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MINI-REVIEW

Novel roles of *Bacillus thuringiensis* to control plant diseases

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Abstract *Bacillus thuringiensis* is well known as an effective bio-insecticidal bacterium. However, the roles of *B. thuringiensis* to control plant diseases are not paid great attention to. In recent years, many new functions in protecting plants from pathogen infection have been discovered. For example, acyl homoserine lactone lactonase produced by *B. thuringiensis* can open the lactone ring of *N*-acyl homoserine lactone, a signal molecule in the bacterial quorum-sensing system. This in turn, significantly silences bacterial virulence. This finding resulted in the development of a new strategy against plant bacterial diseases by quenching bacterial quorum sensing. Another new discovery about *B. thuringiensis* function is zwittermicin A, a linear aminopolyol antibiotic with high activity against the *Oomycetes* and their relatives, as well as some gram-negative bacteria. This paper summarized the relative progresses of *B. thuringiensis* in plant disease control and its favorable application prospects.

Keywords *Bacillus thuringiensis* · Acyl homoserine lactonase (AHL lactonase) · Zwittermicin A

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Introduction

Bacillus thuringiensis is a gram-positive spore-forming bacterium. In *B. thuringiensis*, one or more parasporal crystals with different shapes and insecticidal activity are produced in association with spore formation, which is one of the primary characteristics of the bacterium. Since *B. thuringiensis* was first discovered in 1901, many strains have been isolated worldwide. These strains are divided into 71 serotypes and 84 subspecies based on their serological response differences in their flagellar antigen (Schnepf et al. 1998; Khyami-Horani et al. 2003). Due to its specificity for the control of pests such as *Lepidoptera*, *Diptera*, and *Coleoptera*, over 100 types of insecticides have been developed using natural and genetically engineered strains of *B. thuringiensis* (Roh et al. 2007). In addition, the genes that code for the insecticidal crystal proteins have been successfully transferred into cotton, corn, soybean, and rice, which has led to significant economic benefits (Crickmore 2006). This technology is considered to be one of the most successful models in agricultural biotechnology.

Formerly, only the insecticidal properties of *B. thuringiensis* attracted extensive attention. However, in recent years, the roles of *B. thuringiensis* in plant disease control have been found out. Apart from crystal protein and other insecticidal substances, *B. thuringiensis* also produces other active components with good prospects of application as following: zwittermicin A, which is extremely efficient at preventing the damping-off of alfalfa caused by *Phytophthora medicaginis* (Silo-Suh et al. 1994), and acyl homoserine lactone (AHL) lactonases, which can quench bacterial pathogenicity (Dong et al. 2002). These findings widen the target range of *B. thuringiensis* besides insecticidal activity and help people to better understand its role in soil ecosystem.

AHL lactonase produced by *B. thuringiensis*

The gene encoding AHL lactonase

Many important bacterial pathogens, such as *Agrobacterium tumefaciens*, *Burkholderia cepacia*, *Erwinia chrysanthemi*, *E. carotovora*, *Pantoea stewartii*, and *Pseudomonas aeruginosa*, employ quorum-sensing system with AHLs as signal molecules to sense their population density. This system allows the pathogens to regulate the expression of virulence factors to more effectively attack their host. However, that quorum-sensing system can also be interrupted to protect plants, animals, or humans from bacterial pathogens. Dong et al. (2000) first reported a quorum-quenching enzyme encoded by the *aiiA* gene of *Bacillus* sp. 240B1. This enzyme, which was later characterized as an AHL lactonase, contained 250 amino acids. Sequence alignment revealed that AHL lactonase contains a "$H^{104}XH^{106}XDH^{109}$~H^{169}" zinc-binding motif that is conserved among several groups of metallohydrolases (Dong et al. 2001). Additionally, site-directed mutagenesis indicated that conserved aspartate and most histidine residues were necessary for AHL lactonase activity. Dong et al. (2001) further elucidated that AHL lactonase disturbed quorum sensing via inactivation of AHL signal by hydrolyzing the lactone bond (Fig. 1).

Because the gene encoding AHL lactonase was first cloned from *Bacillus* sp. strain 240B1, it was evaluated whether that gene exists in all *Bacillus* species. Dong et al. (2002) found that *B. cereus* and *B. thuringiensis* as well as *B. mycoides* strains harbor the gene encoding AHL lactonase except for *B. fusiformis* and *B. sphaericus*. Lee et al. (2002) also cloned homologue genes encoding AHL lactonase from 16 subspecies of *B. thuringiensis* and compared the corresponding sequences with those of *Bacillus* sp. strain 240B1. The results revealed high homologies of 89% to 95% and 90% to 96% in the nucleotide sequence and deduced amino acid sequences, respectively. We also checked more than 500 *B. thuringiensis* strains and found that gene in each strain by Southern blotting. Taken together, these results indicated that the gene encoding AHL lactonase is widespread in the *B. thuringiensis* genome.

The mechanism of AHL lactonase to hydrolyze AHL signal

To characterize the enzyme, Wang et al. (2004) purified the recombinant AHL lactonase and its four variants to analyze its kinetic and substrate specificity. The results showed that AHL lactonase had no activity on non-acyl lactones and noncyclic esters, but displayed strong enzyme activity toward all tested AHL signal molecules, even if their length and nature of the substitution at the C3 position of the acyl-chain varied. The amide group and the ketone at the C1 position of the acyl-chain of AHL molecular are important for enzyme–substrate interactions (Fig. 2). Furthermore, mutations of AHL lactonase confirmed that substitution of the His-106, Asp-108, His-109, or His-169 with serine caused conformational changes that led to a significant loss of enzyme activity (Wang et al. 2004). Finally, Lu et al. (2006) found that Tyr-194 of AHL lactonase from marine *B. cereus* strain Y2 was the critical residue in the catalytic activity.

Wang et al. (2004) thought that AHL lactonase did not require zinc or other metal ions for its enzyme activity. However, Thomas et al. (2005) demonstrated that AHL lactonase is a metalloprotein with a dinuclear zinc-binding site by X-ray absorption spectroscopy, which is similar to that structure in glyoxalase II. Removal of both zinc ions resulted in a loss of activity, and reconstitution with zinc restored this activity, which indicated the importance of metal ions for catalytic activity. The results were also confirmed by Kim et al. (2005a). Furthermore, Thomas et al. (2005) certified that metal-bound AHL lactonase hydrolyzed only *N*-hexanoyl-(*S*)-homoserine lactone but not the (R) enantiomer.

