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ApnI, a Transmembrane Protein Responsible for Subtilomycin Immunity, Unveils a Novel Model for Lantibiotic Immunity

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Subtilomycin was detected from the plant endophytic strain *Bacillus subtilis* BSn5 and was first reported from *B. subtilis* strain MMA7. In this study, a gene cluster that has been proposed to be related to subtilomycin biosynthesis was isolated from the BSn5 genome and was experimentally validated by gene inactivation and heterologous expression. Comparison of the subtilomycin gene cluster with other verified related lantibiotic gene clusters revealed a particular organization of the genes *apnI* and *apnT* downstream of *apnAPBC*, which may be involved in subtilomycin immunity. Through analysis of expression of the *apnI* and/or *apnT* genes in the subtilomycin-sensitive strain CU1065 and inactivation of *apnI* and *apnT* in the producer strain BSn5, we showed that the single gene *apnI*, encoding a putative transmembrane protein, was responsible for subtilomycin immunity. To our knowledge, evidence for lantibiotic immunity that is solely dependent on a transmembrane protein is quite rare. Further bioinformatic analysis revealed the abundant presence of ApnI-like proteins that may be responsible for lantibiotic immunity in *Bacillus* and *Paenibacillus*. We cloned the *paeI* gene, encoding one such ApnI-like protein, into CU1065 and showed that it confers resistance to paenibacillin. However, no cross-resistance was detected between ApnI and PaeI, even though subtilomycin and paenibacillin share similar structures, suggesting that the protection provided by ApnI/ApnI-like proteins involves a specific-sequence recognition mechanism. Peptide release/binding assays indicated that the recombinant *B. subtilis* expressing *apnI* interacted with subtilomycin. Thus, ApnI represents a novel model for lantibiotic immunity that appears to be common.

Bacillus subtilis is a Gram-positive, endospore-forming model bacterium. In general, *B. subtilis* grows in association with plants and promotes plant growth (1). However, a recent report revealed that *B. subtilis* is also a saprophytic organism in other environments, such as in an animal's gastrointestinal tract (2). Dozens of antibiotics and enzymes produced by various *B. subtilis* isolates are used in the food industry and agricultural fields. Because of its ability to take up DNA and integrate it into its genome, i.e., natural competence (3), its genetic sequences have been well characterized, including those related to the biosynthesis and regulation mode of most of the antibiotics discovered in this species to date.

B. subtilis is also known to produce lantibiotics (4), which are lanthipeptides with antimicrobial activity (5). Lanthipeptides are a class of ribosomally synthesized and posttranslationally modified peptides (6), which are also referred to as lanthionine (Lan) or 3-methyl-lanthionin (MeLan)-containing peptides. The ring-forming Lan and MeLan peptides are usually generated via a two-step modification process, involving dehydration of the threonine (Thr) and/or serine (Ser) in the precursor peptide to form the double bonds, followed by cyclization mediated by a Michael addition with the thiol bond of cysteine (Cys) in the proper site. This process involves the dehydratase and cyclase, which are, respectively, encoded by either two dependent genes, *lanB* and *lanC*, or a combined gene, *lanM*. Lanthipeptides are subdivided into four classes (I to IV) depending on the specific biosynthetic gene(s) involved. Structurally established lantibiotics can be further subdivided based on sequence similarity, such as the Pep5 group (7). Class I lantibiotics are synthesized as the precursor peptide LanA and are modified by two different enzymes, LanB enzyme (dehydratase) and LanC enzyme (cyclase), and are exported by LanT, and then the leading peptides are removed by the extracellular serine protease LanP.

A lantibiotic producer must possess one or two synergistic immunity mechanisms to protect itself against its own produced lantibiotics. The reported immunity mechanisms usually involve immunity proteins, including ABC transporters (LanFE[G]), lipoproteins or membrane-associated peptides (LanI), and transmembrane proteins (LanH) (8). For lantibiotic immunity, ABC transporters export lantibiotics to the extracellular space by ATP hydrolysis, and in some cases, the transmembrane protein LanH acts as an accessory protein for the ABC transporters involved in substrate recognition (9, 10). In addition, lipoproteins or membrane-associated proteins (LanI) can work either solely or synergistically with ABC transporters by interacting with lantibiotics (11, 12). Most of the LanI proteins thus far reported are located on the external surface of the membrane, such as NisI, SpaI, and PepI. Very few LanI proteins, especially those solely providing immunity, are located within the cell membrane.

Subtilomycin is a recently identified lantibiotic produced by *B. subtilis* strain MMA7 isolated from the marine sponge *Haliclona simulans*, and it shows a broad spectrum of activity against Gram-positive bacteria, including drug-resistant pathogens (13). The relationship between subtilomycin and its gene cluster was established based on the close match between the N-terminal amino

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acid sequence and the 5′-end sequence of the structural gene *subA*. Paenibacillin is a lantibiotic produced by the strain *Paenibacillus polymyxa* OSY-DF (14), and its structure has been elucidated (15); however, the paenibacillin biosynthetic gene cluster has not yet been reported. Since it shows high sequence similarity (54%) to subtilomycin, the structure of subtilomycin has been proposed using the structure of paenibacillin as a template (13). However, no molecular biological evidence has been provided to validate this proposed biosynthetic gene cluster.

In this report, we provide direct evidence to confirm the presence of a subtilomycin gene cluster, termed the *apn* cluster, in the complete genome of *B. subtilis* strain BSn5, which was previously isolated from the plant *Amorphophallus konjac* (16). We compared the gene organization of the *apn* cluster with those of other related gene clusters to determine its specificity and potential function. DNA fragments containing different regions of the gene cluster were expressed in the heterologous host *B. subtilis* strain CU1065 (W168 *att*SPβ *trpC2*) (17), and gene inactivation analysis was performed for the producer BSn5 strain to identify the gene(s) involved in conferring immunity. Our study thus provides valuable information of a rare molecular model underlying lantibiotic immunity.

MATERIALS AND METHODS

Bacterial strains, plasmids, and general methods. The strains and plasmids used in this study are listed in Table 1. *B. subtilis* strains BSn5 and CU1065, along with their derivatives, were cultured in Luria-Bertani (LB) medium at 28°C, and *Escherichia coli* DH5α, which was used as a host for clones, was routinely cultured in LB medium (Invitrogen, Karlsruhe, Germany) at 37°C. For solid culture, LB medium was supplemented with 1.5% agar. For stock preparation, the culture was cultivated overnight at 28°C in LB medium mixed with sterile glycerol (final concentration of 17%) and stored at −80°C. For selective media, the following antibiotics were added: ampicillin, 100 μg/ml (*E. coli*); spectinomycin, 100 μg/ml (*B. subtilis*); and kanamycin, 20 μg/ml (*B. subtilis*). Isolation of plasmids and chromosomal DNA, restriction endonuclease digestion, agarose gel electrophoresis, PCR, and transformation of *E. coli* were performed by following established protocols for molecular biology techniques described by Sambrook and Russell (18).

Plasmid construction and manipulation of *B. subtilis* strains. Gene disruption plasmids were constructed using two approaches. The first was establishment of the *apnB* mutant allele (*apnB*::*spc*) by subcloning of the spectinomycin resistance gene (*spc*) from pIC333 into the PCR-introduced ClaI and XbaI sites of the *apnB* gene in pMD18T-apnB, resulting in plasmid pB1201 (Table 1). The second approach was the establishment of *apnT*, *ybcL*, *ybdG*, and *apnI* mutant alleles (*apnT*::*kan*, *ybcL*::*kan*, *ybdG*::*kan*, and *apnI*::*kan*, respectively) by amplifying the upstream and downstream arms of each gene fragment into the upstream and downstream restriction sites of the integration vector pDG780, respectively, resulting in plasmids pB1202, pB1203, pB1204, and pB1205 (Table 1). The *B. subtilis* competent cell preparation was performed by following the classical nutritional downshift method described by Anagnostopoulos and Spizizen in 1960 (3), with modifications described by Yasbin et al. (19). A series of at least five 10-min interval grades were set to ensure that the t_0 point (i.e., the point at which the culture leaves the logarithmic growth phase) could be captured at every cycle considering the lower transformation efficiency of wild-type strain BSn5 than of *B. subtilis* 168. Chromosomal gene replacement of wild-type *apnB*, *apnT*, *ybcL*, and *ybdG* with the corresponding constructed mutation alleles was carried out by transforming BSn5 with the constructed plasmids, resulting in the mutants B1201, B1202, B1203, B1204, and B1205. B1201 was selected for spectinomycin resistance evaluation, and the other mutants were selected for kanamycin resistance evaluation. Proper allelic replacement was confirmed by PCR and sequencing.

