FEBS Letters 582 (2008) 3125–3131

L-2,3-Diaminopropionate: One of the building blocks for the biosynthesis of Zwittermicin A in *Bacillus thuringiensis* subsp. *kurstaki* strain YBT-1520

Changming Zhao[1], Chunxu Song[1], Yi Luo, Ziniu Yu, Ming Sun*

State Key Laboratory of Agricultural Microbiology, College of Life Science and Technology, Huazhong Agricultural University, Wuhan 430070, PR China

Received 1 July 2008; revised 29 July 2008; accepted 30 July 2008

Available online 8 August 2008

Edited by Aleksander Benjak

Abstract Zwittermicin A (ZwA) is a hybrid polyketide–non-ribosomal peptide that is thought to be biosynthesized from five proposed building blocks, including the 2,3-diaminopropionate. Candidate genes for de novo biosynthesis of 2,3-diaminopropionate, *zwa5A* and *zwa5B*, have been identified in a previous study. In this research, *zwa5A* was interrupted and chemically synthesized 2,3-diaminopropionate was used to feed the *zwa5A*$^-$ mutant. Results showed that feeding with 2,3-diaminopropionate restored the ability of the *zwa5A*$^-$ mutant to produce ZwA. Another non-ribosomal peptide synthase gene, designated *orf3*, was identified. Amino acid dependent PPi release assay showed that the adenylation domain ZWAA2 of ORF3 acyl-adenylated L-2,3-diaminopropionate effectively. Taken together, it can be concluded that L-2,3-diaminopropionate is indeed one of the building blocks for the biosynthesis of Zwittermicin A.

Keywords: Zwittermicin A; L-2,3-Diaminopropionate; Adenylation; *Bacillus thuringiensis*

1. Introduction

Zwittermicin A (ZwA) is a kind of polyketide (PK) and non-ribosomal peptide (NRP) hybrid produced by a variety of *Bacillus thuringiensis* and *B. cereus* strains [1–3]. ZwA inhibits the growth of a wide range of microorganisms and facilitates the insecticidal activity of the protein toxins produced by *B. thuringiensis* [4,5]. The chemical structure of ZwA has been determined [6,7] (Fig. 1A), but the biosynthetic gene cluster itself has not been cloned and many details of the biosynthesis pathway remain poorly understood.

A 16 kb DNA fragment related to the biosynthesis of ZwA from *B. cereus* strain UW85 was reported by Handelsman's group [8]. It was deduced that ZwA was assembled from L-Serine, malonyl, aminomalonyl (AM), hydroxymalonyl (HM) and 2,3-diaminopropionate (Dap) based on the structure of ZwA and on the genetic information contained in the gene cluster [8]. The proposed assembly model featured the activated intermediates L-Serine-PCP and Dap-PCP as NRP extender units, with malonyl-CoA, AM-ACP and HM-ACP as PK extender units, and AM-ACP, HM-ACP and Dap were proposed to be biosynthesized de novo before the assembly process. In a subsequent study, genes related to de novo biosynthesis of AM-ACP and HM-ACP were identified from the 16 kb DNA fragment and in vitro experiments have confirmed the general layout of the biosynthesis pathway [9]. Nevertheless, building blocks labeling studies are necessary to support the details of the ZwA assembly line hypothesis. Unfortunately, few data are presently available regarding the identity of the genes involved.

A gene encoding a putative non-ribosomal peptide synthase (NRPS)-polyketide synthase (PKS) hybrid, designated *nrps–pks*, was identified in the 16 kb DNA fragment. The predicted amino acid sequence of this hybrid synthase was found to consist of seven functional domains, organized as follows: condensation domain (C), adenylation domain (A), peptidyl carrier protein (PCP), ketosynthase (KS), acyl transferase (AT), ketoreductase (KR) and acyl carrier protein (ACP) [8]. This structure should allow it to catalyze loading of one NRP building block and one PK building block. Obviously, there must be some other unrecognized *nrps* genes or *pks* genes located outside of the already identified 16 kb DNA fragment that play an important role since there are five building blocks to account for in the biosynthesis of ZwA.

Candidate genes for de novo biosynthesis of the proposed building block Dap, designated *zwa5A* and *zwa5B*, have been cloned from *B. thuringiensis* strain YBT-1520 as described in our previous report [10]. These genes were located 19.6 kb down stream of the 16 kb DNA fragment identified by Handelsman and coworkers. In this study, we identified another *nrps* gene, designated *orf3*, within this 19.6 kb gap and we demonstrated that L-Dap is one of the building blocks for the biosynthesis of ZwA.

2. Materials and methods

2.1. Bacterial strains, plasmids and primers

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

2.2. Insertional inactivation of zwa5A gene

A 2419 bp blunt end fragment harboring the temperature sensitive *Bacillus* replicon from plasmid pDG491 was inserted into the HincII site of plasmid pDG780 to create *Escherichia coli*/*Bacillus* shuttle vector

*Corresponding author. Fax: +86 27 87280670.
E-mail address: m98sun@mail.hzau.edu.cn (M. Sun).

[1]These authors contributed equally to this work.