Despite the significant progresses that have been made in the biochemical nature of AHL lactonase, the ring opening mechanism and the role that metal ions play in catalysis were not elucidated. Kim et al. (2005b) first reported preliminary crystallographic analysis of *B. thuringiensis* AHL lactonase, containing two Zn^{2+} ions. Additionally, Liu et al. (2005) and Kim et al. (2005a) also reported the similar crystallographic structure to elucidate catalysis mechanism. The enzyme holds two Zn^{2+} ions, with the Zn1 ion being coordinated by His-104, His-106, and His-169, and the Zn2 ion being coordinated by His-109, His-235, and Asp-108. Two Zn^{2+} ions were found to be bridged by one water molecule (or a hydroxide ion) and the Oδ2 atom of Asp-191 (Fig. 2). When the enzyme and the AHL signal reacted, the hydroxyl ion of Tyr-194 acted as a donor in a hydrogen bond.

Based on the three-dimensional structures of AHL lactonase and the complex containing AHL lactonase and its substrate C_8-homoserine lactone (HSL), as well as the

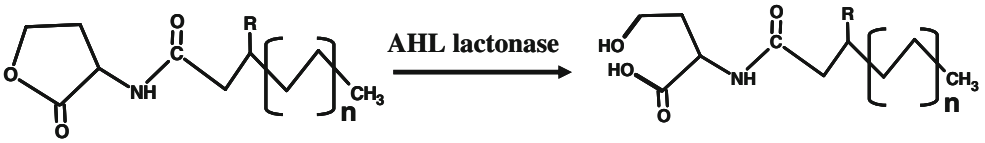

Fig. 1 The mode for AHL lactonase to open lactone ring of AHL signal

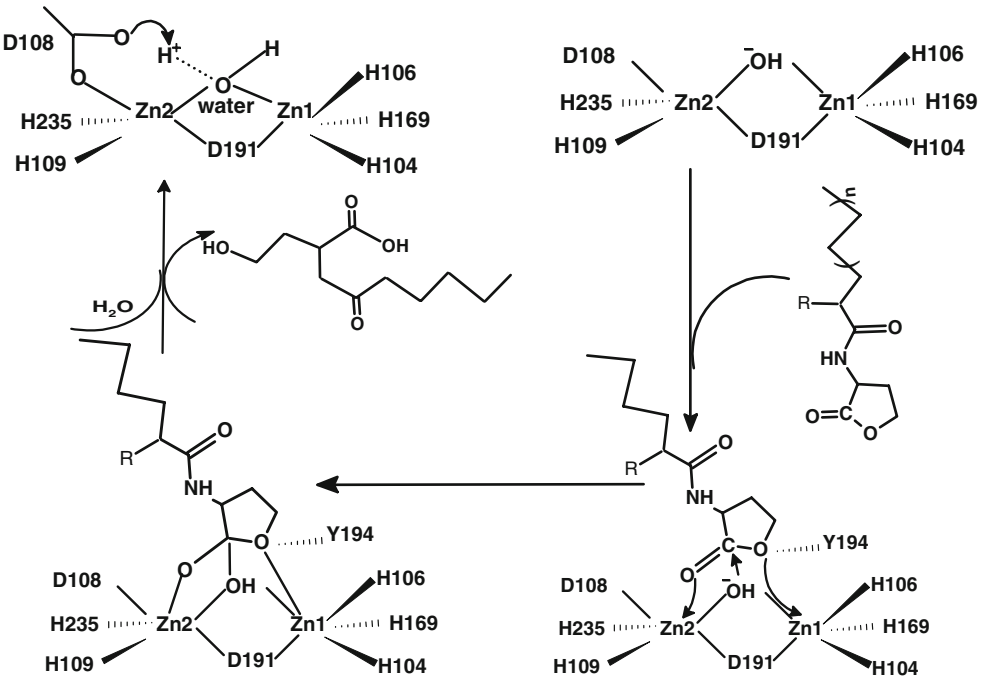

Fig. 2 Proposed catalysis mechanism for AHL lactonase to degrade AHL-based zinc catalysis mechanisms (Kim et al. 2005a)

structures of other enzymes in the metallo-β-lactamase superfamily, Kim et al. (2005a) suggested a catalytic pathway for AHL lactonase (Fig. 2). The reaction involved a nucleophilic attack by the water hydroxide bridging the two Zn^{2+} ions on the substrate's carbonyl carbon. The lactone ring and carbonyl oxygens of the HSL then interacted with Zn1 and Zn2, respectively, resulting in enhanced polarization of the carbonyl bond and making it more susceptible to a nucleophilic attack. Concurrently, due to its strong hydrogen bond with the ring oxygen of AHL, Tyr-194 was believed to act as a general acid protonating the leaving group.

Momb et al. (2006) used $H_2{}^{18}O$ to evaluate the nucleophilic attack and found that the isotope labeling patterns were most consistent with an addition/elimination mechanism attacking a solvent-derived hydroxide at the carbonyl carbon and eliminating an alcohol-leaving group. In addition, they demonstrated that the dinuclear metal site of the AHL lactonase was essential for both structural stabilization and catalysis.

Unusually, Momb et al. (2006) found that the Cd^{2+}-substituted enzyme exhibited catalytic rates that were 1,600- to 24,000-fold faster than those of other quorum-quenching enzymes. This finding indicated that, through an understanding of the structure and mechanism of this enzyme family, more effective quorum-quenching agents could be developed as antimicrobial agents.

Potential application of AHL lactonase

To assay the potential of AHL lactonase to destroy a bacterial quorum-sensing system, the gene encoding that enzyme was transferred into *E. carotovora* SCG1, the pathogen of Chinese cabbage soft rot disease (Dong et al. 2000). The results showed that expression of AHL lactonase sharply reduced the AHL signal of *E. carotovora* SCG1, in turn, decreased the extracellular pectolytic enzyme activities and attenuated its pathogenicity. Additionally, Dong et al. (2004) certified that *B. thuringiensis* only abolished the AHL signal produced by *E. carotovora*, but not interfered with the normal growth of *E. carotovora* when two microorganisms were cocultured. Whereas *B. fusiformis* and *E. coli* strains without AHL degradation enzyme did not exhibit that effect. When the gene encoding AHL lactonase was mutated, *B. thuringiensis* lost its ability to control pathogens (Dong et al. 2004; Zhou et al. 2006). These results suggested that the gene encoding AHL lactonase can be utilized to resist infection of *E. carotovora*.

Beyond that, Dong et al. (2001) transformed the gene encoding AHL lactonase with or without fusing the coding sequence of a 27-amino-acid secretion signal peptide into the genome of tobacco and potato plants. The AHL lactonase activities could be detected in both transformed plants. This in turn enhanced the resistance against *E. carotovora* infection. Furthermore, the ability of transgenic plants to resist *E. carotovora* infection was consistent with the level of AHL lactonase expression. However, expression of AHL lactonase fusing with the secretion signal peptide was generally lower than that enzyme fusing without signal peptide in transgenic plants.

After Dong et al. (2000 and 2001) certified this quorum-quenching strategy against bacterial infection, we synthesized the gene encoding AHL lactonase based on the codon

preference of *Amorphallus konjac* and then transformed it into *A. konjac* to resist soft rot disease caused by *E. carotovora*. The results demonstrated that transgenic lines of konjac only slightly improved the resistance against *E. carotovora* (unpublished data). This disease has resulted in serious loss of konjac harvest, and we need to do more research to protect *A. konjac* from soft rot.