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
E. coli DH5α	Cloning host	42
Paenibacillus ATCC 842	Wild type; paenibacillin producer strain	28
B. subtilis		
BSn5	Wild type; subtilomycin producer	16
B1201 (Δ*apnB*)	Strain BSn5 derivative; Δ*apnB*::*spc*	This study
B1202 (Δ*apnT*)	Strain BSn5 derivative; Δ*apnT*::*kan*	This study
B1203 (Δ*ybcL*)	Strain BSn5 derivative; Δ*ybcL*::*kan*	This study
B1204 (Δ*ybdG*)	Strain BSn5 derivative; Δ*ybdG*::*kan*	This study
B1205 (Δ*apnI*)	Strain BSn5 derivative; Δ*apnI*::*kan*	This study
CU1065	Strain168 derivative; *trpC2 attSP*β	17
B1301	CU1065 *amyE*::(*spc* subtilomycin gene cluster)	This study
B1302	CU1065 *amyE*::(*spc* fragment *apnI*)	This study
B1303	CU1065 *amyE*::(spc fragment *apnT*)	This study
B1304	CU1065 *amyE*::(*spc* fragment *apnI-apnT*)	This study
B1305	CU1065 *amyE*::(*spc* fragment pro_{apnA} *apnI*)	This study
B1306	CU1065 *amyE*::(*spc* fragment pro_{apnA} *apnT*)	This study
B1307	CU1065 *amyE*::(*spc* fragment pro_{apnA} *apnI-apnT*)	This study
B1308	CU1065 *amyE*::(*spc* fragment pro_{apnA} *paeI*)	This study
B1309	CU1065 *amyE*::(*spc* fragment pro_{paeA} *paeI*)	This study
Plasmids		
pDG780	pBluescript KS^+ harboring *kan* (*amp kan*)	43
pMD18T simple	Clone vector for *E. coli* (*amp*)	TaKaRa
pIC333	Mini-Tn*10* transposons delivery vector harboring *spc* and *erm*	44
pDG1730	Integration vector harboring *amyE*::*spc* (*amp erm*)	45
pB1201	pMD18T harboring mutation of Δ*apnB*::*spc*	This study
pB1202	pDG780 harboring mutation of Δ*apnT*::*kan*	This study
pB1203	pDG780 harboring mutation of Δ*ybcL*::*kan*	This study
pB1204	pDG780 harboring mutation of Δ*ybdG*::*kan*	This study
pB1205	pDG780 harboring mutation of Δ*apnI*::*kan*	This study
pB1301	pDG1730 harboring *amyE*::(*spc apn* gene cluster)	This study
pB1302	pDG1730 harboring *amyE*::(*spc apnI*)	This study
pB1303	pDG1730 harboring *amyE*::(*spc apnT*)	This study
pB1304	pDG1730 harboring *amyE*::(*spc apnI-apn*T)	This study
pB1305	pDG1730 harboring *amyE*::(*spc* pro_{apnA} apnI)	This study
pB1306	pDG1730 harboring *amyE*::(*spc* pro_{apnA} apnT)	This study
pB1307	pDG1730 harboring *amyE*::(*spc* pro_{apnA} *apnI-T*)	This study
pB1308	pDG1730 harboring *amyE*::(*spc* pro_{apnA} *paeI*)	This study
pB1309	pDG1730 harboring *amyE*::(*spc* pro_{paeA} *paeI*)	This study

Different fragments containing the whole subtilomycin gene cluster, *apnI*, *apnT*, or *apnI-apnT* were amplified using the primers listed in Table 2. Then, these fragments were cut using proper restriction endonuclease and cloned into another integration vector, pDG1730, resulting in the heterologous expression alleles *amyE*::(*spc* subtilomycin gene cluster), *amyE*::(*spc* fragment *apnI*), *amyE*::(*spc* fragment *apnT*), and *amyE*::(*spc* fragment *apnI-apnT*) in plasmids pB1301, pB1302, pB1303, and pB1304, respectively. The fragment containing the predicted promoter region of

TABLE 2 Primer sequences used in this study

Primer	Sequence (5′→3′)[a]	Use
ybcL UpF	CTAACTAGTCAGAGATCAGTAAGTCC	*ybcL* gene disruption
ybcL UpR	TGTCTGCAGAGGTTCCAACTAAAAAGC	*ybcL* gene disruption
ybcL DownF	GGACTCGAGAGTTTGGTTGGATCAAAG	*ybcL* gene disruption
ybcL DownR	TGAGGTACCCTCAGTAATCTTATTAGCC	*ybcL* gene disruption
ApnBUpF	TTTGCATGCTTAGTATTCTCGAGCAG	*apnB* gene disruption
ApnBDownR	TGCGGATCCATTAAATAGGGATAGTG	*apnB* gene disruption
ApnBUpR	CCTTCGTATCCTATTACTCGCATTT	*apnB* gene disruption
ApnBDownF	AGAATCTAGAGCAATCACCTATTCTAAGAGC	*apnB* gene disruption
SpcF	AGTATCGATGCGGTGCTACAGAGTTCTTG	Amplification of spectinomycin resistance gene
SpcR	GGGTCTAGAGTAAACGCTGAATATCGTGTT	Amplification of spectinomycin resistance gene
ApnTUpF	TTTGGATCCGGTTGTTGAAGACGCAG	*apnT* gene disruption
ApnTDownR	TCCTTTCCCATACTCTTC	*apnT* gene disruption
ApnTDownF	CAACTCGAGCGAGTTCTCTAAGTTA	*apnT* gene disruption
ApnTDownR	TTTGGTACCTGGTCAGAGACCGCATCT	*apnT* gene disruption
YbdGUpF	GGGACTAGTTCGAGATAG	*ybdG* gene disruption
YbdGUpR	CACCTGCAGATTATATACAGCGGAAG	*ybdG* gene disruption
YbdGDownF	TATCTCGAGGGTTCGCTAGGATGAAGG	*ybdG* gene disruption
YbdGDownR	TGAGGTACCGGTTTAAACATGCTGTC	*ybdG* gene disruption
ApnIUpF	GAAACTAGTTCTAGAGGCGGAACAAGAT	*apnI* gene disruption
ApnIDownR	GGGGAATTCATGAAAAGAACTGCGAACC	*apnI* gene disruption
ApnIDownF	TAACTCGAGAGGTTATACATATGGGC	*apnI* gene disruption
ApnIDownR	GCAACAAAGGTACCTGGTGAAATAG	*apnI* gene disruption
apnF	CCTGGATCCGCTCTGTTTCCGTTGTCG	Clone of apn cluster
apnR	TGAGGATCCTACCCAAGTGGTCTCATTATTT	Clone of apn cluster
ApnIF	TTCGGATCCGGTCAAGATTCGTGGGAC	Clone of fragment *apnI-apnT* (I)
ApnIR	GGCGAATTCGTTATTGCCCATATGTATAAC	Clone of fragment *apnI*
ApnTF	AGTGGATCCTAGCGAAAGTGTTCATTC	Clone of fragment *apnT*
ApnTR	GACGAATTCAAGCAAAACCAAAGCAGCC	Clone of fragment *apnI-apnT* (T)
ProapnAF	TTTGGATCCGCGAGCTGAAGTACAGTACG	Clone of the *apnA* promoter region
ProapnAR	CTTGGATCCTTCCATTTCCTCCTTTT	Clone of the *apnA* promoter region
ProapnAF2	TTTGGATCCGCGAGCTGAAGTACAGTACG	Clone of the *apnA* promoter region
ProapnAR2	CTTGAATTCCTTAGCTCGAGTTCCATTTCCTCCTTTT	Clone of the *apnA* promoter region
PropaeAF	AACGGATCCATCGGGACCAAAGTGATTCGACTCA	Clone of the *paeA* promoter region
PropaeAR	CTTGAATTCTGCCTCTCGAGTTTCCTCTTCCTCTCTGAATTAAAT	Clone of the *paeA* promoter region
PaeIF	TTTGGATCCCTTAGCTCGAGCAGATGCTGGTATTTAGCT	Clone of the *paeI* gene
PaeIR	CTCCGAATTCGAATGGGCCGTTTGTTTT	Clone of the *paeI* gene

[a] Restriction sites are underlined.

apnA (pro_{apnA}) was amplified with primers containing BamHI restriction sites and cloned into all of the constructed vectors, except for that containing the whole *apn* cluster. PCR was used to identify the inserted direction of the pro_{apnA} fragment. The same procedure as described above was used to prepare the competent cells of *B. subtilis* CU1065. The heterologous expression recombinants were selected for spectinomycin resistance and were confirmed by PCR and sequencing.

To express the gene *paeI* for putative paenibacillin immunity in CU1065, the fragments containing *paeI* and the predicted promoter regions of *apnA* and *paeA* (pro_{apnA} and pro_{paeA}, respectively) were amplified using the primers listed in Table 2 and were T/A cloned into the pMD18T Simple vector. Then, the *paeI* fragments were cut and inserted into the downstream regions of the pro_{apnA} and pro_{paeA} fragments, resulting in insertion of pro_{apnA}-paeI and pro_{paeA}-paeI, respectively, in the vector pMD18T Simple. Finally, the two fragments were cut using BamHI and EcoRI and cloned into the integration vector pDG1730, resulting in the heterologous expression alleles *amyE*::(*spc* fragment pro_{apnA}-paeI) and *amyE*::(*spc* fragment pro_{paeA}-paeI) in plasmids pB1308 and pB1309 (Table 1). The *B. subtilis* CU1065 transformation and the confirmation of recombinants were performed by following the procedures described above.