0014-5793/$34.00
doi:10.1016/j.febslet.2008.07.054

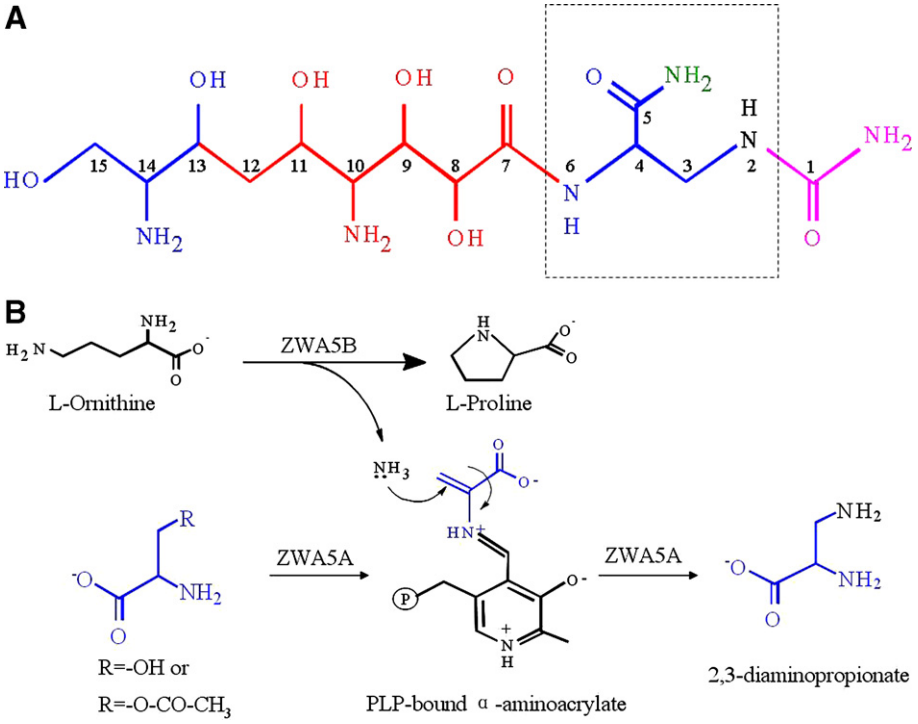

Fig. 1. Chemical structure of ZwA (part A) and the proposed biosynthesis pathway of 2,3-diaminopropionate (part B). Box of broken line indicates the proposed building block of 2,3-diaminopropionate.

Table 1
Bacteria strains and plasmids used in this study

Strains/plasmids	Description	References
Bacillus thuringiensis		
YBT-1520	Wild-type, ZwA producing	[10]
BMB0143	YBT-1520 carries recombinant plasmid pBMB0141	This study
BMB0144	*zwa5A*$^-$ mutant strain of YBT-1520	This study
BMB0145	YBT-1520 carries recombinant plasmid pBMB0145	This study
BMB0146	YBT-1520 carries recombinant plasmid pBMB0146	This study
Bacillus cereus UW030	UW85 derivate, ZwA non-producing	[1]
Erwinia herbicola LS005	Standard bacteriological test strain for ZwA	[1]
Escherichia coli BL21 (DE3)	*hsdS gal*(λcI*ts*857 *ind*1 *sam*7 *nin*5 *lac UV-5-T7*gene1)	Merck
EMB1300	*E. coli* BL21 carries plasmid pGEX-6p-1	This study
EMB1301	*E. coli* BL21 carries recombinant plasmid pBMB1301	This study
EMB1302	*E. coli* BL21 carries recombinant plasmid pBMB1302	This study
E. coli DH10B-BAC1F8	clone 1F8 of YBT-1520 BAC library	[10]
Plasmids		
pDG491	Amp^r, Cm^r, rep^{ts}	[23]
pDG780	Amp^r, Kan^r	Offered by BGSC
pDG646	Amp^r, harbors *erm*^{R} gene	Offered by BGSC
pBMB0631	Amp^r, Kan^r, *E. coli*/*Bacillus* (rep^{ts}) shuttle vector	This study
pBMB0141	pBMB0631 harbors *zwa5A′-erm*^{R}	This study
pBMB0145	pHT304 harbors *zwa5A*	This study
pBMB0146	pHT304 harbors *zwa5A-5B*	This study
pGEX-6p-1	GST · Tag, Amp^r	GE Healthcare
pBMB1301	pGEX-6p-1 harbors *zwaA1*	This study
pBMB1302	pGEX-6p-1 harbors *zwaA2*	This study

ZwA, Zwittermicin A; Amp^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Erm^r, erythromycin resistant; Kan^r, kanamycin resistant; rep^{ts}, temperature sensitive replicon for *B. thuringiensis*; BGSC, Bacillus Genetic Stock Center (The Ohio State University, USA).

pBMB0631. Primer pair 6B0813/6B0814 was used to amplify a 613 bp fragment of the *zwa5A* gene, designated *zwa5A′* (HindIII fragment; the first base pair is 23 bp down stream from the initiation codon and the last base pair is 342 bp up stream from the termination codon), from *B. cereus* UW85, which is a ZwA producing strain. The 1601 bp *erm*^{R} gene (BamHI fragment) from plasmid pDG646 was inserted into the BamHI site (21 bp up stream of the last base pair) of *zwa5A′*, yielding a *zwa5A′–erm*^{R} construct. This *zwa5A′–erm*^{R} construct with HindIII extremities was then ligated to the 5489 bp *Hin*dIII fragment from shuttle vector pBMB0631 to create recombinant plasmid pBMB0141.