Except for transgenic crops, engineered bacteria expressing AHL lactonase are receiving attention because they can directly contribute the effects in the rhizosphere of crops. *B. thuringiensis* has the potential to be a soil antagonism. However, these isolates are not fit to use direct in the field for two reasons: (1) The expression level of AHL lactonase in *B. thuringiensis* is low, and (2) *B. thuringiensis* only produces AHL lactonase during vegetative phase and no activity during the spore phase.

To overcome the low yield of AHL lactonase in native strains of *B. thuringiensis*, Zhu et al. (2006) fused the gene encoding AHL lactonase with the promoter of the insecticidal crystal protein coding gene *cry3A*, which is active during vegetative phase and sporulation, and then transformed that gene into strain BMB-005, a bacterium harboring several *cry* genes with high toxicity against *Helicoverpa armigera*, *Plutella xylostella*, and *Spodoptera exigua*. The ability of the transformant to degrade AHL molecules was 2.4-fold higher than that of BMB-005. In addition, the transformant induced strong resistance to *E. carotovora* infection on potato slices and cactus stems. However, this transformant did not confer enough resistance to soft rot of *A. konjac* grown in pots. The results indicated that the effects of engineered *B. thuringiensis* were affected in the soil ecological environment. One reason might be that AHL lactonase in *B. thuringiensis* is an intracellular enzyme (Dong et al. 2002) and that AHL lactonase had little chance to meet the AHL signal of bacterial pathogen.

To overcome this defect, the surface layer (S-layer) protein, which belongs to a family of excretive proteins that can anchor to the bacterial cell surface of *B. thuringiensis*, was employed to carry AHL lactonase out of cell for exploiting the potential of *B. thuringiensis*. To achieve the desired result, Zhang et al. (2006) created an acceptor, *B. thuringiensis* Tr5, to express AHL lactonase. *B. thuringiensis* Tr5 harbors expression cassettes of S-layer protein, but the gene encoding S-layer protein was mutated, which can avoid the competition for the same promoter between the native S-layer protein and fusion protein. When the fusion protein that included part of the S-layer protein and AHL lactonase was expressed in *B. thuringiensis* Tr5, the transformants displayed much higher extracellular AHL-degrading activity than wild-type *B. thuringiensis* strains (Zhang et al. 2007).

These engineering bacteria are hardly to use in practice due to many deficiencies. Endophytic bacteria may provide an optimal target for the expression of AHL lactonase because they are known to thrive within some plants by promoting plant growth and suppressing plant pathogens. Endophytic bacteria expressing AHL lactonase should have a sustainable ability to inhibit bacterial pathogens that employ quorum sensing to regulate toxin synthesis. Endophytic *Burkholderia* sp. KJ006, which has no pathogenicity toward rice, but a broad spectrum of antifungal properties, was isolated from the roots of rice plants (Cho et al. 2007; Eberl 2006). The engineered strain KJ006, which contained an expression cassette of the gene encoding AHL lactonase, was constructed to control *Burkholderia glumae*, a casual pathogen of seedling rot and grain rot of rice that also employs quorum sensing to regulate its major virulence factor. The results indicated that the engineered strain reduced the disease incidence of rice seedling rot caused by *B. glumae* in situ. Therefore, this strategy can be used as a novel biological control agent against pathogenic bacteria, especially within the same ecological niche.

Besides *E. carotovora*, many other bacterial pathogens also employ quorum-sensing system to regulate toxin expression, such as *Agrobacterium*, *Erwinia*, *Pectobacteria*, and *Pseudomonas*, which result in serious diseases of crops (Barnard and Salmond 2007; Girard and Bloemberg 2008; Von Bodman et al. 2003; White and Winans 2007). Until now, some bacterial disease could not be effectively controlled by spraying germicide in the field. The discovery of AHL lactonase provides a novel strategy to control these bacterial pathogens.

Role debate of AHL lactonase in *B. thuringiensis*

The actual role of AHL lactonase in *B. thuringiensis*, as well as the reason that all *B. cereus* groups contain the gene encoding AHL lactonase are still unknown (Dong et al. 2002; Lee et al. 2002; Zhou et al. 2006). It has been suggested that insecticidal *B. thuringiensis* strains might compete with gram-negative bacteria in natural ecosystems by autoinducer-degrading activity (Dong et al. 2002; Lee et al. 2002). However, some facts contradict this suggestion. For example, the ecological environment and nutritional sources of the *B. cereus* group are not the same as those of gram-negative bacteria, such as *E. carotovora*. That is, *E. carotovora* specializes in degrading plant-derived nutrition, while the *B. cereus* group prefers protein and amino acid substrates (Jensen et al. 2003). Therefore, competition seems unlikely. In addition, many bacteria that do not contain the gene encoding AHL lactonase can survive in the same ecological environment as that of *E. carotovora*. Taken together, these findings suggest that AHL lactonase may have a self-regulating function and

tune some unknown behaviors to benefit *B. thuringiensis*. Zhang et al. (2002, 2004) confirmed that the quorum-sensing-dependent Ti plasmid conjugal transfer system of *A. tumefaciens* was regulated by degradation of AHL by an AHL lactonase (AttM). This finding can partially hint the actual role of that AHL lactonase plays in *B. thuringiensis*. However, it is not yet understood why no AHL signal molecules is found in *B. thuringiensis.*

Zhou et al. (2006) mutated, reverse mutated, and overexpressed the gene encoding AHL-lactonase in the *B. thuringiensis* 171, nonplasmid mutant, to assay its function. The results indicated that an elevated expression of AHL lactonase reduced ethanol tolerance, increased melanin production, inhibited swarming, and delayed sporulation of *B. thuringiensis*. These results suggested that AHL lactonase might regulate the expression of upstream and downstream genes thereby improving the membrane permeability and rapid transportation of nutrition or other substances. However, further studies are needed to elucidate the mechanisms underlying the roles of AHL lactonase in *B. thuringiensis*.

Zwittermicin A from *B. thuringiensis*

Discovery of zwittermicin A and its target range

P. megasperma f. sp. *medicaginis* (Drechs.) can cause the serious damping-off of alfalfa seedlings in poorly drained soils. Handelsman et al. (1990) screened alfalfa root-associated bacteria for inhibitory effects on damping-off disease and isolated approximately 700 bacterial strains. The ability of these bacteria to protect alfalfa seedling from *P. megasperma* was then evaluated; however, only the *B. cereus* UW85 exhibited strong activity toward *P. megasperma* (Handelsman et al. 1990). The active component analysis from *B. cereus* UW85 indicated that this compound with activity to resist the damping-off of alfalfa was zwittermicin A (Silo-Suh et al. 1994). He et al. (1994) further elucidated that the structure of zwittermicin A is a linear aminopolyol antibiotic, which represented a new class of antibiotics. And then, Stabb et al. (1994) provided a way to identify bacteria with zwittermicin A activity because most *Bacillus* strains that produced zwittermicin A were found to inhibit the growth of *E. herbicola* or to be sensitive to phage P7. Subsequently, some *B. thuringiensis* and *B. mycoides* strains were reported with zwittermicin A activity, and these strains harbor the zwittermicin A self-resistance gene (Emmert et al. 2004; Nair et al. 2004; Zhang et al. 2006; Zhao et al. 2007; Shao et al. 2008).