Isolation and purification of subtilomycin. To prepare subtilomycin, a single colony of BSn5 was inoculated from the culture plate into 5 ml of LB medium with agitation, and then the overnight culture was transferred into a 500-ml flask containing 100 ml of LB medium at the ratio of 1/100 (vol/vol). The flask was incubated at 28°C for 12 h in a rotary shaker with agitation at 200 rpm. After centrifugation at 10,000 × *g* for 10 min at 4°C using the centrifuge Sorvall ST 16R (Thermo Fisher Scientific; USA), the cell-free supernatant was filtered through a 0.22-μm membrane filter. Ammonium sulfate was added intermittently to the cell-free supernatant to 30% saturation during stirring using a magnetic stirrer. The mixture was set at 4°C for 2 h. The resulting precipitate was collected by centrifugation at 12,000 × *g* for 30 min at 4°C, followed by dialyzation desalination. The extract was used as the active ammonium sulfate crude extract (ASCE) of the subtilomycin preparation for further bioassays, high-performance liquid chromatography (HPLC), and matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrum (MS) analysis. The subtilomycin extracts from BSn5 mutants were prepared by following the same protocol.

Crude subtilomycin extracts were condensed by filtering through a Millipore Amicon Ultra-15 3K device and adjusted to 25% acetonitrile (0.1% trifluoroacetic acid [TFA]), followed by centrifugation at 12,000 × *g* for 5 min to remove insoluble material. Subtilomycin was purified by preparative HPLC using a Waters 1528 binary HPLC pump, Waters 2489 UV/visible detector, and Waters 2707 auto-sample system equipped with an Agilent Eclipse XDB-C18 PrepHT column (5 μm, 21.2 by 150 mm). The mobile phase consisted of chromatography-grade water containing 0.1% trifluoroacetic acid (TFA) in pump A and 80% chromatography-

grade acetonitrile containing 0.086% TFA in pump B. The elution step was performed under an acetonitrile gradient of 30 to 70% over 20 min at a flow rate of 9.0 ml/min. Subtilomycin was eluted within 14.3 min (see Fig. S4 in the supplemental material). The purified peptide was lyophilized and used for subtilomycin quantification by HPLC.

Bioassay for detection of subtilomycin. Agar well diffusion assays were used to detect the antimicrobial activities of the isolates as previously described (20). Thirty milliliters of LB agar (1.0%, wt/vol) medium was poured into 100- by 100-mm^2 plastic dishes and inoculated (1%, vol/vol) with the indicator suspension. After drying for 30 min, nine 6-mm wells were bored in each plate. Approximately 60 μl of crude extract samples from the supernatants of *B. subtilis* strains was pipetted into each well, and the plates were maintained at 4°C for 4 h for diffusion and then incubated overnight at 28°C. The diameter of the inhibition zones was measured to determine antimicrobial activity.

Detection of subtilomycin by HPLC and liquid chromatography (LC)-MS. The same HPLC system as described above for purification was used for subtilomycin detection. The data were displayed and analyzed by using a Breeze system (Waters Corporation, USA). An Agilent HC-C18 reverse-phase column (250 by 4.6 mm; particle size of 5 μm) was employed (Agilent, USA). The mobile phase consisted of chromatography-grade water containing 0.1% TFA in pump A and chromatography-grade acetonitrile in pump B. The crude lantibiotic preparation was dissolved in 20% acetonitrile (TFA, 0.1%), and 5 to 20 μl was loaded. The elution step was performed under an acetonitrile gradient of 20 to 80% over 15 min at a flow rate of 1 ml/min. The peaks were detected by measuring the absorbance at 235 nm and 254 nm.

Liquid chromatography quadrupole-time of flight (LC-Q-TOF) MS was carried out using an Agilent 1260 LC device equipped with a C_{18} reverse-phase column (100 by 1.8 mm; particle size of 3.5 μm) under chromatography detection conditions as the flow rate was decreased by 0.3 ml/min; the injection volume was 1 μl, and the diode array detection was performed at 254 nm. MS was performed using the Q-TOF MS G6540A system (Agilent) equipped with a dual-source electrospray ionization (ESI) ion source and was operated in positive-ion mode. Calibration was carried out with standard references of mass 121.0509 and 922.0098. The source parameters were as follows: gas temperature of 350°C, gas flow rate of 9 liters/min, and nebulizer stress of 40 lb/in^2 (gauge). The capillary, fragmenter, skimmer, and octopole radio frequency (RF) peak voltage were set at 4,000 V, 250 V, 65 V, and 750 V, respectively, for the scan source. The quadrupole was set to pass ions from *m/z* 200 to 2,000. The MS scan rate was set to 1.5 spectra/s.

After determination of the ions of subtilomycin at *m/z* 1,079.1666 by MS, target tandem MS (MS/MS) mode was chosen for acquisition of the fragmental ions of subtilomycin. The MS and MS/MS scan rates were set to 1.5 spectra/s and 0.8 spectrum/s, respectively. The MS and MS/MS scan ranges were set to *m/z* 200 to 2,000 and *m/z* 100 to 2,000, respectively. The target ions were isolated and fragmented in the collision cell by adding optimized collision energy at 34 eV. Data were analyzed using Agilent MassHunter qualitative analysis software.

Detection of subtilomycin by MALDI-TOF MS. The MS analysis of the peptide preparations was performed by using the 4800 Plus MALDI TOF/TOF analyzer (Applied Biosystems, USA). Aliquots of 0.5-μl extracts were mixed with a 0.5-μl sample of matrix (α-cyano-4-hydroxycinnamic acid in acetonitrile and 0.1% TFA in water, 1:3). The samples were spotted onto the MALDI target and dried in air. Mass spectra were measured in positive ion-mode in the range of *m/z* 800 to 4,000 for searching and 2,000 to 5,000 Da for comparison and analyzed using ProteinPilot software.

Preparation of peptide antibiotics for sensitivity assay. Vancomycin and nisin were purchased from Sigma-Aldrich, USA. By searching the National Center for Biotechnology Information (NCBI) database using the paenibacillin structural sequence, we found that a Bacillus Genetic Stock Center (BGSC) preserved isolate, *Paenibacillus* strain ATCC 842, with complete genome sequence information, may produce paenibacillin. Based on a previously reported isolation and detection strategy (14), we aimed to detect and prepare crude extracts of paenibacillin. Briefly, a single colony of ATCC 842 was activated in 5 ml of tryptic soy broth yeast extract and incubated at 28°C for 24 h. The resulting culture was inoculated into a 500-ml flask containing 100 ml of tryptic soy broth yeast extract. The flask was incubated at 28°C for 24 h in a rotary shaker with agitation at 200 rpm. After centrifugation at 9000 × *g* for 10 min, the cell-free supernatant was mixed with Amberlite XAD-7 resin (Sigma) at a 10% (wt/vol) ratio, and the mixture was set for static adsorption overnight at 4°C. The absorbed paenibacillin resin was washed sequentially with 1 liter of distilled water and 500 ml of 30% (vol/vol) ethanol. Finally, the resin was eluted with 250 ml of 75% (vol/vol) ethanol (pH 2.0). The fraction was condensed with a rotary evaporator at 35°C under vacuum, and the concentrate was adjusted to pH 7.0 with phosphate buffer and then was freeze-dried. The powder was resolved in distilled water, followed by centrifugation at 12,000 × *g* for 5 min. The resulting supernatant was considered to be the paenibacillin crude extract and was used to detect paenibacillin under LC-MS by following the same procedure as described above for subtilomycin detection.

Subtilomycin sensitivity assay. The subtilomycin sensitivity assay of *B. subtilis* CU1065 and recombinants was performed using agar diffusion tests according to a modification of a previously described protocol (21). Stationary-phase-grown CU1065 and its recombinant cultures with an optical density at 600 nm (OD_{600}) of 0.8 were each inoculated at 1/1,000 into diluted LB agar (1.0%, wt/vol) at 40°C and then poured into 90-mm petri dishes (20 ml) and dried for 30 min. Seven 6-mm wells were bored into each plate. Serially diluted (0.125 to 200 μM) subtilomycin extracts (60 μl) were loaded into the wells of test plates. The plates were maintained at 4°C for 4 h to allow sufficient diffusion and then incubated overnight at 28°C. The response was calculated as the mean area of the inhibition halos. Each test was independently replicated three times. The same procedure was employed for vancomycin, nisin, and paenibacillin sensitivity tests of *B. subtilis* CU1065 and the *apnI*-expressing recombinants (pro_{apnA}apnI).

Peptide-binding assay for subtilomycin. The peptide-binding assay for subtilomycin was performed according to a previously described method, with some modifications (21). *B. subtilis* CU1065 and its recombinant expressing *apnI* were cultured overnight. The cells were harvested and washed twice with 50 mM sodium phosphate buffer (pH 7.0) containing 1% glucose. The cell concentration was adjusted to an OD_{600} of 10 with 1 ml of incubation buffer containing 50 mM sodium phosphate, 1% glucose, and 1 M NaCl. The cell suspension was incubated with subtilomycin at concentrations of 15 μg/ml and 25 μg/ml with shaking (150 rpm) for 30 min at 30°C. After incubation, the cells were centrifuged at 17,000 × *g* for 10 min. The supernatants were transferred to the HPLC system with the same parameters as described above. The harvested cell pellets were gently mixed with 1 ml of 20% acetonitrile in water containing 0.1% trifluoroacetic acid and incubated with shaking (150 rpm) for 5 min at 30°C. The cells were removed by centrifugation at 17,000 × *g* for 10 min, and the remaining supernatants were loaded into the HPLC system. Subtilomycin amounts in cell-free supernatants and in cell extracts were quantitatively determined.

