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Restoration of Bioactive Lantibiotic Suicin from a Remnant *lan* Locus of Pathogenic *Streptococcus suis* Serotype 2

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Lantibiotics are ribosomally synthesized, posttranslationally modified antimicrobial peptides. Their biosynthesis genes are usually organized in gene clusters, which are mainly found in Gram-positive bacteria, including pathogenic streptococci. Three highly virulent *Streptococcus suis* serotype 2 strains (98HAH33, 05ZYH33, and SC84) have been shown to contain an 89K pathogenicity island. Here, on these islands, we unveiled and reannotated a putative lantibiotic locus designated *sui* which contains a virulence-associated two-component regulator, *suiK-suiR*. *In silico* analysis revealed that the putative lantibiotic modification gene *suiM* was interrupted by a 7.9-kb integron and that other biosynthesis-related genes contained various frameshift mutations. By reconstituting the intact *suiM* in *Escherichia coli* together with a semi-*in vitro* biosynthesis system, a putative lantibiotic named suicin was produced with bactericidal activities against a variety of Gram-positive strains, including pathogenic streptococci and vancomycin-resistant enterococci. Ring topology dissection indicated that the 34-amino-acid lantibiotic contained two methyllanthionine residues and one disulfide bridge, which render suicin in an N-terminal linear and C-terminal globular shape. To confirm the function of *suiK-suiR*, SuiR was overexpressed and purified. *In vitro* analysis showed that SuiR could specifically bind to the *suiA* gene promoter. Its coexpression with *suiK* could activate *suiA* gene promoter in *Lactococcus lactis* NZ9000. Conclusively, we obtained a novel lantibiotic suicin by restoring its production from the remnant *sui* locus and demonstrated that virulence-associated SuiK-SuiR regulates its production.

Lantibiotics, an abbreviated term for lanthionine-containing antibiotics, are gene-encoded antimicrobial peptides that are mainly produced by Gram-positive bacteria and are characterized for the presence of unusual amino acids such as lanthionine (Lan) and methyllanthionine (MeLan) in their sequences. Nisin, the prototype lantibiotic, has been widely used as a food preservative in the food industry without substantial occurrence of resistance (1). Lantibiotics are active at nanomolar concentrations against a wide range of Gram-positive bacteria, including some clinically important pathogens (2). Thus, they have been regarded as potential alternatives for conventional antibiotics, with promising applications in both the food and pharmaceutical industries (3).

The lantibiotic biosynthesis apparatus is encoded by gene clusters, which contain genes encoding precursor peptides, modification and processing enzymes, translocation proteins, regulatory components, and immunity proteins. Lantibiotics are initially synthesized as precursor peptides that consist of N-terminal leader peptides and C-terminal core peptides. Certain Ser/Thr residues in the core peptides are dehydrated and converted to dehydroalanine (Dha) and dehydrobutyrine (Dhb), which are subsequently conjugated with Cys via covalent bonds to form thioether bridges, namely, Lan and MeLan (4). Based on their structural features, lantibiotics are classified into type A and B groups, among which type A lantibiotics are elongated whereas type B lantibiotics are globular in structure (5). Type A lantibiotics are further divided into two subgroups. Type AI lantibiotics such as nisin, subtilin, and epidermin are elongated and amphipathic screw-shaped cationic peptides catalyzed by the cooperative actions of two distinct enzymes—dehydratase LanB and cyclase LanC (6). Type AII lantibiotics such as lacticin 481 and nukacin ISK-1 are N-terminal linear and C-terminal globular cationic peptides promoted by a bifunctional enzyme (LanM) that shows both dehydratase and cyclase activities (7). Mature lantibiotics are released following cleavage of the leader peptides by specific proteases. Leader peptides of type AII lantibiotics usually contain a double glycine (GG) motif that is processed by the LanT dedicated transporter protein (8). In typical cases, the production of lantibiotics is autoinduced via a two-component system (TCS) containing a LanK sensor histidine kinase and a LanR response regulator (4).