The target range of activity for zwittermicin A was very wide. Zwittermicin A was found to be a high activity against the *Oomycetes* and a moderate activity against some gram-negative bacteria and many plant pathogenic fungi such as *Alternaria*, *Fusarium*, *Helminthosporium*, and *Ustilago* (Silo-Suh et al. 1998).

Mechanism of sensitivity or resistance to zwittermicin A

To determine why zwittermicin A is active against lower eukaryotes, such as *Oomycetes*, as well as a variety of bacteria, but not against the bacteria that produce it, Milner et al. (1996) constructed a genomic library from *B. cereus* UW85 to investigate the mechanism. The results revealed the presence of a 1.2-kb fragment of DNA from *B. cereus* UW85, designated *zmaR*, that conferred zwittermicin A resistance. The *zmaR* gene encoded a 43.5-kDa protein. Stohl et al. (1999) further demonstrated that ZmaR was an acetyltransferase that catalyzed the acetylation of zwittermicin A using acetyl coenzyme A (CoA) as a donor group. However, the ZmaR protein had no homologous motifs with other known acetyltransferases, which indicated that it belonged to a new class of acetyltransferases. In addition, when the *zmaR* gene of *B. cereus* UW85 was knocked out, the mutant was sensitive to zwittermicin A, which suggested that ZmaR protein conferred important self-resistance for the *Bacillus* group that produces zwittermicin A (Stohl et al. 1999).

Shang et al. (1999) reported that zwittermicin A provided the strongest inhibition of germination of the cysts and elongation of germ tubes in *P. torulosum*. However, the cellular target for zwittermicin A and the reason for which zwittermicin A sometimes exerted little or no effect on prokaryotic and eukaryotic cells that did not contain the *zmaR* gene were still unknown. To enhance the fundamental understanding and applications of zwittermicin A, Stabb and Handelsman (1998) used *Escherichia coli* mutants as targets to detect the mechanism of prokaryotic cells without response to zwittermicin A. He found two classes of zwittermicin A-resistant mutants of *E. coli*. One class was related to genes such as *hemA*, *hemB*, *hemL*, *cydAB*, or *atp*, which are responsible for the electrical membrane potential ($\Delta\psi$) component of a proton motive force (PMF) and are able to resist aminoglycoside antibiotics. The other class, which is sensitive to aminoglycoside, was affected by the presence of *rpoB* and *rpoC* genes that encoded subunits of RNA polymerase. These results deduced that zwittermicin A entered into the target cell via PMF driving and then disturbed DNA replication, DNA gyrase, or topoisomerase I, which are related to the function of *rpoB* and *rpoC* genes. The mechanisms for a bacteria-resistant zwittermicin A, as mentioned above, were only the tip of the submerged iceberg, whereas the facts for zwittermicin A to kill the target cell of fungi or bacteria are still an enigma.

Biosynthesis gene cluster for Zwittermicin A

To make better use of zwittermicin A, it is very important to know the pathway for biosynthesis of zwittermicin A in the *Bacillus* group. Emmert et al. (2004) analyzed the structure of zwittermicin A and deduced that it required five precursors for its biosynthesis: L-serine, malonyl-CoA, aminomalonyl-acyl carrier protein (ACP), hydroxymalonyl-ACP, and 2,3-diaminopropionate (Fig. 3). In addition, carbamoyltransferase was found to confer a carbamoyl residue (Zhao et al. 2007). Of these precursors, L-serine and malonyl-CoA can be provided by metabolic intermediate products in most bacteria. However aminomalonyl-ACP, hydroxymalonyl-ACP, and 2,3-diaminopropionate are special precursors for zwittermicin A biosynthesis that only occur in the *Bacillus* group. Therefore, the zwittermicin A gene cluster must be responsible for the synthesis of these three precursors. Emmert et al. (2004) suggested the synthetic pathway of two precursors, aminomalonyl-ACP and hydroxymalonyl-ACP (Fig. 4a and b) based on the enzyme encoded by the gene located in the zwittermicin A biosynthetic cluster. At that time, the synthetic pathway of 2,3-diaminopropionate was not elucidated because no gene encoding enzyme was cloned to be relative with biosynthesis that precursor from *Bacillus* group. It was gradually clear until the gene cluster sequence was known more than 38 kb (Zhao et al. 2007).

Research for the biosynthesis pathway of zwittermicin A began from exploring self-resistance genes of *B. cereus* UW85 to that antibiotic. It was well known that organisms producing antibiotic require antibiotics self-resistant genes to protect themselves from the action of their toxic metabolites, and that genes are often found in the antibiotic biosynthetic cluster (Cundliffe 1989). This indicates that the biosynthetic cluster of zwittermicin A in the *Bacillus* group

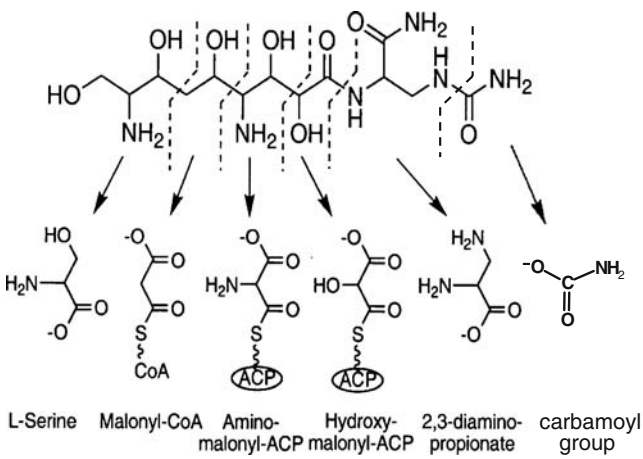

Fig. 3 Chemical structure of zwittermicin A and predicted precursors for its biosynthesis. The *dashed lines* hint the individual precursors in the final molecule, while the *arrows* point to the corresponding precursor shown at the bottom (modified from Emmert et al. 2004)