RESULTS

Production of subtilomycin in *B. subtilis* strain BSn5. In accordance with a previously reported method for purifying subtilomycin (13), we prepared ASCEs to detect the production of subtilomycin from the 12-h supernatant of *B. subtilis* BSn5. LC-electrospray ionization MS of the crude extracts revealed that BSn5 can produce a compound with the same characteristics as subtilomycin. The corresponding main peak was detected at a retention time of 7.9 min under the UV monitor at 254 nm using similar mobile-phase conditions (Fig. 1A), and the MS extracted from this peak showed that the monoisotopic mass of the quadruply charged $[M + 4H]^{4+}$, triply charged $[M + 3H]^{3+}$, and doubly

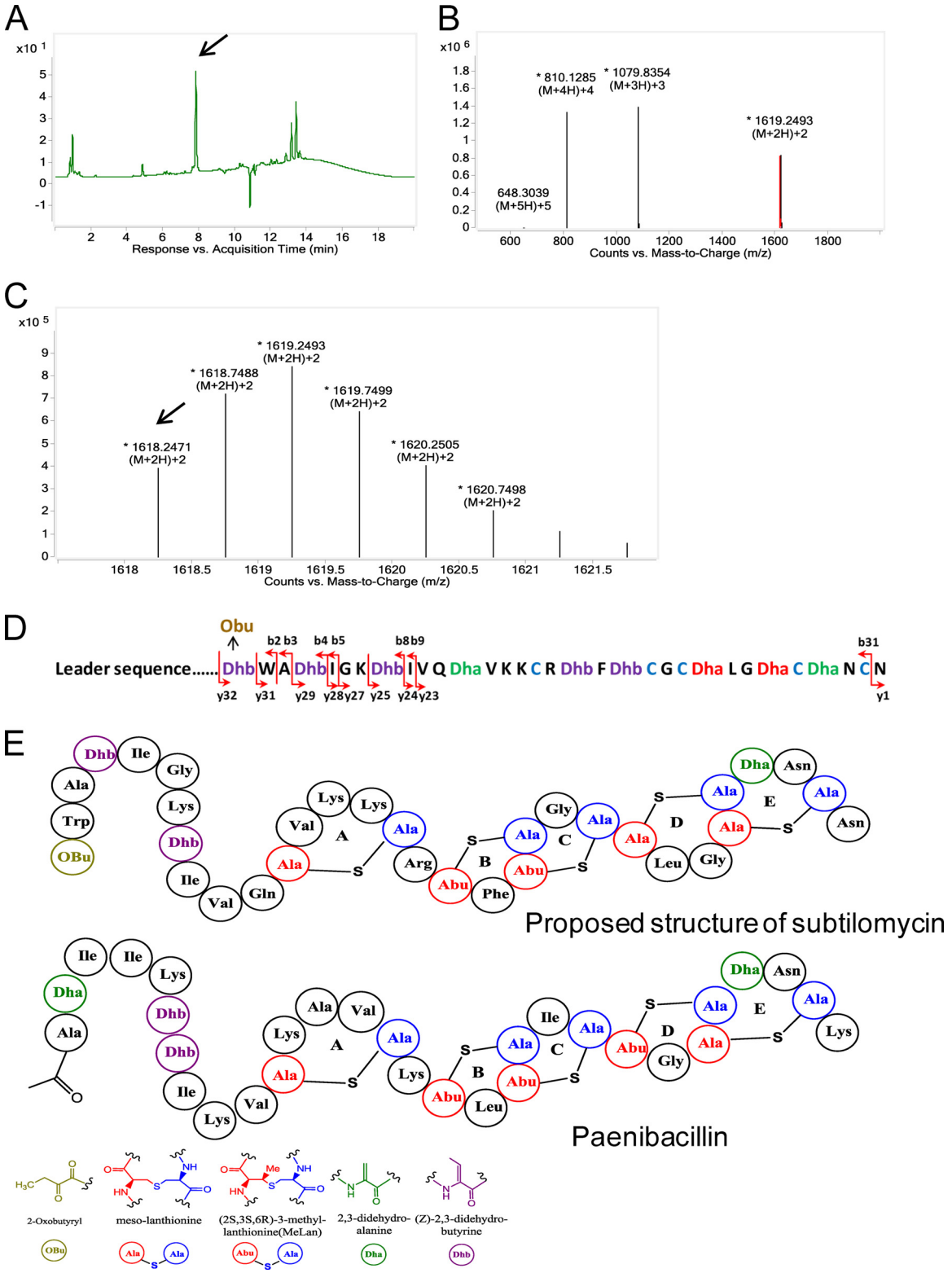

FIG 1 Detection of the production of subtilomycin by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). (A) Chromatography profile of active ammonium sulfate crude extracts of the *Bacillus subtilis* BSn5 culture supernatant. The target compound is indicated by an arrow. (B) ESI-MS of the compound indicated by the arrow in panel A. (C) Magnification of the doubly charged ions $[M + 2H]^{2+}$ in panel B; the monoisotopic mass peak is indicated by an arrow. (D) Fragmental ions obtained from target tandem mass spectrometry of the peptide with a mass of 3,234.48 Da. Ions y and b indicate the fragmented peptides extended from the carboxyl terminus and amino terminus, respectively. (E) Predicted structure of subtilomycin and established structure of paenibacillin (15). The predicted structure motifs are shown in different colors. Obu refers to 2-oxobutyryl.

charged $[M + 2H]^{2+}$ ions present in the sample were *m/z* 809.6273, *m/z* 1,079.1666, and *m/z* 1,618.2471, respectively, which are all equivalent to the mass of 3,234.48 Da, which corresponds extremely well to the theoretical mass of subtilomycin of 3,234.4806 Da (Fig. 1B and C).

Based on the MS/MS results, we further verified the predicted N-terminal modification of 2-oxobutyrate. Similar to the reported N-terminal modification of Pep5 (22), the N-terminal Dhb (C_4H_6NO—) is spontaneously deaminated into 2-oxobutyrate ($C_4H_5O_2$—) in subtilomycin, resulting in an accurate mass gain of 0.9843 Da. All the measured b ions (extending from the amino terminus) are consistent with the mass of peptides with an N-terminal 2-oxobutyrate modification (see Table S1 in the supplemental material). Previously, Li et al. used tandem mass spectrometric analysis to determine the ring topology of lanthipeptides that do not contain overlapping rings (23). As the proposed subtilomycin structure does contain overlapping rings, we could not directly determine the subtilomycin structure based on our MS/MS results. However, significantly increased fragmentation was observed at the N-terminal region, which contains no predicted thioether rings, thereby providing substantial support for the predicted subtilomycin structure (Fig. 1D and E).

Identification and cloning of the subtilomycin biosynthetic gene cluster. Through scanning the whole genome sequence of strain BSn5, a putative subtilomycin gene cluster (BSn5_12550 to BSn5_12575), termed the *apn* cluster, that spans positions 2428600 to 2436982 was identified (16). Although the relationship between subtilomycin and its biosynthetic gene cluster was recently established based on the single match between the primary amino acid sequence of the N-terminal region of subtilomycin and the 5′ end of the structural gene *subA* (13), no direct molecular biological evidence has yet been provided to validate the function of the proposed subtilomycin biosynthetic gene cluster. Therefore, we explored the effect of gene deletion of *apnB*, which encodes a LanB-like dehydratase, and *apnT*, which encodes a putative transporter, to identify the function of the gene cluster (Fig. 2A).

Bioassays and HPLC were used to detect the change in subtilomycin production in the mutants (Fig. 2B). In contrast to the wild-type strain, the culture supernatant from the Δ*apnB* mutants failed to inhibit the target indicator strain *B. subtilis* CU1065, and no production of subtilomycin was detected by HPLC from the ASCEs of the Δ*apnB* mutant (Fig. 2B). Therefore, *apnB* is necessary for subtilomycin production. The results from bioassays and HPLC showed that very little subtilomycin could be detected from the supernatant of the Δ*apnT* mutant. The *apnT* gene, which encodes a putative ABC transporter, was predicted to be a transporter for the export of subtilomycin. We prepared intracellular extracts from the 10-h cultured cells of the wild-type BSn5 and the Δ*apnT* mutant by liquid nitrogen grinding to detect the precursor peptide of subtilomycin by high-resolution LC-MS. However, no predicted mass (6,250.35 Da) of precursor peptide or significant accumulation of masses from 5,500 to 6,500 Da could be observed in BSn5 or the Δ*apnT* mutant (data not shown). In order to confirm the presence of the minimal subtilomycin gene cluster, two flanking genes of the cluster were deleted as described in Materials and Methods. The *ybcL* gene, which encodes a putative efflux transporter, and *ybdG*, which encodes a putative hydrolase, located upstream and downstream of the cluster, respectively, were disrupted (Fig. 2A). No significant change was observed with respect to the production of subtilomycin (Fig. 2B). The detection of subtilomycin production was also evaluated using MALDI-TOF MS, and the results were consistent with those from the bioassay and HPLC analyses (see Fig. S1 in the supplemental material).