Table 2
Primers used in this study

Primers	Sequence
6B0813	CGCAAGCTTAGAAAGGGTAATCGGCAA HindIII
6B0814	GGGAAGCTTATCACCAAAAATAATGGA HindIII
AK5G1025	CCGAAAAGTATTGTATTTAC
6E2171	GGAACCATACTTAATATAGA
6F0876	ATGAATTCATGCATGACCTCCCAGC EcoRI
6F0877	CCGAATTCATTGAAATGTTTAGTATT EcoRI
P5AB0	CCGAATTCGAAAACATTTAATTACTC EcoRI
P5AB1	CCGAATTCTAAGCTTATCAAATAAC EcoRI
A1P0	AACGGATCCAAATCTGCAATGGATCTAGA BamHI
A1P6	CCCGAATTCTTTCCAAATCTCAACTAATTT EcoRI
A2P0	ACCGGATCCTTGCGTAATATTAATATGTT BamHI
A2P5	ACCCTCGAGTAAACTACTATCTATGTCATTTC XhoI

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

Plasmid pBMB0141 was introduced into *B. thuringiensis* strain YBT-1520 by electroporation with a Bio-Rad GenePulser instrument. Transformants were cultured in Luria Bertani (LB) medium at 42 °C, with 25 μg/mL erythromycin as selective antibiotic. The *zwa5A*$^-$ mutant strain BMB0144 was selected out by PCR screening with primer pair AK5G1025 (locates 187 bp up stream of the initiation codon of *zwa5A* gene, in *zwa6* gene)/6E2171 (locates in *erm*^{R} gene).

Genes *zwa5A* and *zwa5A-5B* were amplified by primer pairs 6F0876/6F0877 and P5AB0/P5AB1 and then inserted into the EcoRI site of shuttle vector pHT304 to create recombinant plasmids pBMB0145 and pBMB0146, respectively. These recombinant plasmids were introduced into strain BMB0144 to complement the *zwa5A*$^-$ mutation.

2.3. In vivo feeding experiment

Overnight cultured *B. thuringiensis* YBT-1520 mutant strain BMB0144 were transferred into Tryptic Soy Broth (TSB) (Becton, Dickinson and Company) medium and cultured at 28 °C. Chemically synthesized Dap was used to feed mutant strain BMB0144 with the concentration of 100 μg/mL at 0 h, 12 h, 24 h, 36 h, 48 h or 60 h after cell growth. Culture filtrates were collected after the strains had been cultured for 72 h in total and then used to evaluate ZwA yield.

2.4. Purification and identification of ZwA

Culture filtrates of *B. thuringiensis* YBT-1520 and the mutant strains were filtered by MF-Millipore™ Filters (pore diameter, 0.22 μm) and were then used to test the inhibition of *Erwinia herbicola* strain LS005, which is an indicator strain of ZwA, by the method described previously [1,11]. Culture filtrates were further filtered by Biomax® Filters (Millipore, NMWL 5 kDa). High-performance liquid chromatography combined with ion trap/time-of-flight mass spectrometry (LC/MS-ITTOF) (Shimadzu) was used to detect ZwA according to the modified method described previously [12]. The separation was performed on an ODS-C18 column using a gradient elution consisting of mobile phase A (0.1% formic acid) and mobile phase B (acetonitrile/water (20:80)).

2.5. Cloning of the nrps gene

Bacterial artificial chromosome (BAC) clone 1F8 [10], which harbored a DNA fragment covering a part of the ZwA biosynthetic gene cluster extending from the 16 kb DNA fragment identified by Handelsman and coworkers to the region encoding the *zwa6–zwa5A–zwa5B* genes, was sequenced by the shotgun method. The amino acid sequence predicted for the potential *nrps* gene *orf3* identified in this region was analyzed for the presence of functional domains using Ansari's method as documented at the following address: http://www.nii.res.in/nrps-pks.html [13].

2.6. Over-expression and purification of adenylation domains ZWAA1 and ZWAA2

The adenylation domain encoding fragment *zwaA1* was amplified from *orf3* by primer pair A1P0/A1P6 from *orf3* and inserted into the BamHI/EcoRI site of plasmid pGEX-6P-1(a vector for making glutathione-*S*-transferase fusions), while *zwaA2* was amplified by A2P0/A2P5 and inserted into the BamHI/XhoI site, yielding plasmids pBMB1301 and pBMB1302, respectively. Both recombinant plasmids were introduced into *E. coli* strain BL21 (DE3) to over-express their respective GST-adenylation domain fusion proteins. The expressed fusion proteins were purified by GST · Bind™ Purification Kits (Novagen) and cleaved by PreScission™ Protease (GE Healthcare) to prepare the adenylation domains ZWAA1 and ZWAA2.