could be highly correlated with the presence of the *zmaR* gene. Milner et al. (1996) first constructed a cosmid library from the *B. cereus* UW85 genome and cloned the *zmaR* gene, which conferred zwittermicin A resistance. Next, the *zmaR* gene was confirmed to exist ubiquitously in the members of the *Bacillus* group that produced zwittermicin A (Raffel et al. 1996). In addition, Stohl et al. (1999) used the *zmaR* sequence to hybridize corresponding library transformants, which revealed the genes that flanked the *zmaR* gene and allowed exploration of the zwittermicin A biosynthetic cluster. Three genes, *zmaD*, *zmaE*, and *zmaN*, were found. The genes of *zmaD* and *zmaE* were upstream of *zmaR*, and *zmaN* was downstream (Fig. 5). ZmaE was found to have a sequence similar to that of acyl-CoA dehydrogenases, whereas ZmaN revealed that it may encode a polyketide synthase (PKS; Stohl et al. 1999). In addition, ZmaD, which only encodes a predicted 10.2-kDa protein, was believed to be an ACP (Emmert et al. 2004; Chan et al. 2006). Nair et al. (2004) constructed a cosmid library from *B. thuringiensis* subsp. *kurstaki* HD1 and then screened the library for the zwittermicin A biosynthetic cluster using the *zmaR* gene. They discovered a longer zwittermicin A biosynthetic sequence approximately 4.5 kb that contained five open reading frames (ORFs). Two of the ORFs flanking the *zmaR* genes shared high homology with *B. cereus* UW85. In addition, the *zmaG* and *zmaH*, which are located downstream from the *zmaR* gene, were also reported for the first time (Fig. 5). These results suggested that the zwittermicin A biosynthetic cluster within the *Bacillus* group were very similar. That is, they might share similar biosynthetic way.

Emmert et al. (2004) found an approximately 16-kb sequence of the zwittermicin A biosynthetic cluster in a bacterial artificial chromosome (BAC) genomic library of *B. cereus* UW85. Sequence analysis revealed the presence of some of the ORFs (from *zmaD* to *zmaJ*) responsible for biosynthetic zwittermicin A. In addition, all of the ORFs were found to have the same transcriptional orientation as *zmaR* (Molnar et al. 1996; Recktenwald et al. 2002). Furthermore, the function of each ORF in biosynthetic zwittermicin A was analyzed as part of these studies. ZmaD, ZmaE, ZmaN, and ZmaG participate in the hydroxymalonyl-ACP synthetic pathway (Fig. 5). In this synthetic pathway, the initiative substrate is glycolytic intermediate, and 1,3-bisphosphoglycerate is the substrate by which ZmaN-catalyzed modification of holo-ZmaD occurs (Emmert et al. 2004; Chan et al. 2006). This proposed pathway was the first example of aminomalonyl-ACP as an extender unit the biosynthesis of a polyketide biosynthesis (Emmert et al. 2004; Chan et al. 2006).

However, neither gene encoded an enzyme that resulted in the production of 2,3-diaminopropionate, and the carbamoyltransferase gene was not included in this 16-kb

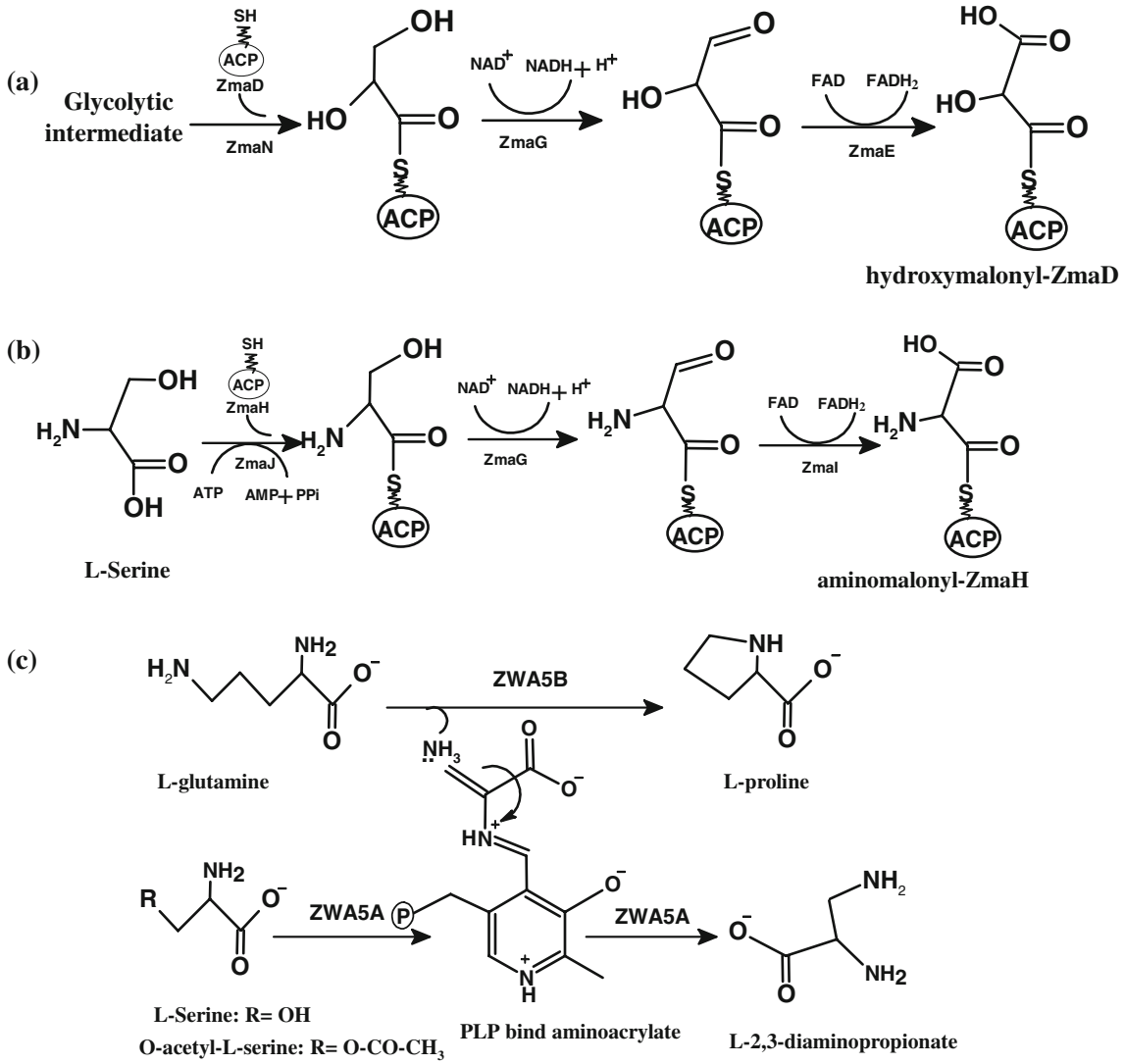

Fig. 4 The biosynthetic pathway for three proposed precursors. **a** Hydroxymalonyl-ACP synthetic pathway; **b** aminomalonyl-ACP synthetic pathway; **c** 2,3-diaminopropionate synthetic pathway (Emmert et al. 2004; Chan et al. 2006; Zhao et al. 2007)