In addition, the *B. subtilis* CU1065 recombinant harboring the whole *apn* cluster was constructed. Subtilomycin production was evaluated in the 12- to 16-h cultured supernatant of the recombinant by LC-MS (Fig. 2C; see also Fig. S2 in the supplemental material), which further verified that the minimal *apn* cluster was responsible for subtilomycin biosynthesis.

The putative transmembrane protein-encoding gene *apnI* confers subtilomycin immunity. A structurally elucidated lantibiotic, paenibacillin, produced by *Paenibacillus polymyxa* OSY-DF was identified with the highest identity (54%) to subtilomycin; however, its biosynthetic gene cluster has not been published. In addition, other lantibiotics from the Pep5 group showed homology with subtilomycin, including epilancin 15X, epilancin K7, Pep5, and epicidin 280. We compared the subtilomycin biosynthetic cluster with four reported and related lantibiotic gene clusters from the Pep5 group (Fig. 3). Four genes homologous to *lanA*, *lanP*, *lanB*, and *lanC* were found to be relatively conserved in all of the gene clusters examined, including the subtilomycin cluster. However, the genes responsible for lantibiotic export, immunity, and N-terminal modification showed variations in terms of their arrangement. The genes responsible for lantibiotic export in the Pep5 group are located upstream of the genes *lanAPBC* in the direction opposite to that observed in the subtilomycin cluster. The transporter gene is missing in the gene cluster for epicidin 280, which was proposed to be replaced by other transporters in the host (24). The immunity genes *pepI* and *eciI* are located closely upstream of the structural genes *pepA* and *eciA*, respectively. The putative epilancin 15X immunity gene *elxI* is located downstream and next to *elxC*.

In the *apn* cluster, the gene products of *apnAPBC* were predicted, respectively, as structural peptide ApnA, leader peptide-cleaving protease ApnP, and lanthionine synthetases ApnB and ApnC. The gene organization *apnI-apnT*, which is located downstream of *apnAPBC*, is different from that of the other reported gene clusters (Fig. 3). As deletion of *apnT* led to a considerable loss of subtilomycin production in the supernatant, *apnT*, which encodes an ABC transporter, likely plays a key role in subtilomycin export. Through protein BLAST using the sequence of the *apnI*-encoded protein, no functionally validated homolog could be found in the NCBI database. Membrane helix prediction using TMHMM (25), HMMTOP (26), and the DAS-TM filter (27) revealed the presence of five transmembrane domains in the putative protein ApnI. Immunity genes are usually present in lantibiotic biosynthetic clusters and are necessary for lantibiotic production. Therefore, the fact that no immunity-related gene was found in the subtilomycin gene clusters indicated that *apnI* and/or *apnT* is likely involved in subtilomycin immunity.

To investigate which genes, *apnI* and/or *apnT*, could confer resistance on the subtilomycin-sensitive host *B. subtilis* CU1065 (*B. subtilis* 168 background), we first predicted two putative promoters in the *apn* cluster, which are located on the upstream genes *apnA* and *apnT*. Different fragments that contained the whole cluster, *apnI-apnT*, *apnT*, or *apnI* were cloned into the integration vector pDG1730. The promoter region of *apnA* (pro_{apnA}) was

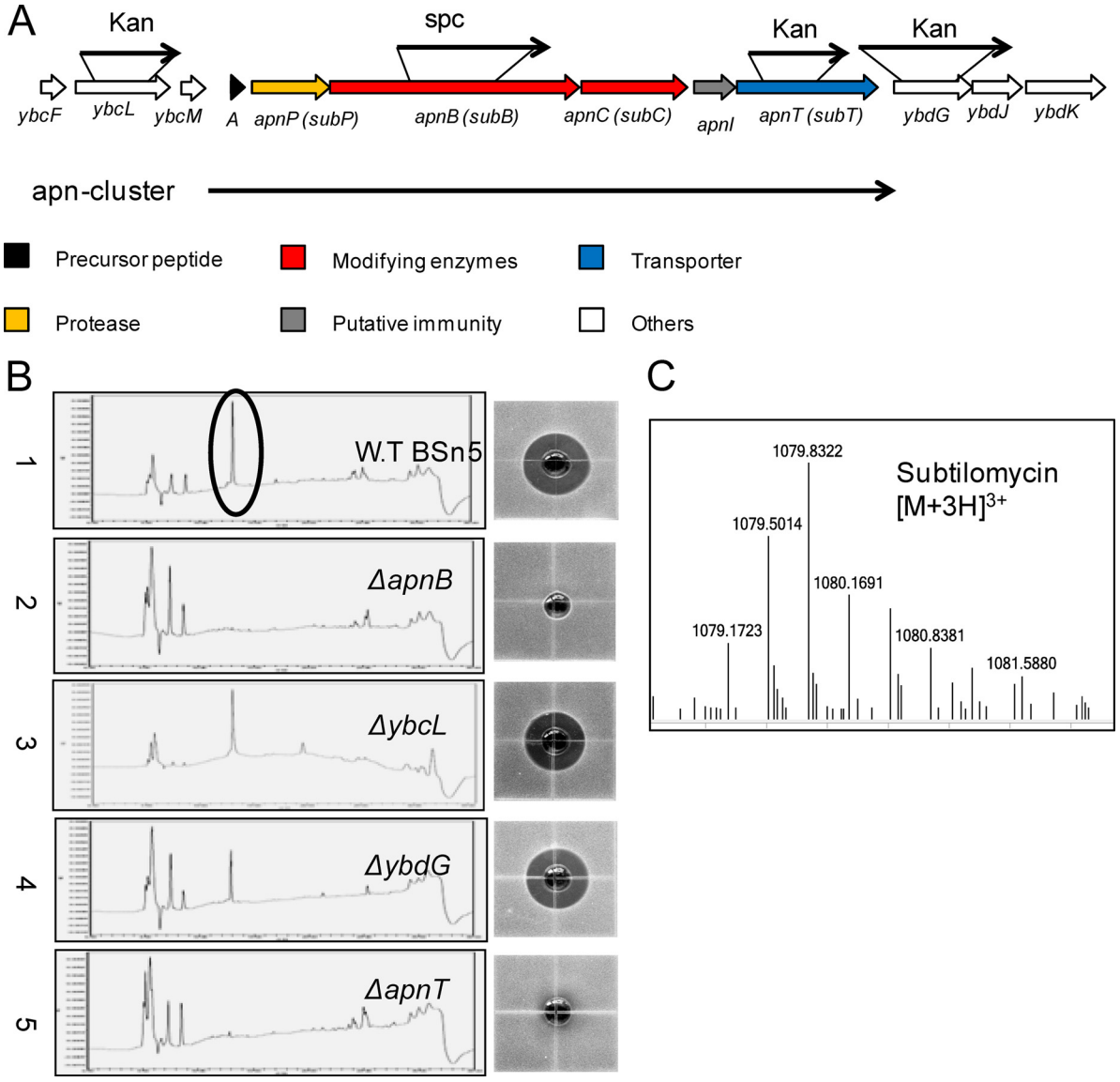

FIG 2 The subtilomycin gene cluster and inactivation analysis of related genes. (A) The *apn* cluster genes are shown in different colors: the precursor peptide encoded by *apnA* is shown in black, the genes encoding modifying enzymes are shown in red, the gene encoding protease is shown in yellow, the gene involved in putative immunity is shown in gray, and the gene encoding the transporter is shown in blue. The flanking genes are shown in white and are numbered. The black arrow indicates related genes interrupted by spectinomycin (Spc) or kanamycin (Kan) resistance genes. (B) Bioassay and high-performance liquid chromatography results for detection of subtilomycin from active ammonium sulfate crude extracts of wild-type strain BSn5 and its mutants. 1, wild-type strain BSn5; 2, Δ*apnB* mutant; 3, Δ*ybcL* mutant; 4, Δ*ybdG* mutant; 5, Δ*apnT* mutant. The black ring refers to the respective target peaks of subtilomycin in high-performance liquid chromatography. (C) ESI-MS detection of subtilomycin production in the 12-h supernatant of the recombinant harboring the *apn* cluster. The triply charged ion $[M + 3H]^{3+}$ is shown.

cloned in front of each of the constructed fragments *apnI-apnT*, *apnT*, and *apnI*, resulting in the vectors pro_{apnA}apnI-T, pro_{apnA}apnT, and pro_{apnA}apnI, respectively, to ensure the expression of each gene (Fig. 4A). The constructed vectors were transformed and integrated into the *amyE* locus of the *B. subtilis* CU1065 chromosome, resulting in corresponding recombinants.

Subtilomycin sensitivity tests were conducted to determine if the cloned fragments conferred resistance on different *B. subtilis* recombinants. The fragments containing *apnI* with pro_{apnA} resulted in the same CU1065 resistance level as that observed for fragments containing the whole cluster (Fig. 4B and C). The recombinants normally expressing *apnT* showed the same level of sensitivity to subtilomycin as wild-type CU1065 (Fig. 4B and C), which suggested that *apnT* does not confer subtilomycin resistance on the host. Our results indicated that the single gene *apnI* could confer protection against subtilomycin on the sensitive host strain *B. subtilis* CU1065.