2.7. Amino acid-dependent PPi release assay

PPi levels were measured using the continuous spectrophotometric assay furnished by the EnzChek Pyrophosphate Assay Kit (Molecular Probes) as described by Ehmann[14]. Reactions were carried out at 30 °C in 500 μL total volume and contained 1× Buffer, 0.2 mM MesG, 10 mM $MgCl_2$, 5 mM ATP, 10 mM amino acid, 0.3 U inorganic pyrophosphatase (IP), 1 U purine nucleoside phosphorylase (PNP), 2 μM adenylation domain. Reactions were initiated by addition of amino acid substrate after a 10 min incubation at 30 °C to remove any contaminated PPi or Pi by PPi/IP/PNP/MesG couple. Nucleic Acid/Protein Analyzer DU800 (Beckman) was used to monitor absorbance at 360 nm every 30 s for 8 min. A standard curve for the pyrophosphate assay was generated using the pyrophosphate standard as a source of PPi according to the protocol of the EnzChek Pyrophosphate Assay Kit. The concentration of PPi in solutions was determined by conjunction with the standard curve for PPi.

2.8. Specificities prediction of the adenylation domains

In silico prediction of the adenylation domain specificities was performed using Rausch's NRPSpredictor program as documented at the following address: http://www-ab.informatik.uni-tuebingen.de/software [15].

3. Results and discussion

3.1. Creation of a zwa5A$^-$ mutant

A genomic DNA fragment from *B. thuringiensis* YBT-1520 (also present in *B. cereus* UW85) encoding the genes *zwa6*, *zwa5A* and *zwa5B* had been identified in the course of a previous study in our laboratory [10]. The gene products corresponding to the latter two, ZWA5A and ZWA5B, were found to be homologs of cysteine synthase and ornithine cyclodeaminase, respectively. Interestingly, it has been hypothesized that during the biosynthesis of viomycin in *Streptomyces* sp., L-Dap is synthesized by the concerted actions of cysteine synthase and ornithine cyclodeaminase homologs [16]. Precursor labeling studies performed on viomycin have determined that L-Serine is the precursor for L-Dap [17]. Based on all of these observations, we deduced that L-Dap biosynthesis may follow the same pathway during the biosynthesis of ZwA as during that of viomycin (Fig. 1B).

In order to evaluate the importance of the *zwa5A* gene for the production of ZwA, a knocked out version of *zwa5A* was produced by insertion of an erythromycin resistance marker (*erm*^{R}), and the resultant *zwa5A′* construct was used to promote homologous recombination with the corresponding DNA sequence of the *B. thuringiensis* YBT-1520 genome, resulting in the *erm*^{R}, *zwa5A*$^-$ knockout mutant strain BMB0144. PCR with primer pair AK5G1025 (locates in *zwa6* gene)/6E2171 (locates in *erm*^{R}