sequence from *B. cereus* UW85. This problem was not resolved until Zhao et al. (2007) reported a much longer sequence of approximately 38.6 kb from the genomic BAC library of *B. thuringiensis* subsp. *kurstaki* strain YBT-1520 (Fig. 5). In the 38.6-kb sequence, an ORF that was responsible for carbamoyltransferase, *zwa6*, was identified. There is a 19-kb intermediate distance between the ORF *zwa6* and downstream orf9, reported by Emmert et al. (2004). Therefore, it was suggested that ZWA6 was responsible for the catalysis of premature zwittermicin A into zwittermicin A in the last step of zwittermicin A biosynthesis (Zhao et al. 2007). Next, two downstream ORFs, *zwa5A* and *zwa5B*, were identified proximal to *zwa6* by walking sequencing. Interestingly, no obvious promoter was recognized upstream of *zwa5B*, which indicates that these two genes are in the same operon as *zwa5A* and share a common promoter. Zhao et al. (2007) predicted the synthesis pathway of 2,3-diaminopropionate based on analysis of the role of the ORFs zwa5A–zwa5B (Fig. 5). The synthesis pathway, all precursors for assembly zwittermicin A, were gradually uncovered.

Although these predictions did not perfectly describe the synthesis of the three special precursors that are necessary to assemble zwittermicin A, the biosynthesis pathway they describe is reasonable due to the gene function analysis and examples of other antibiotic. Resolution of these questions provided insight into the mechanism by which zwittermicin A could be developed in the *Bacillus* group step by step. Emmert et al. (2004) described a preliminary model for the assembly of zwittermicin A antibiotic using a mixed nonribosomal peptide synthetase (NRPS)/PKS pathway because several genes encoding the enzyme were homologous to NRPS and PKS. Combing the pathway suggested by Zhao et al. (2007) with the model described by Emmert

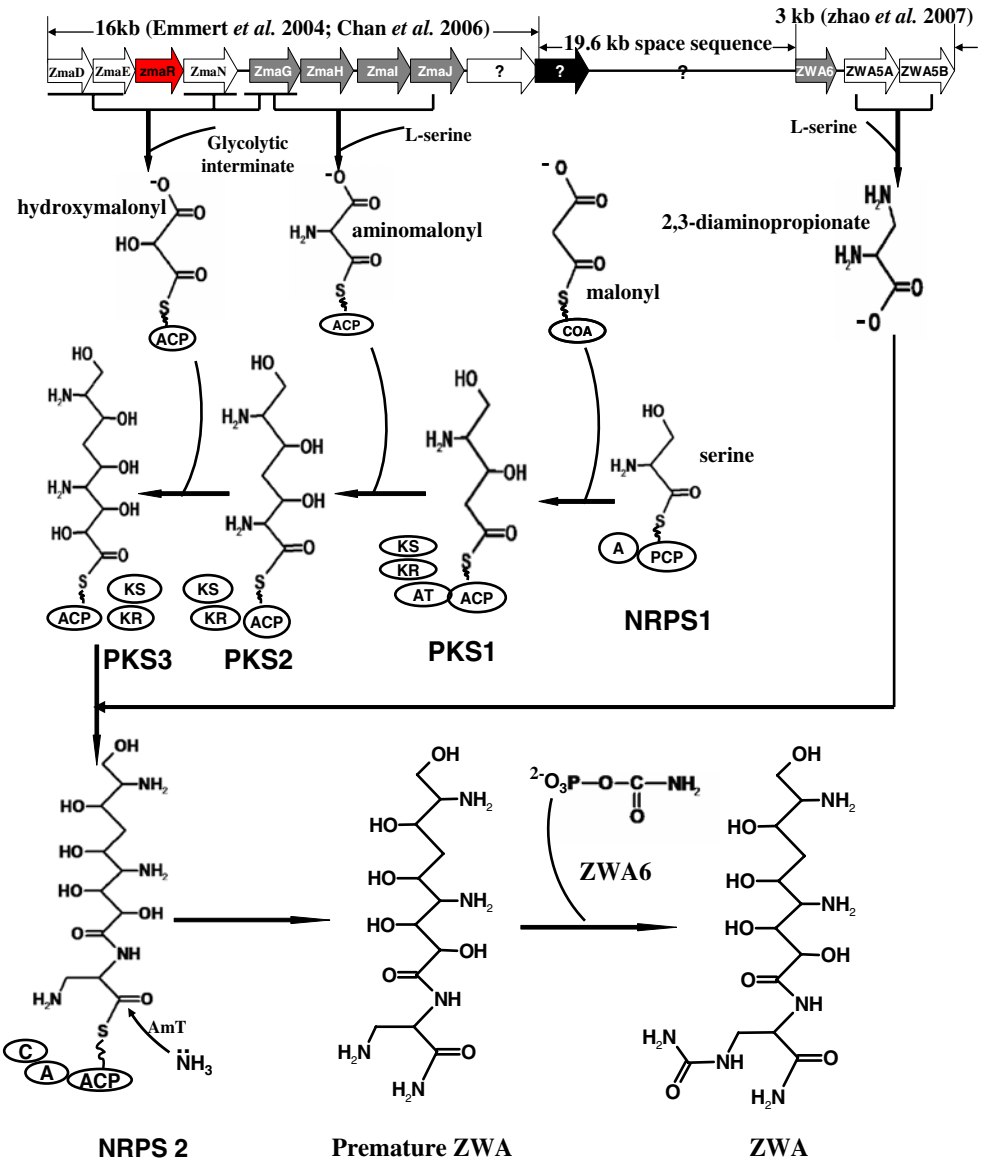

Fig. 5 Framework for the biosynthetic pathway from zwittermicin A gene cluster to product assembly. The zwittermicin A gene cluster is arranged and some sequence yet to know its role in biosynthetic zwittermicin A. ZmaD, ZmaE, ZmaN, and ZmaG are responsible for hydroxymalonyl. ZmaG, ZmaH, ZmaI, and ZmaJ are responsible for aminomalonyl. While ZWA5A and ZWA5B are responsible for 2,3-diaminopropionate. Monomers incorporated into zwittermicin A according to NRPS/PKS megasynthase routes. *A* adenylation, *PCP* peptidyl carrier protein, *KS* ketosynthase, *KR*, ketoreductase, *AT* acyltransferase, *C* condensatione (modified from Emmert et al. 2004; Chan et al. 2006; and Zhao et al. 2007)

et al. gave a total of seven necessary steps (Fig. 5). Briefly, NRPS1 may activate and tether serine to its peptidyl carrier protein domain. PKS1 could then tether a malonyl moiety from malonyl-CoA to its ACP domain and subsequently condense the upstream serine with the covalently bound malonyl moiety. The ketoreductase domain of PKS1 would then reduce the carbonyl from serine, generating the intermediate shown on PKS1 (Fig. 5). An analogous manner then associates with aminomalonyl and hydroxymalonyl to generate the intermediate shown on PKS2 and PKS3. The other enzyme then activates 2,3-diaminopropionate using the PKS3-tethered intermediate to generate the intermediate on NRPS2, at which point the premature zwittermicin A backbone comes into being. Finally, a hypothetic carbamoyltransferase (ZWA6) catalyzes the premature zwittermicin A into zwittermicin A (Fig. 5).