To determine whether the *apnI* gene plays a role in immunity in a producer strain, subtilomycin sensitivity tests were conducted using wild-type BSn5 and its Δ*apnI* and Δ*apnT* mutants. The results showed that wild-type strain BSn5 and the Δ*apnT* mutant could tolerate high subtilomycin concentrations (up to approximately 0.3 mM), whereas the Δ*apnI* mutant was significantly sensitive to subtilomycin (Fig. 4D and E).

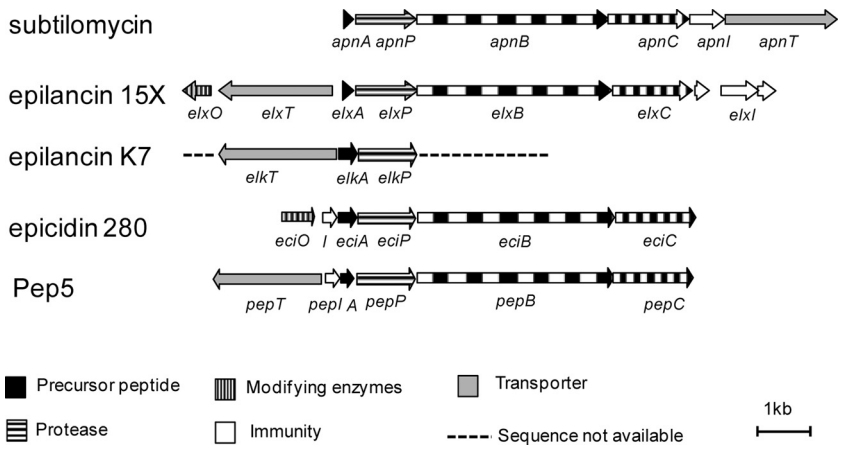

FIG 3 Comparison of the organization of subtilomycin gene clusters with biosynthetic gene clusters of Pep5 group lantibiotics, epilancin 15X (39), epilancin K7 (40), epicidin 280 (24), and Pep5 (41). Genes are labeled differently depending on the predicted function of their products: precursor peptides (black), proteases (horizontal stripes), modification enzymes (vertical stripes), immunity (white), and transporters (gray). The unavailable sequences in strain K7 are shown as a broken line.

Discovery of the ApnI-like proteins for lantibiotic immunity. As subtilomycin shares structural similarity with paenibacillin, we thus wondered what gene does play a role in paenibacillin immunity. Using the reported draft genome sequence (28), we found that the annotated genes of the putative paenibacillin biosynthetic gene cluster are present in the order *paeNAPBCIT*. We then compared the proposed paenibacillin gene cluster to the *apn* cluster (Fig. 5). Interestingly, a putative transmembrane protein-encoding gene, *paeI*, is present downstream of the conserved *lanAPBC* gene organization. Despite the low sequence identity (27%) between ApnI and PaeI, their similar topological structures and the same gene arrangement of the cluster as for the *apn* cluster suggested that PaeI was responsible for paenibacillin immunity.

Furthermore, we obtained more uncharacterized putative lantibiotic gene clusters from the *Bacillus cereus* group (including 5 *B. cereus* strains and 5 *Bacillus thuringiensis* strains) based on running protein BLAST using the sequences of ApnA and ApnI on the NCBI website. Comparison of these gene clusters with the *apn* cluster showed that they shared the same gene arrangement, *lanAPBC* followed by *lanI* and *lanT*. The LanI proteins from the *B. cereus* group showed low similarity (27 to 29%) to ApnI. The LanIs from these gene clusters were predicted to have six transmembrane domains, which had similar topology with ApnI. Accordingly, we named these LanI proteins as ApnI-like proteins and predicted that they were responsible for the immunity of the corresponding lantibiotics.

We predicted that the paenibacillin structural gene *paeA* would also exist in the genome sequence of *Paenibacillus* strain ATCC 842, which is the strain most closely related genetically to the paenibacillin producer strain *P. polymyxa* OSY-DF (28), and paenibacillin production was detected from the crude extracts of the 24-h-cultured supernatant of strain ATCC 842, according to a previously reported method (see Fig. S3 in the supplemental material) (14). We estimated that the purity of paenibacillin in the crude extracts was about 8% based on the relative abundance of the paenibacillin peak under the detection of UV 254 nm by LC-MS. To validate the function of *paeI*, which encodes an ApnI-like protein, we cloned the fragment containing the *paeI* and the downstream promoter regions of *apnA* (pro_{apnA}) and *paeA* (pro_{paeA}) into the integrated vector pDG1730, respectively, and transformed them into *B. subtilis* CU1065. A concentration gradient of crude paenibacillin was employed for the sensitivity test of the three strains. The results showed that the fragment containing *paeI* under the control of pro_{apnA} could confer certain resistance on CU1065 at the low concentration dilutions of paenibacillin (the 2nd and 3rd dilutions), compared with the other two strains (Fig. 6). At the increasing paenibacillin concentrations (the 5th and 6th dilutions), no significant difference could be observed among the three strains (Fig. 6A), which indicates that the paenibacillin resistance level conferred by PaeI was much lower than the ApnI-conferred subtilomycin resistance level in the background of *B. subtilis* CU1065.

Immunity provided by ApnI is specific toward subtilomycin. Since the sole putative transmembrane protein, ApnI, conferred significant resistance to the sensitive host CU1065, we next evaluated whether ApnI could confer cross-resistance against other bacteriocins. Toward this end, we selected the following peptide antibiotics based on their relationship with subtilomycin, listed from more distantly to closely related: vancomycin, nisin, and paenibacillin. We first carried out a sensitivity test with vancomycin and nisin. As shown in Fig. 7A, CU1065 with and without the integrated gene *apnI* (with the pro_{apnA}-*apnI* fragment) showed an inhibition zone of the same size with various vancomycin and nisin concentrations, suggesting that ApnI could not recognize vancomycin and nisin.

Since subtilomycin and paenibacillin showed high similarity (54%) and have the same five-thioether ring backbone (Fig. 2B), we next evaluated whether ApnI and PaeI could confer cross-resistance against paenibacillin and subtilomycin, respectively. The sensitivity tests showed that despite the fact that these antibiotics are structurally similar, no cross-resistance was observed between the pro_{apnA}-*apnI* and the pro_{apnA}-*paeI* recombinants (Fig. 7B). Together, these results imply that ApnI provides immunity that may involve a specific sequence recognition mechanism.

The recombinant *B. subtilis* expressing ApnI could bind subtilomycin. We characterized the function of ApnI by conducting a quantitative peptide-binding assay using 15 μg/ml and 25 μg/ml of subtilomycin (Fig. 8). As addition of NaCl to the assay solution could decrease the amount of cell-binding lantibiotics by reducing the lantibiotic affinity to the cytoplasmic membrane (21), to in-