Lantibiotics are most frequently found to be produced by probiotic lactic acid bacteria for their traditional use in the food and diary industries (9). However, some of them have been lately discovered in various pathogenic micoorganisms (10). A typical instance is the two-component lantibiotic cytolysin that possesses both bactericidal and hemolytic activity, acting as an important virulence factor for *Enterococcus faecalis* (11). In addition, streptococci are prevalently known as producers of lantibiotics, many of which are pathogens. For example, *Streptococcus pyogenes* produces streptin and streptococcin A-FF22, *S. mutans* produces the three lantibiotics mutacin I, II, and III, and *S. uberis* produces the nisin-like lantibiotic nisin U (12). Although *S. pneumoniae* has not been found to produce any lantibiotics, a two-component lantibiotic from *S. pneumoniae* R6 has been synthesized by using nisin synthetic machinery (13). With increasing available genomic information, genome mining with LanM, LanBC, or BAGEL has unearthed a great deal of otherwise undefined lantbiotic loci in streptococci (14–16). The prevalence of lantibiotic gene clusters in pathogenic streptococci might improve their competiveness

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among related species and promote their colonization and infection of the human host, thus facilitating their pathogenesis (17).

As an important zoonotic pathogen, *S. suis* has 35 identified serotypes. *S. suis* serotype 2 (SS2), the most prevalent and virulent one, has been found in 20 countries and has caused several human infection outbreaks (18). Two recent large-scale outbreaks of human streptococcal toxic-shock-like syndrome (STSLS) caused by SS2 infections in China have already raised considerable international concern (19). The genomes of three STSLS-causing SS2 strains (98HAH33, 05ZYH33, and SC84) have been sequenced, and a unique 89-kb pathogenicity island (PAI), designated 89K, was located by comparative genomic analysis performed with other serotype 2 strains (20). On this 89K PAI, a two-component regulation system, *salK-salR*, was identified and shown to be crucial for the high virulence of SS2 because knockout of *salK-salR* eliminates its lethality, which could be restored by complementary expression of *salK-salR* (21). However, the mechanism underlying the function of SalK-SalR remains unknown.

Previous research indicated that SalK-SalR played a role in lantibiotic production, as it is operonically associated with the lantibiotic gene cluster (22). In the present study, we analyzed and characterized the genes neighboring *salK* and *salR* and reannotated a putative and novel lantibiotic gene cluster locating on 89K PAI in the aforementioned STSLS-causing SS2 strains. We recommended the gene cluster to be designated *sui*, in which a putative lantibiotic modification gene *suiM* was found to be interrupted by a 7.9-kb integron, suggesting its dysfunction in lantibiotic modification. Here, we recovered the function of intact *suiM* and biosynthesized a novel bioactive lantibiotic named suicin. Structure dissection elucidated that suicin was a type AII lantibiotic which showed an N-terminal linear and C-terminal globular structure. We also demonstrated that the virulence-associated SuiK-SuiR system was involved in regulating suicin biosynthesis, implying that role of SuiK-SuiR in SS2 virulence might be associated with its role in suicin production in SS2.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* DH5α and MC1061 were used as hosts for DNA cloning and BL21(DE3) for protein expression. The bacteria were incubated in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 1% sodium chloride) at 37°C, and 200 μg/ml erythromycin (Em) or 50 μg/ml kanamycin (Kan) was added if necessary. *Lactococcus lactis* NZ9000 was incubated in M17 medium (0.5% tryptone, 0.25% yeast extract, 0.5% soya peptone, 0.5% beef extract, 1.9% β-glycerophosphate disodium, 0.05% vitamin C, 0.025% magnesium sulfate) supplemented with 0.5% glucose (GM17), and 5 μg/ml Em was included if needed. *S. suis* HAbb was isolated from an STSS patient from the epidemic in Jiangsu (China), and its genomic DNA was provided by the Research Institute for Medicine of Nanjing Command in China. TCEP [tris(2-carboxyethyl) phosphine], DL-dithiothreitol (DTT), NEM (*N*-ethylmaleimide), and DiBAC4(3) [bis-(1,3-dibutylbarbituric acid) trimethine oxonol] were purchased from Sigma-Aldrich. Crude nisin Z was obtained from the Silver Elephant company (Zhejiang, China) and was applied to reverse-phase high-pressure liquid chromatography (RP-HPLC) using a C_{18} column to obtain pure nisin Z.