This hypothesis is not perfect because it is still unknown which enzyme acts as the incorporation of different precursors. Shao et al. (2008) found a new gene *tzw1* that located upstream of *zmaD*. Namely, the gene *tzw1* is beyond the known sequence for zwittermicin A biosynthesis gene cluster. But *tzw1* mutant abolished zwittermicin A production. Complementation of *tzw1* mutant restored zwittermicin A productivity. These results suggested that *tzw1* is required for zwittermicin A biosynthesis. Shao et al. (2008) further speculated that Tzw1 is probably involved in the activation and incorporation of the 2,3-diaminopropionate extender unit to form the complete zwittermicin A backbone.

In spite of these advances, some important work is still required to complete our understanding of zwittermicin A: (1) The new genes relative with zwittermicin A biosynthesis should be discovered; (2) the function of each gene located in

the zwittermicin A gene cluster must be certified and the mechanism by which this cluster function in the *Bacillus* group must be better elucidated; (3) the crystal structure of zwittermicin A must be evaluated to determine its actual configuration; (4) the mechanism by which zwittermicin A is degraded in nature must be elucidated so that this antibiotic can be successfully used in the field. Only after these questions have been answered will this antibiotic have a promising future.

Potential application of zwittermicin A

Because *B. cereus* UW85 produce the novel and unusual antibiotic, zwittermicin A, which inhibits growth of protists and some gram-negative and gram-positive bacteria, this strain exhibited tremendous biological control potential to suppress plant disease. Both Silo-Suh et al. (1994) and Shang et al. (1999) demonstrated that *B. cereus* UW85 could suppress alfafa seedling damping-off diseases caused by *P. medicaginis*. Furthermore, Zhao et al. (2008) constructed a genetically modified *B. cereus* UW85 by combining the gene encoding AHL lactonase. This strain exhibited better prevention efficacy than the activity of each one against plant pathogens.

In addition to this unusually broad spectrum of antibiotic activity, Broderick et al. (2000, 2003) confirmed that there was synergy between zwittermicin A and *B. thuringiensis* toxin. This result is very useful if we can prepare zwittermicin A very easy.

Although great progresses have been made, so many questions are yet unclear for zwittermicin A. There is still a long way for it in practical use.

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References

Barnard AM, Salmond GP (2007) Quorum sensing in *Erwinia* species. Anal Bioanal Chem 387:415–423

Broderick NA, Goodman RM, Raffa KF, Handelsman J (2000) Synergy between zwittermicin A and *Bacillus thuringiensis* subsp. *Kurstaki* against gypsy moth (*Lepidoptera*: *Lymantriidae*). Environ Entomol 29:101–107

Broderick NA, Goodman RM, Handelsman J, Raffa KF (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 32:387–391

Chan YA, Boyne MT, Podevels AM, Klimowicz AK, Handelsman J, Kelleher NL, Thomas MG (2006) Hydroxymalonyl-acyl carrier protein (ACP) and aminomalonyl-ACP are two additional type I polyketide synthase extender units. Proc Natl Acad Sci USA 103:14349–14354

Cho HS, Park SY, Ryu CM, Kim JF, Kim JG, Park SH (2007) Interference of quorum sensing and virulence of the rice pathogen *Burkholderia glumae* by an engineered endophytic bacterium. FEMS Microbiol Ecol 60:14–23

Crickmore N (2006) Beyond the spore—past and future developments of *Bacillus thuringiensis* as a biopesticide. J Appl Microbiol 101:616–619

Cundliffe E (1989) How antibiotic-producing organisms avoid suicide. Annu Rev Microbiol 43:207–233

Dong YH, Xu JL, Li XZ, Zhang LH (2000) AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*. Proc Natl Acad Sci USA 97:3526–3531

Dong YH, Wang LH, Xu JL, Zhang HB, Zhang XF, Zhang LH (2001) Quenching quorum-sensing-dependent bacterial infection by an *N*-acyl homoserine lactonase. Nature 411:813–817

Dong YH, Gusti AR, Zhang Q, Xu JL, Zhang LH (2002) Identification of quorum-quenching *N*-acyl homoserine lactonases from *Bacillus species*. Appl Environ Microbiol 68:1754–1759

Dong YH, Zhang XF, Xu JL, Zhang LH (2004) Insecticidal *Bacillus thuringiensis* silences *Erwinia carotovora* virulence by a new form of microbial antagonism, signal interference. Appl Environ Microbiol 70:954–960

Eberl L (2006) Quorum sensing in the genus *Burkholderia*. Int J Med Microbiol 296:103–112

Emmert EA, Klimowicz AK, Thomas MG, Handelsman J (2004) Genetics of zwittermicin a production by *Bacillus cereus*. Appl Environ Microbiol 70:104–113

Girard G, Bloemberg GV (2008) Central role of quorum sensing in regulating the production of pathogenicity factors in Pseudomonas aeruginosa. Future Microbiol 3:97–106

Handelsman J, Raffel S, Mester EH, Wunderlich L, Grau CR (1990) Biological control of damping-off of alfalfa seedlings with *Bacillus cereus* UW85. Appl Environ Microbiol 56:713–718

He H, Silo-Suh LA, Handelsman J, Clardy J (1994) Zwittermicin A, an antifungal and plant protection agent from *Bacillus cereus*. Tetrahedron Lett 35:2499–2502

Jensen GB, Hansen BM, Eilenberg J, Mahillon J (2003) The hidden lifestyles of *Bacillus cereus* and relatives. Environ Microbiol 5:631–640

Khyami-Horani H, Hajaij M, Charles JF (2003) Characterization of *Bacillus thuringiensis* ser. *jordanica* (serotype H71), a novel serovariety isolated in Jordan. Curr Microbiol 47:26–31

Kim MH, Choi WC, Kang HO, Lee JS, Kang BS, Kim KJ, Derewenda ZS, Oh TK, Lee CH, Lee JK (2005a) The molecular structure and catalytic mechanism of a quorum-quenching *N*-acyl-L-homoserine lactone hydrolase. Proc Natl Acad Sci USA 102:17606–17611

Kim MH, Kang HO, Kang BS, Kim KJ, Choi WC, Oh TK, Lee CH, Lee JK (2005b) Crystallization and preliminary crystallographic analysis of *Bacillus thuringiensis* AHL-lactonase. Biochim Biophys Acta 1750:5–8