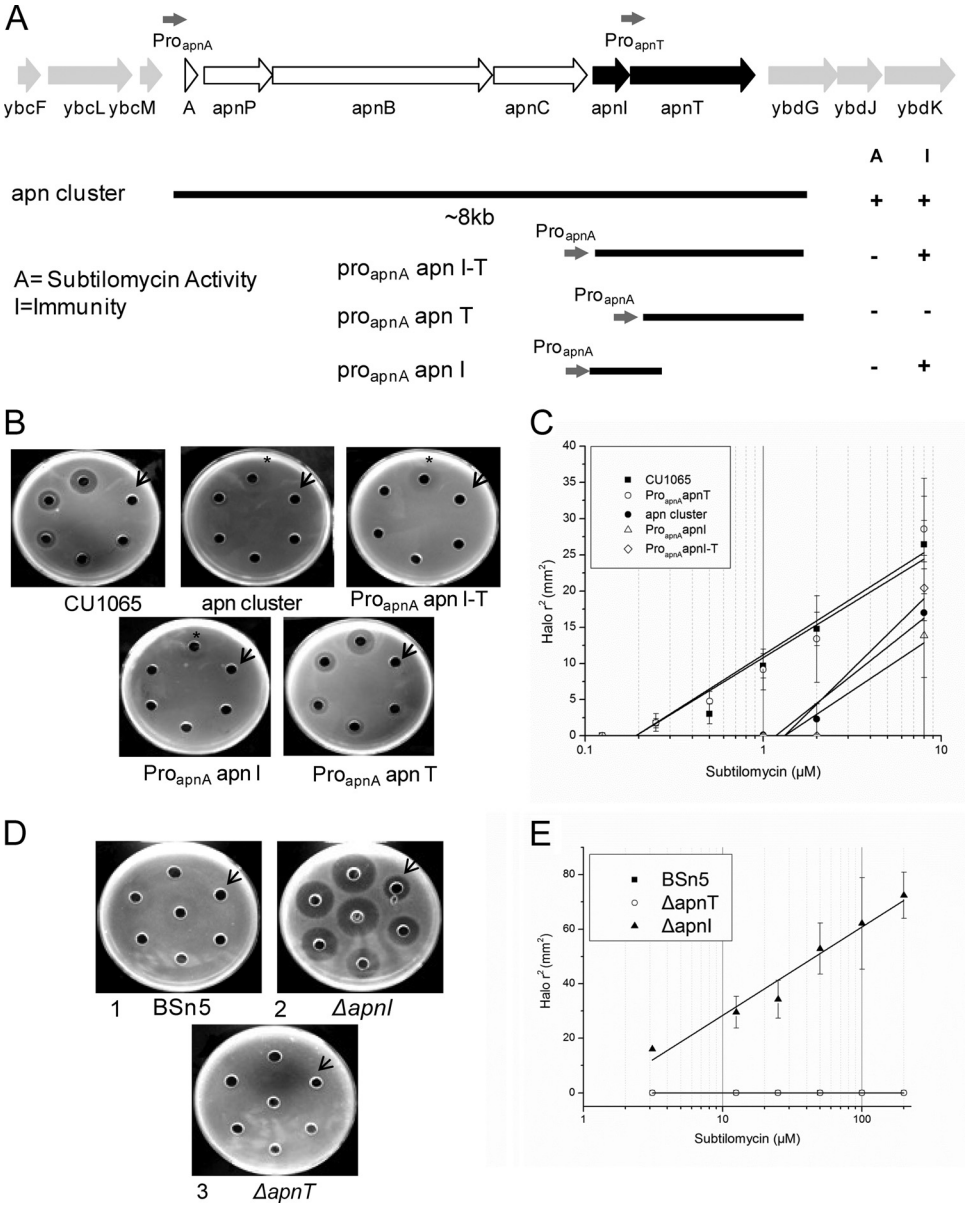

FIG 4 Functional analysis of subtilomycin immunity by gene expression in *B. subtilis* CU1065 and gene interruption in *B. subtilis* BSn5. (A) Map displaying essential DNA fragments for subtilomycin immunity. Each construction was cloned into the vector pDG1730 and integrated into the *amyE* locus of the chromosome of the subtilomycin-sensitive strain *B. subtilis* CU1065. The predicted fragments containing the *apnA* and *apnT* promoter regions are indicated by gray arrows. (B) Subtilomycin sensitivities of *B. subtilis* CU1065 strains harboring different fragments of the subtilomycin gene cluster were investigated in agar diffusion tests. *B. subtilis* CU1065 and its recombinants were transformed with the vector containing pro_{apnA}-apnI-T, pro_{apnA}-apnI, pro_{apnA}-apnT, or the whole subcluster. Starting from the arrow and moving clockwise, the subtilomycin concentrations applied were as follows: 0.125 μM, 0.25 μM, 0.5 μM, 1 μM, 2 μM, and 8 μM. The asterisk indicates a blurry inhibition zone. (C) Linear dependencies between the square of the radius of the halos shown in panel B and the natural logarithm of the applied subtilomycin concentration with 60 μl of subtilomycin and diffusion for at least 4 h. Results for *B. subtilis* CU1065 transformed with vectors containing *apnI*, *apnT*, *apnI-apnT*, and subgene clusters are shown. (D) Subtilomycin immunity of strain BSn5 (plate 1) with interruption of *apnI* (plate 2) and *apnT* (plate 3). Starting from the arrow and moving clockwise, the applied concentrations of subtilomycin were 3.125 μM, 12.5 μM, 25 μM, 50 μM, 100 μM, and 200 μM. The well in the middle of the plate was not counted. (E) Subtilomycin immunity of BSn5 with interruption of *apnT* and *apnI*. The immunity response was calculated corresponding to the square of the radius of the halos shown in panel D.

vestigate the target specificity of ApnI against subtilomycin, the assay was performed in the presence of 1 M NaCl. For *B. subtilis* CU1065 expressing *apnI*, ~7 μg of subtilomycin was found to be attached to the cells independently of the quantity of applied subtilomycin (15 or 25 μg/ml) (Fig. 8). Correspondingly, the amount of subtilomycin remaining in the supernatant of the *apnI*-expressing recombinant decreased significantly (Fig. 8). This result suggests that the *apnI*-expressing strain confers resistance by sequestering subtilomycin.

DISCUSSION

Although a link between subtilomycin and its gene cluster was proposed based on a match between the *N*-terminal region and a functional gene at the 5′ end, its biosynthetic gene cluster has not

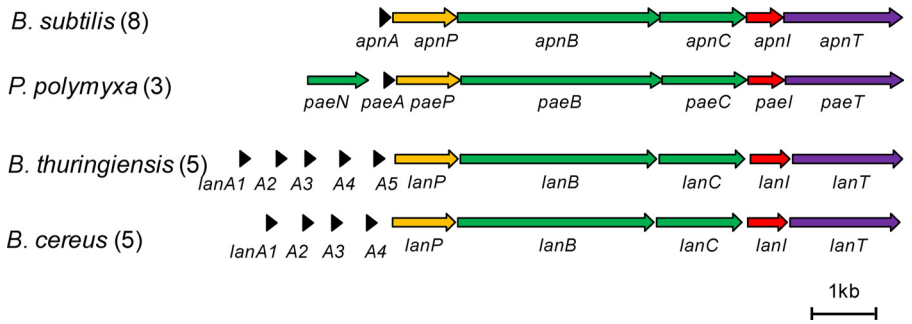

FIG 5 The potential ApnI-like immunity protein-encoding gene from the uncharacterized lantibiotic gene clusters. Genes are color-coded by the predicted function of their products: genes for precursor peptides (black), genes for proteases (yellow), genes for modifying enzymes (green), genes for transporters (purple), and genes for immunity (red). The bar represents 1 kbp. The number of independent isolates that have the potential cluster is shown. Details for these sequences are shown in Table S2 in the supplemental material.

yet been functionally validated. In this study, we experimentally identified the *apn* cluster responsible for subtilomycin biosynthesis and provided evidence that *apnI* does indeed confer subtilomycin immunity on its host. When we BLAST searched the NCBI database using the ApnI amino acid sequence, no functionally validated homolog could be found. We thus first determined the mechanism of self-resistance of a lantibiotic producer toward Pep5, which is homologous with subtilomycin. The self-protection against Pep5 is solely dependent on functional expression of the 69-amino-acid PepI, which was demonstrated through a series of cloning experiments (29, 30). Further functional analysis indicated that PepI is a lipoprotein with an apolar N-terminal segment for its localization at the membrane-cell wall interface and a hydrophilic C terminus, which confers immunity (11). However, the predicted *apnI* gene product is a membrane protein with five transmembrane domains, which is vastly different from PepI.

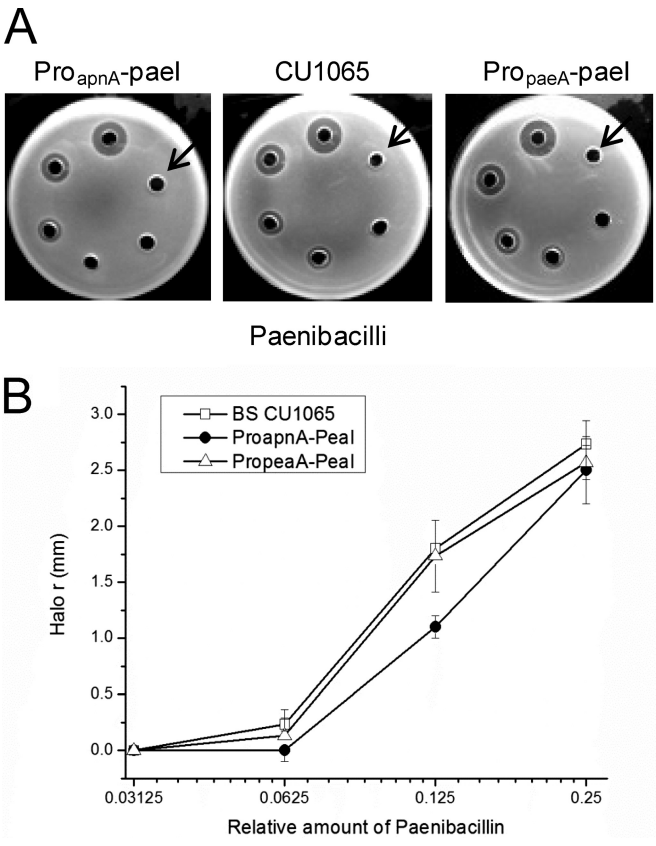

FIG 6 Functional analysis of the *paeI* gene in *B. subtilis* CU1065. (A) Paenibacillin sensitivity test results of *B. subtilis* CU1065 and the recombinants harboring fragments pro$_{apnA}$-paeI and pro$_{paeA}$-paeI. The concentrations of crude paenibacillin were applied with a series of 2-fold dilutions and were not quantified. The concentrations of paenibacillin increased starting from the arrow and moving clockwise. (B) The response as the length of the radii of the inhibition halos versus the corresponding relative amount of paenibacillin as shown in panel A. Two high concentrations of paenibacillin (the 5th and 6th dilutions) were removed. This experiment was independently performed at least 3 times, and the results were consistent.

Other known models for lantibiotic immunity usually involve the *lanFE*(*G*) genes, encoding an ABC transporter complex (Fig. 9). LanFE(G) can independently provide lantibiotic immunity, such as LctFEG for lacticin 481 immunity (31), or work synergistically with a membrane-associated protein, such as NisI (32), or a transmembrane protein, such as NukH (9, 10) and LtnI (33). Lantibiotic immunity that is solely provided by transmembrane proteins is very rare. We found only one report of such a protein, CylI, which is different from other transmembrane immunity proteins, as it contains a conserved zinc metalloprotease (MEROPS family M50A) domain, which suggests that it plays a role in immunity by cleaving one or both of the cytolysin subunits (34, 35).

