chromosome. Some *B. thuringiensis* and *B. cereus* can produce ZwA, but not all of them (Silo-Suh et al. 1994; Nair et al. 2004). No ZwA biosynthesis cluster was found from the genome sequence of *B. cereus* group (Ivanova et al. 2003; Read et al. 2003). This tells us that ZwA biosynthesis cluster is not a constitutive component of their chromosome.

Pulse field gel electrophoresis showed that ZwA producing strains (e.g., *B. thuringiensis* strain YBT-1520, HD1 and *B. cereus* strain UW85 and UW56) have gigantic plasmids (Fig. 5). To determine the location of the ZwA biosynthesis genes of these strains, the gigantic plasmids were separated from their chromosomes by PFGE and southern hybridization was performed with a 1,058-bp DNA fragment containing *zwa5B* as the probe. Results showed that *zwa5B* locates on the chromosome, but not on the plasmid (Fig. 5). Antibiotic biosynthesis genes are usually organized in a

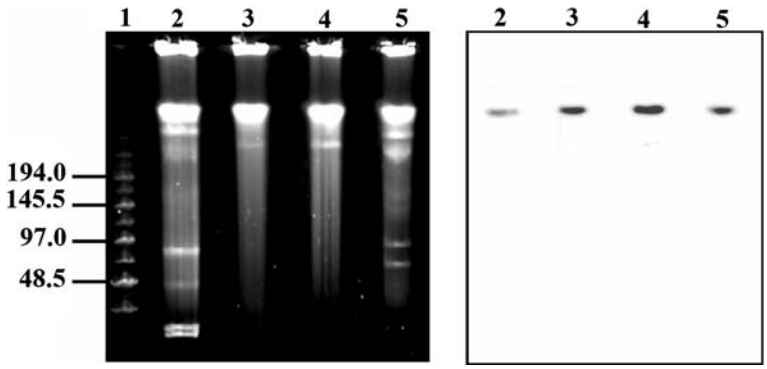

Fig. 5 Southern blot analysis of four ZwA-producing strains. Intact total DNA of four ZwA-producing strains prepared from plugs were separated by PFGE (*left panel*; 1% agarose gel, 6 V/cm, 14°C for 18 h; switch times ramped from 1 to 25 s), transferred onto a nylon membrane and followed by hybridization with a 1,029-bp DNA fragment containing *zwa5B* as probe. The autoradiograph is as shown on the *right panel*. *Lane 1* MidRange II PFG markers (size in kb), *lane 2 B. thuringiensis* HD1, *lane 3 B. cereus* UW56, *lane 4 B. cereus* UW85, *lane 5 B. thuringiensis* YBT-1520

gene cluster (Du et al. 2000; Wu et al. 2000; Ahlert et al. 2002). If it is also true in these strains we tested, the ZwA biosynthesis clusters should locate on their chromosomes.

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