Lee SJ, Park SY, Lee JJ, Yum DY, Koo BT, Lee JK (2002) Genes encoding the *N*-acyl homoserine lactone-degrading enzyme are widespread in many subspecies of *Bacillus thuringiensis*. Appl Environ Microbiol 68:3919–3924

Liu D, Lepore BW, Petsko GA, Thomas PW, Stone EM, Fast W, Ringe D (2005) Three-dimensional structure of the quorum-quenching *N*-acyl homoserine lactone hydrolase from *Bacillus thuringiensis*. Proc Natl Acad Sci USA 102:11882–11887

Lu X, Yuan Y, Xue XL, Zhang GP, Zhou SN (2006) Identification of the critical role of Tyr-194 in the catalytic activity of a novel *N*-acyl-homoserine lactonase from marine *Bacillus cereus* strain Y2. Curr Microbiol 53:346–350

Milner JL, Stohl EA, Handelsman J (1996) Zwittermicin A resistance gene from *Bacillus cereus*. J Bacteriol 178:4266–4272

Molnar I, Aparicio JF, Haydock SF, Khaw LE, Schwecke T, Konig A, Staunton J, Leadlay PF (1996) Organisation of the biosynthetic gene

cluster for rapamycin in *Streptomyces hygroscopicus*: analysis of genes flanking the polyketide synthase. Gene 169:1–7

Momb J, Thomas PW, Breece RM, Tierney DL, Fast W (2006) The quorum-quenching metallo-gamma-lactonase from *Bacillus thuringiensis* exhibits a leaving group thio effect. Biochemistry 45:13385–13393

Nair JR, Narasimman G, Sekar V (2004) Cloning and partial characterization of zwittermicin A resistance gene cluster from *Bacillus thuringiensis* subsp. *kurstaki* strain HD1. J Appl Microbiol 97:495–503

Raffel SJ, Stabb EV, Milner JL, Handelsman J (1996) Genotypic and phenotypic analysis of zwittermicin A-producing strains of *Bacillus cereus*. Microbiology 142:3425–3436

Recktenwald J, Shawky R, Puk O, Pfennig F, Keller U, Wohlleben W, Pelzer S (2002) Nonribosomal biosynthesis of vancomycin-type antibiotics: a heptapeptide backbone and eight peptide synthetase modules. Microbiology 148:1105–1118

Roh JY, Choi JY, Li MS, Jin BR, Je YH (2007) *Bacillus thuringiensis* as a specific, safe, and effective tool for insect pest control. J Microbiol Biotechnol 17:547–559

Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler DR, Dean DH (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol Mol Biol Rev 62(3):775–806

Shang H, Chen J, Handelsman J, Goodman RM (1999) Behavior of *Pythium torulosum* zoospores during their interaction with tobacco roots and *Bacillus cereus*. Curr Microbiol 38:199–204

Shao T, Bai L, Zhang J, Wang G, Liu D, Li Z, Liu J, Song F, Huang D (2008) A nonribosomal peptide synthetase gene tzw1 is involved in zwittermicin A biosynthesis in *Bacillus thuringiensis* G03. Curr Microbiol 57(1):61–65

Silo-Suh LA, Lethbridge BJ, Raffel SJ, He H, Clardy J, Handelsman J (1994) Biological activities of two fungistatic antibiotics produced by *Bacillus cereus* UW85. Appl Environ Microbiol 60:2023–2030

Silo-Suh LA, Stabb EV, Raffel SJ, Handelsman J (1998) Target range of zwittermicin A, an aminopolyol antibiotic from *Bacillus cereus*. Curr Microbiol 37:6–11

Stabb EV, Handelsman J (1998) Genetic analysis of zwittermicin A resistance in *Escherichia coli*: effects on membrane potential and RNA polymerase. Mol Microbiol 27:311–322

Stabb EV, Jacobson LM, Handelsman J (1994) Zwittermicin A-producing strains of *Bacillus cereus* from diverse soils. Appl Environ Microbiol 60:4404–4412

Stohl EA, Brady SF, Clardy J, Handelsman J (1999) ZmaR, a novel and widespread antibiotic resistance determinant that acetylates zwittermicin A. J Bacteriol 181:5455–5460

Thomas PW, Stone EM, Costello AL, Tierney DL, Fast W (2005) The quorum-quenching lactonase from *Bacillus thuringiensis* is a metalloprotein. Biochem 44:7559–7569

Von Bodman SB, Bauer WD, Coplin DL (2003) Quorum sensing in plant-pathogenic bacteria. Annu Rev Phytopathol 41:455–483

Wang LH, Weng LX, Dong YH, Zhang LH (2004) Specificity and enzyme kinetics of the quorum-quenching *N*-acyl homoserine lactone lactonase (AHL-lactonase). J Biol Chem 279:13645–13651

White CE, Winans SC (2007) Cell–cell communication in the plant pathogen *Agrobacterium tumefaciens*. Philos Trans R Soc Lond B Biol Sci 7362:1135–1148

Zhang HB, Wang LH, Zhang LH (2002) Genetic control of quorum-sensing signal turnover in *Agrobacterium tumefaciens*. Proc Natl Acad Sci USA 99:4638–4643

Zhang HB, Wang C, Zhang LH (2004) The quormone degradation system of *Agrobacterium tumefaciens* is regulated by starvation signal and stress alarmone (p)ppGpp. Mol Microbiol 52:1389–1401

Zhang Y, Fernando WG, de Kievit TR, Berry C, Daayf F, Paulitz TC (2006) Detection of antibiotic-related genes from bacterial biocontrol agents with polymerase chain reaction. Can J Microbiol 52(5):476–481

Zhang L, Ruan L, Hu C, Wu H, Chen S, Yu Z, Sun M (2007) Fusion of the genes for AHL-lactonase and S-layer protein in *Bacillus thuringiensis* increases its ability to inhibit soft rot caused by *Erwinia carotovora*. Appl Microbiol Biotechnol 74:667–675

Zhao C, Luo Y, Song C, Liu Z, Chen S, Yu Z, Sun M (2007) Identification of three Zwittermicin A biosynthesis-related genes from *Bacillus thuringiensis* subsp. *kurstaki* strain YBT-1520. Arch Microbiol 187:313–319

Zhao C, Zeng H, Yu Z, Sun M (2008) *N*-acyl homoserine lactonase promotes prevention of Erwinia virulence with zwittermicin A-producing strain *Bacillus cereus*. Biotechnol Bioeng 100(3):599–603

Zhou Y, Ye WX, Zhou Y, Zhu CG, Sun M, Yu Z (2006) Ethanol tolerance, yield of melanin, swarming motility and growth are correlated with the expression levels of *aiiA* gene in *Bacillus thuringiensis*. Enzyme Microb Technol 38:967–974

Zhu C, Yu Z, Sun M (2006) Restraining *Erwinia* virulence by expression of *N*-acyl homoserine lactonase gene *pro3A-aiiA* in *Bacillus thuringiensis* subsp *leesis*. Biotechnol Bioeng 95:526–532