Through bioinformatics analysis, many ApnI-like proteins in the uncharacterized lantibiotic gene clusters that are related to the *apn* cluster were found in the NCBI database (see Table S2 in the supplemental material). Although low identity (27 to 29%) is present between the sequences of ApnI and the LanI proteins, they all are predicted to have a topological structure similar to that of ApnI, with five or six transmembrane domains. The LanA structural peptide genes in these gene clusters were also shown to be closely related, with a sequence identity of 47% to 58% to ApnA (Fig. 5; see also Table S2). In total, 21 strains of *B. subtilis*, *Paenibacillus*, *B. cereus*, and *B. thuringiensis* have the putative lantibiotic biosynthetic gene cluster with ApnI-like proteins. We successfully introduced one such ApnI-like protein, PaeI, in *B. subtilis* CU1065 from *Paenibacillus* strain ATCC 842 and found that PaeI indeed confers resistance to paenibacillin. These results indicated that the novel model represented by ApnI for lantibiotic immunity is widely present among the *Bacillus* and *Paenibacillus* taxa. In addition, in the *B. subtilis* background, *paeI* could be expressed under

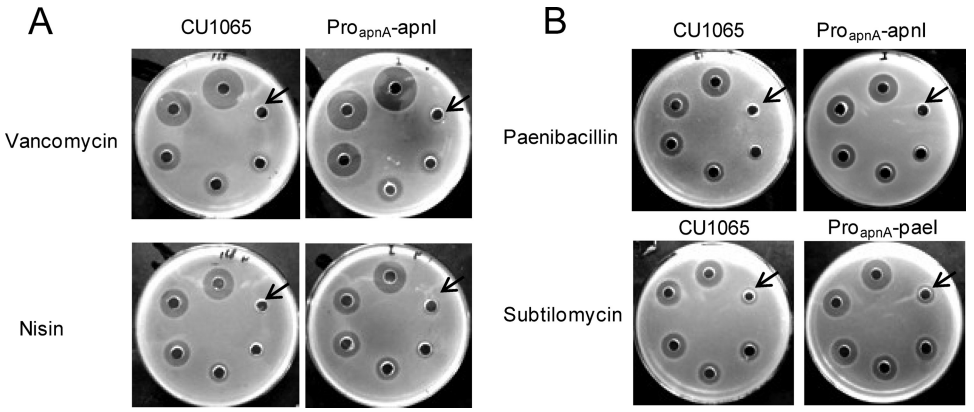

FIG 7 (A) Specificity analysis of the subtilomycin immunity gene *apnI* by sensitive test in CU1065. Gradient amounts of antibiotics were added from the arrow and moving clockwise. Vancomycin concentrations were 0.3375 nM, 0.675 nM, 1.35 nM, 2.7 nM, 5.4 nM, and 10.8 nM; nisin concentrations were 0.41 μM, 0.82 μM, 1.64 μM, 3.28 μM, 6.54 μM, and 13.08 μM. (B) Detection of cross-resistance to paenibacillin and subtilomycin in CU1065 conferred by genes *apnI* and *paeI*, respectively. The amounts of crude paenibacillin were applied with diluted series 2 times and not quantified. Gradient amounts of subtilomycin were added from the arrow and moving clockwise: 0.5 μM, 1 μM, 2 μM, 4 μM, 8 μM, and 16 μM. Each assay was independently repeated at least three times.

the control of the promoter of *apnA* derived from *B. subtilis*. However, it could not be expressed under the control of the promoter of *paeA* derived from *Paenibacillus*, indicating that expression of subtilomycin and paenibacillin might be involved in different regulation mechanisms. We found that *B. subtilis* became relatively resistant to paenibacillin when *paeI* was introduced into the sensitive strain CU1065. However, the paenibacillin resistance level afforded by PaeI was much lower than the ApnI-conferred subtilomycin resistance level (Fig. 4B and C and Fig. 6). In addition, we tested if *paeI*-expressing cells could bind paenibacillin in the peptide-binding assay. The result showed that no interaction between paenibacillin and the recombinant expressing *paeI* could be observed (data not shown). We inferred that the protein PaeI was not well expressed in CU1065 on the basis of its poor incorporation into the membrane of *B. subtilis*. On the other hand, other factors, like the protein PaeT, which was encoded by *paeT* immediately downstream of *paeI*, might be necessary for full immunity against paenibacillin.

Immunity genes often provide protection that is specific to corresponding lantibiotics and structurally related analogs. Conferred cross-protection against other bacteriocins is rare and has been found only among very closely related lantibiotics, such as Pep5 and epicidin 280 (24) as well as lacticin 3147 and C55 (36). The results of the sensitivity tests showed that PaeI and ApnI did not provide cross-protection against subtilomycin and paenibacillin, respectively, even though paenibacillin is structurally similar to subtilomycin (Fig. 2B). We inferred that the sequence similarity of 54% between paenibacillin and subtilomycin is not suitably high compared with that of lantibiotics that show cross-immunity, such as Pep5 and epicidin 280 (75% similarity) (24) and subunits LtnA1 and C55α (86% similarity) and LtnA2 and C55β (55% similarity), components of the lantibiotics lacticin 3147 and C55, respectively (36, 37). These results suggest that ApnI confers subtilomycin immunity through a specific sequence recognition mechanism. Using the peptide-binding assay (21), we detected the interaction of subtilomycin with the cells that expressed *apnI*, which suggested that ApnI proteins play a role in immunity by sequestering subtilomycin from its site of action to the cell membrane. Therefore, the fact that the transmembrane protein ApnI solely provided protection against subtilomycin indicates that this case represents a novel model for lantibiotic immunity (Fig. 9).

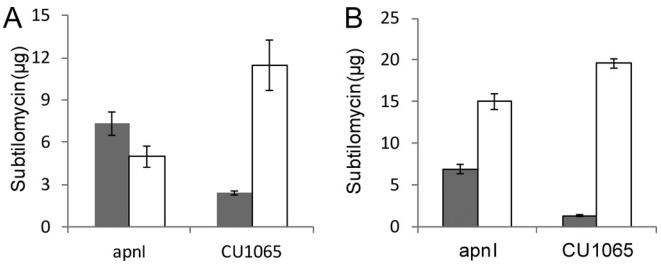

FIG 8 Quantitative peptide-binding assay for ApnI with subtilomycin. The cell suspensions (OD_{600} = 10) of stationary-phase-grown *B. subtilis* CU1065 and its recombinant expressing *apnI* were incubated with subtilomycin at concentrations of 15 μg/ml (A) and 25 μg/ml (B) in the presence of 1 M NaCl under the conditions described in Materials and Methods. The amounts of subtilomycin remaining in the supernatants (open bars) and the amounts remaining in the cell-associated samples (filled bars) were quantified by reverse-phase HPLC. Values represent means, with error bars representing standard deviations.

The lantibiotic subtilomycin is produced by *B. subtilis* strain MMA7, isolated from the marine sponge *Haliclona simulans*. The presence of the proposed subtilomycin gene cluster was detected from all sponge-derived *B. subtilis* strains by PCR screening (13). The proposed subtilomycin cluster was also determined to be present in *B. subtilis* strain BSn5, which was isolated from the plant *Amorphophallus konjac*, by mining the complete genome sequence (16). In the present study, we used high-resolution LC-MS analysis to confirm the production of subtilomycin in strain BSn5 and provided experimental evidence relating the production of subtilomycin to its biosynthetic gene cluster. The fact that the plant endophytic strain BSn5 can produce subtilomycin further suggests that the production of subtilomycin might be specific to *B. subtilis* strains originating from an animal or plant host. Therefore, it would be interesting to investigate subtilomycin production from more isolates from different hosts and nonhost environments. Subtilomycin may provide some benefits to the producer with respect to host colonization, which is in line with the concept

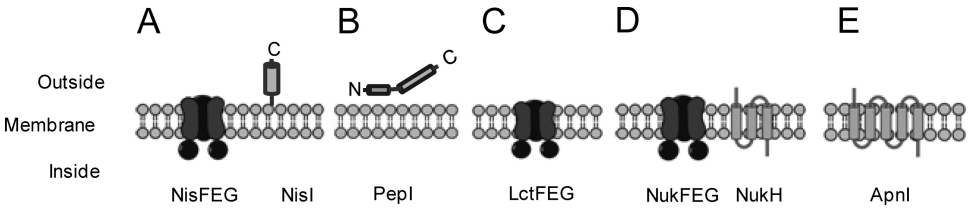

FIG 9 Models of lantibiotic immunity systems. A, NisFEG and NisI for nisin immunity; B, PepI for Pep5 immunity; C, LctFEG for lacticin 481 immunity; D, NukFEG and NukH for nukacin ISK-1 immunity; E, ApnI for subtilomycin immunity.

of bacteriocin production as a probiotic trait (38). Therefore, further investigations of the relationship between host colonization ability and subtilomycin production are merited.

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