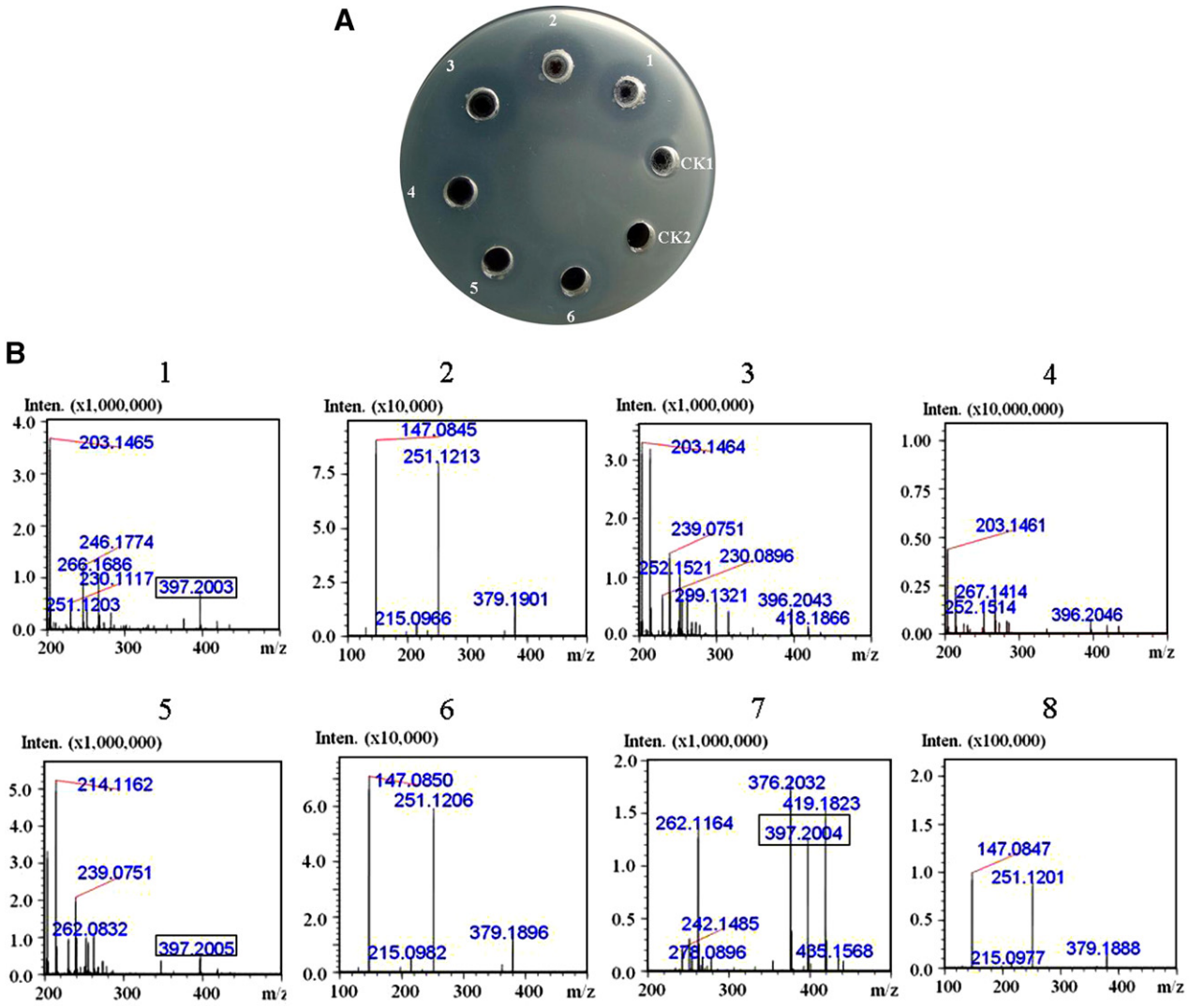

Fig. 2. Inhibition activity and LC/MS-ITTOF analysis of ZwA produced by strain YBT-1520 and *zwa5A*$^-$ mutant strain BMB0144. (Part A) the inhibition activity of *zwa5A*$^-$ mutant strain to *Erwinia herbicola* strain LS005 when 2,3-aminopropionate was fed at different stage, such as (1) at the same time with inoculation, (2) 12 h after, (3) 24 h after, (4) 36 h after, (5) 48 h after, (6) 60 h after. Hole CK1 meant mutant strain without 2,3-aminopropionate fed, while hole CK2 meant 100 μg/ml 2,3-aminopropionate solution, which was the concentration used in the feeding experiment. (Part B) Accurate MS, and MS/MS spectra of ZwA. (1) MS of YBT-1520, (2) MS/MS of precursor ion 397.2003, (3) MS of BMB0144, (4) MS of BMB0145, (5) MS of BMB0146, (6) MS/MS of precursor ion 397.2005, (7) MS of BMB0144 fed with Dap 12 h after, and (8) MS/MS of precursor ion 397.2004.

gene) amplified a 1.5 kb DNA fragment showed that the *zwa6* gene and *erm*^{R} gene were located together in a single DNA molecule, confirming the insertion of the *zwa5A*$^-$ knockout construct. The *zwa5A*$^-$ mutant strain BMB0144 showed no activity against *E. herbicola* LS005, which is an indicator strain of ZwA (Fig. 2A).

In LC/MS-ITTOF analysis, ZwA eluted at retention time of 4.5 min and showed a protonated molecule ion at *m/z* 397 ($[M+H]^+$). The MS/MS spectrum of precursor ion 397 showed fragment ions at *m/z* 147, 251 and 379 (Fig. 2B), which were proposed to result from breakage of the N_6–C_7 bond and from dehydration, respectively. No ZwA molecule ion was observed in the MS spectra of strain BMB0144. These results indicated that the *zwa5A*$^-$ mutant strain BMB0144 had been rendered unable to produce ZwA.

Genetic complementation experiments were performed with intact *zwa5A* and *zwa5A-5B* genes, yielding the revertant mutant strains BMB0145 and BMB0146, respectively. Culture filtrates of strain BMB0145 were unable to inhibit growth of the indicator strain *E. herbicola* LS005 while those from strain BMB0146 were able to do so indicating that ZwA was produced in the culture filtrates of strain BMB0146 but not in those of strain BMB0145. It was according with the LC/MS-ITTOF analysis result that no ZwA molecule ion was observed in the MS spectra of strain BMB0145 while a molecule ion at *m/z* 397 was found in that of strain BMB0146 (Fig. 2B). These results showed clearly that the production of ZwA was negatively affected by the insertional disruption of the *zwa5A* gene. However, a polarity effect on *zwa5B* could not be ruled out from contributing to the observed effects, since only the combined *zwa5A-5B* genes restored the ability of the *zwa5A*$^-$ mutant to produce ZwA, possibly linked to the fact that no obvious promoter could be recognized between *zwa5A* and *zwa5B*.

3.2. Dap restored the ability of the *zwa5A*$^{-}$ mutant to produce ZwA

Culture filtrates of strain BMB0144 fed with Dap were able to effectively inhibit growth of *E. herbicola* LS005 (Fig. 2A). Samples from cultures to which Dap was fed after 24 h or 36 h showed the best inhibition efficacy out of all six tests performed. LC/MS-ITTOF analysis result showed that strain BMB0144 fed with Dap after 24 h produced more ZwA than the wild-type strain, *B. thuringiensis* YBT-1520 (Fig. 2B). These observations indicated that chemically synthesized Dap was a suitable substitute for the product of the *zwa5A* gene, which is necessary for the biosynthesis of ZwA.

According to results obtained by Handelsman and coworkers, ZwA was first detected after 48 h, and production reached maximal levels after 72 h [18]. Expression of ZmaR, the protein conferring self-resistance to ZwA, was first detected after 12 h, and the level of ZmaR increased concomitantly with ZwA production, reaching maximal levels after 72 h [19]. Since antibiotic self-resistance genes clustered with antibiotic biosynthetic genes often shares similar regulatory pathways with the biosynthetic genes [20,21], it seems reasonable to assume that the expression of the ZwA biosynthetic gene cluster was initiated after 12–24 h. The observation that expression of the ZwA cluster starts after 12–24 h explains why Dap is most effective when added in the period that follows (24–36 h), as this is likely to correspond to the period when L-Dap would have been naturally produced.

Gene interruption and feeding experiments indicated that Dap is the building block for the biosynthesis of ZwA. Absolutely, identification of NRPS catalysis loading of Dap into ZwA assembly line is a conclusive evidence to support this standpoint.

3.3. Identification of nrps gene orf3 and function domain analysis of ORF3

A hypothetical 6528 bp gene, *orf3*, was recognized as a potential *nrps* gene within the gap between the 16 kb DNA fragment identified by the Handelsman group and the region encoding the *zwa6–zwa5A–zwa5B* genes (GenBank accession number EU520420). The predicted amino acid sequence of the gene product of *orf3* showed that the corresponding protein, designated NRPS ORF3, consists of six functional domains, organized as follows: A (ZWAA1), PCP, C, A (ZWAA2), PCP and TE (thioesterase domain). In this context, thioesterase domain fuses to the C-terminal of the NRPS protein and catalyzes antibiotic backbone release from assembly line, which is why it is also referred to as a termination domain [22]. Interestingly, no protein with a thioesterase domain has been recognized in the previously characterized 16 kb DNA fragment, and among all of the five proposed building blocks for ZwA, Dap is the last one in the assembly line model [8]. In fact, *orf3* is the only gene encoding an intact NRPS in the genome of strain YBT-1520 (genome sequence not shown here). Taken together, these informations suggest that ORF3 may be the NRPS that catalyzes loading of Dap during the biosynthesis of ZwA.

3.4. Adenylation domain ZWAA2 acyl-adenylated L-Dap effectively

The adenylation domain is one of the three core domains (the other two domains are PCP and C) involved during the loading of an NRP extender unit in an NRP assembly line. It catalyzes the formation of an aminoacyl adenylate intermediate at the expense of Mg^{2+}-ATP and release of PPi[22]. This adenylation reaction is amino acid specific and necessary for the amino acids to be assembled.

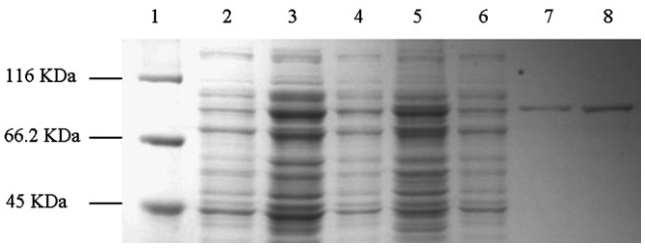

Fig. 3. SDS–PAGE analysis of the GST · adenylation-domain fusion protein. Lane 1, protein molecular weight marker; lane 2, total protein of strain EMB1301; lane 3, total protein of strain EMB1301 (induced with 0.1 mM IPTG); lane 4, total protein of strain EMB1302; lane 5, total protein of strain EMB1302 (induced with 0.1 mM IPTG); lane 6, total protein of strain EMB1300 (induced with 0.1 mM IPTG); lane 7, fusion protein of GST · ZWAA1; lane 8, fusion protein of GST · ZWAA2.

The two adenylation domains identified in ORF3 (ZWAA1 and ZWAA2) were over expressed as GST-tagged fusion proteins, which were purified and analyzed by SDS–PAGE as shown in Fig. 3. The GST tags were then removed from the fusion proteins by protease cleavage and further purification, yielding purified proteins corresponding to the two respective adenylation domains.

Amino acid-dependent PPi release experiment was employed to test adenylation of L-Dap in the presence of the purified adenylation domains. PPi levels analysis results showed that adenylation domain ZWAA2 did acyl-adenylate L-Dap effectively. Three kinds of L-Dap similar amino acid, L-Ala, L-Cys and L-Ser, were used as control in the PPi release experiment. Under the same conditions, 15 μM PPi was produced when L-Dap was used as substrate while only 3 μM PPi was produced when L-Ser was used (Fig. 4A). During the in vitro adenylation reactions, ZWAA2 acyl-adenylated L-Ala and L-Cys in some measure. Adenylation domain ZWAA2 can thus be expected to necessarily acyl-adenylate L-Dap during the biosynthesis of ZwA in vivo when competing non-specific amino acids concentrations are far lower than in in vitro experiments.

In the analysis of ZWAA1, it seemed that the most appropriate substrate was L-Cys, but the process was not as effective in comparison to the acyl-adenylation of L-Dap by ZWAA2 (Fig. 4B). Many more amino acids should be tested to determine the most appropriate substrate of ZWAA1. At this point, however, it seems unlikely that L-Dap itself could be the specific substrate of ZWAA1.

In silico prediction of the adenylation domain specificities showed that the probable preferred substrate of ZWAA2 is a gly = ala = val = leu = ile = abu = iva-like amino acid (Fig. S1). Although the program used, NRPSpredictor, is known to yield poor results with the glycine/alanine model due the small size of the substrates [15], this prediction did fit with our biochemical specificity test results since Dap is a kind of small-sized amino acid and shares a similar carbon backbone with the predicted amino acid.

In contrast, it was quite unexpected that the substrate predicted for ZWAA1 was also a gly = ala = val = leu = ile = abu = iva-like amino acid. However, the detected activity of ZWAA1

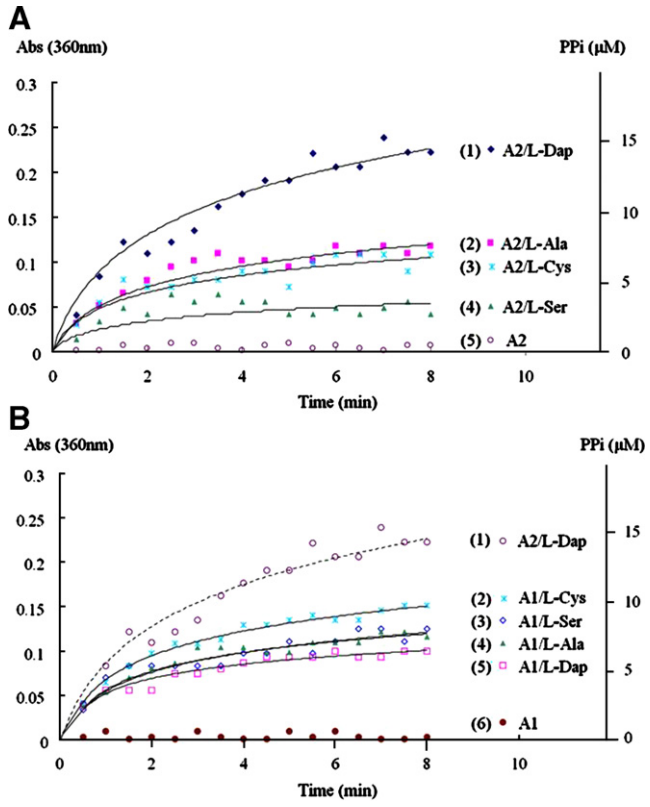

Fig. 4. PPi levels of the adenylation reaction measured by continuous spectrophotometric assay. Part A, analysis of adenylation domain ZWAA2. Amino acid (1) L-Dap, (2) L-Ala, (3) L-Cys and (4) L-Ser used as substrate, no amino acid was used in reaction (5). Part B, analysis of adenylation domain ZWAA1, ZWAA2 acyl-adenylated L-Dap used as control. (1) Amino acid (2) L-Cys, (3) L- Ser, (4) L-Ala and (5) L-Dap used as substrate, no amino acid was used in reaction (6).

on L-Cys was very weak and we cannot rule out that ZWAA1 is actually inert in vivo where amino acids are found in much lower concentrations than in the assay. It is possible that ZWAA1 is in fact a degenerated Dap adenylation domain and that ZWAA2 is the truly active one. If so, where this inert domain originated or how ZWAA1 might have lost its activity remains unknown. Another possibility is that ZWAA1 belongs to a skipped module in the ZwA assembly line since two potential modules can be aligned out (if C is an iteratively acting domain) from NRPS ORF3, whose functional domains are organized as follows: A (ZWAA1) – PCP – C – A (ZWAA2) – PCP – TE. Our data and analysis suggested that the latter four domains are responsible for the assembling of building block 2,3-diaminopropionate and releasing of the carbon skeleton. It seems that the former three domains constitute an intact functional module (maybe a skipped one), while their real role remains obscure. Further investigation is definitely necessary to confirm the mechanism of NRPS ORF3 in ZwA assembly.

Two main conclusions can thus be drawn from the mutation-feeding experiment and adenylation analysis results. Firstly, that L-Dap is one of the building blocks for the biosynthesis of ZwA and is de novo biosynthesized under the catalysis of ZWA5A. Secondly, that L-Dap is acyl-adenylated by ZWAA2 of NRPS ORF3 and may be loaded onto the ZwA assembly line under the catalysis of NRPS ORF3.

Acknowledgements: We thank Jo Handelsman for providing *B. cereus* strain UW030 and *E. herbicola* strain LS005 and Géraldine A Van der Auwera for the strict proofreading of the manuscript. This project was funded by National Natural Science Foundation of China (30770020), the National High Technology Research and Development Program (863 Program) of China (2006AA02Z174 and 2006AA03A243) and the National Basic Research Program (973 Program) of China (2003CB114201).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.07.054.

References

[1] Stabb, E.V., Jacobson, L.M. and Handelsman, J. (1994) Zwittermicin A-producing strains of *Bacillus cereus* from diverse soils. Appl. Environ. Microbiol. 60 (12), 4404–4412.
[2] Raffel, S.J., Stabb, E.V., Milner, J.L. and Handelsman, J. (1996) Genotypic and phenotypic analysis of zwittermicin A-producing strains of *Bacillus cereus*. Microbiology 142 (Pt 12), 3425–3436.
[3] Nair, J.R., Narasimman, G. and Sekar, V. (2004) Cloning and partial characterization of zwittermicin A resistance gene cluster from *Bacillus thuringiensis* subsp. *kurstaki* strain HD1. J. Appl. Microbiol. 97 (3), 495–503.
[4] Silo-Suh, L.A., Stabb, E.V., Raffel, S.J. and Handelsman, J. (1998) Target range of zwittermicinA, an aminopolyol antibiotic from *Bacillus cereus*. Curr. Microbiol. 37, 6–11.
[5] Broderick, N.A., Goodman, R.M., Handelsman, J. and Raffa, K.F. (2003) Effect of host diet and insect source on synergy of gypsy moth (Lepidoptera: Lymantriidae) mortality to *Bacillus thuringiensis* subsp. *kurstaki* by zwittermicin A. Environ. Entomol. 32 (2), 387–391.
[6] He, H.Y., Silo-Suh, L.A., Handelsman, J. and Clardy, J. (1994) Zwittermicin A, an antifungal and plant protection agent from *Bacillus cereus*. Tetrahedron Lett. 35 (16), 2499–2502.
[7] Manker D.C., Lidster W.D., Starnes R.L., and Macintosh S.C. (1994) Potentiator of Bacillus pesticidal activity. USA Patent, US 6,406,691 B1.
[8] Emmert, E.A., Klimowicz, A.K., Thomas, M.G. and Handelsman, J. (2004) Genetics of Zwittermicin A production by *Bacillus cereus*. Appl. Environ. Microbiol. 70.1, 104–113.
[9] Chan, Y.A., Boyne 2nd., M.T., Podevels, A.M., Klimowicz, A.K., Handelsman, J., Kelleher, N.L. and Thomas, M.G. (2006) Hydroxymalonyl-acyl carrier protein (ACP) and aminomalonyl-ACP are two additional type I polyketide synthase extender units. Proc. Natl. Acad. Sci. USA 103 (39), 14349–14354.
[10] Zhao, C.M., Luo, Y., Song, C.X., Liu, Z.X., Chen, S.W., Yu, Z.N. and Sun, M. (2007) Identification of three Zwittermicin A biosynthesis-related genes from *Bacillus thuringiensis* subsp. *kurstaki* strain YBT-1520. Arch. Microbiol. 187 (4), 313–319.
[11] Silo-Suh, L.A., Lethbridge, B.J., Raffel, S.J., He, H., Clardy, J. and Handelsman, J. (1994) Biological activities of two fungistatic antibiotics produced by *Bacillus cereus* UW85. Appl. Environ. Microbiol. 60 (6), 2023–2030.
[12] Liu, Z.Y., Huang, L.L., Dai, M.H., Chen, D.M., Wang, Y.L., Tao, Y.F. and Yuan, Z.H. (2008) Metabolism of olaquindox in rat liver microsomes: structural elucidation of metabolites by high-performance liquid chromatography combined with ion trap/time-of-flight mass spectrometry. Rapid Commun. Mass Spectrom. 22, 1009–1016.
[13] Ansari, M.Z., Yadav, G., Gokhale, R.S. and Mohanty, D. (2004) NRPS-PKS: a knowledge-based resource for analysis of NRPS/PKS megasynthases. Nucleic Acids. Res. 32 (Web Server issue), W405–W413.
[14] Ehmann, D.E., Shaw-Reid, C.A., Losey, H.C. and Walsh, C.T. (2000) The EntF and EntE adenylation domains of *Escherichia*

coli enterobactin synthetase: sequestration and selectivity in acyl-AMP transfers to thiolation domain cosubstrates. Proc. Natl. Acad. Sci. USA 97 (6), 2509–2514.
[15] Rausch, C., Weber, T., Kohlbacher, O., Wohlleben, W. and Huson, D.H. (2005) Specificity prediction of adenylation domains in nonribosomal peptide synthetases (NRPS) using transductive support vector machines (TSVMs). Nucleic Acids. Res. 33 (18), 5799–5808.
[16] Thomas, M.G., Chan, Y.A. and Ozanick, S.G. (2003) Deciphering tuberactinomycin biosynthesis: isolation, sequencing and annotation of the viomycin biosynthetic gene cluster. Antimicrob. Agents. Chemother. 47 (9), 2823–2830.
[17] Carter, J.H., Du Bus, R.H., Dyer, J.R., Floyd, J.C., Rice, K.C. and Shaw, P.D. (1974) Biosynthesis of viomycin. I. Origin of alpha, beta-diaminopropionic acid and serine. Biochemistry 13, 1221–1227.
[18] Milner, J.L., Raffel, S.J., Lethbridge, B.J. and Handelsman, J. (1995) Culture conditions that influence accumulation of zwittermicin A by *Bacillus cereus* UW85. Appl. Microbiol. Biotechnol. 43, 685–691.
[19] Stohl, E.A., Milner, J.L. and Handelsman, J. (1999) Zwittermicin A biosynthetic cluster. Gene 237, 403–411.
[20] Cundliffe, E. (1989) How antibiotic-producing organisms avoid suicide. Annu. Rev. Microbiol. 43, 207–233.
[21] Matsuo, H., Mochizuki, H., Davies, J. and Sugiyama, M. (1997) Production of bleomycin *N*-acetyltransferase in *Escherichia coli* and *Streptomyces verticillus*. FEMS Microbiol. Lett. 153, 83–88.
[22] Sieber, S.A. and Marahiel, M.A. (2005) Molecular mechanisms underlying nonribosomal peptide synthesis: approaches to new antibiotics. Chem. Rev. 105, 715–738.
[23] Baum, J.A. (1994) Tn5401, a new class II transposable element from *Bacillus thuringiensis*. J. Bacteriol. 176, 2835–2845.
[24] Stachelhaus, T., Mootz, H.D. and Marahiel, M.A. (1999) The specificity conferring code of adenylation domains in nonribosomal peptide synthetases. Chem. Biol. 6 (8), 493–505.