Indicator strains for antimicrobial assay were maintained in separate media as follows. Bacilli (*Bacillus cereus*, *B. thuringiensis*, *B. subtilis*, and *B. lentus*) were inoculated into LB medium at 37°C; *Micrococcus flavus* NCIB8166 was inoculated into S1 medium (0.8% tryptone, 0.5% yeast extract, 0.5% glucose, 0.2% disodium hydrogen phosphate, 0.5% sodium chloride, 0.1% Tween 20) at 30°C; streptococci (*S. gordonii*, *S. mutans*, and *S. bovis*) and enterococci (*E. pernyi*, *E. faecalis*, and *E. durans*) were cultured with BHI medium (1.75% brain heart infusion, 1% enzymatic digest of gelatin, 0.2% dextrose, 0.5% sodium chloride, 0.25% disodium phosphate) at 37°C; *Lactococcus lactis* were grown in GM17 medium at 30°C; lactobacilli (*Lactobacillus acidophilus*, *Lb. casei*, and *Lb. curvatus*) and *Leuconostoc dextranicum* were incubated into MRS medium (1% beef extract, 1% casein peptone, 0.5% yeast extract, 2% glucose, 0.5% sodium acetate, 0.2% ammonium citrate, 0.1% Tween 80, 0.01% magnesium sulfate, 0.005% manganese sulfate) at 30°C; and staphylococci (*Staphylococcus aureus* and *S. epidermidis*), *Shigella dysenteriae*, *Shigella flexneri*, and *Pseudomonas aeruginosa* were maintained with LB medium at 37°C. Solid media were prepared with 1.5% agar.

Plasmid construction and site-directed mutagenesis. Molecular cloning techniques were carried out according to standard protocols (23). PCR was performed using TransStart *FastPfu* DNA polymerase (Transgene, China). Plasmids, PCR products, or DNA fragments were prepared or purified by the use of Axygen kits according to the respective instructions.

suiAM$_1$ was amplified from genomic DNA of *S. suis* Habb with primers containing recognition sites of NdeI and BamHI and *suiM*$_2$ with primers containing recognition sites of BamHI and XhoI, respectively. First, *suiAM*$_1$ was ligated to pET28a to obtain pET-*suiAM*$_1$. *suiM*$_2$ was then introduced into pET-*suiAM*$_1$ with a BamHI site between *suiAM*$_1$ and *suiM*$_2$. After the BamHI site was eliminated by site-specific mutagenesis as described later, pET-*suiAM* was obtained with intact *suiM*. The 477-bp fragment of *suiT* encoding the 159-amino-acid (aa) N-terminal protease domain was amplified with primers containing recognition sites of NcoI and XhoI and then introduced into pET-28a to obtain plasmid pET-*suiT*$_{159}$. *suiR* was amplified with primers containing recognition sites of BamHI and XhoI and then introduced into pET28a to obtain pET-*suiR*. SuiT$_{159}$ was N-terminally and SuiR was C-terminally 6×His tagged. A *suiA* promoter (P_{suiA}) was amplified from genomic DNA of *S. suis* Habb and *gfp* from pGFP (24). P_{suiA} and *gfp* were fused by overlapping PCR and introduced into pMG36e to obtain pMG36e-*PsuiAgfp*. *suiKR* and *suiR* were then amplified and introduced into pMG36e-*PsuiAgfp* in opposite orientations and were under the control of constitutive promoter P32, thus obtaining pMG36e-*suiKRPsuiAgfp* and pMG36e-*suiRPsuiAgfp*.

Site-specific ligase-independent mutagenesis (SLIM) was performed via a PCR method with primers containing corresponding mutations as referred to the protocols described by Chiu et al. (25).

Protein expression and purification. *E. coli* BL21(DE3) transformed with expression vectors were incubated in 1 liter of LB broth at 37°C. At an optical density at 600 nm (OD_{600}) of 0.6 to 0.7, IPTG (isopropyl β-D-1-thiogalactopyranoside) was added to the cell culture to a final concentration of 0.5 mM, and the cells were then induced for another 20 h at 16°C. Induced cells were harvested by centrifugation at 5,000 × *g* for 20 min and resuspended in 30 ml binding buffer (50 mM Na_2HPO_4, 500 mM NaCl, pH 7.4) plus 20 mM imidazole. After cells were lysed by sonication, centrifugation was performed at 10,000 × *g* for 30 min to separate supernatant and inclusion bodies. The supernatant was applied to immobilized metal affinity chromatography (IMAC) of an Ni^{2+} column in a stepwise wash with binding buffer plus 40 mM imidazole, and, finally, the target protein was eluted with binding buffer plus 500 mM imidazole. The inclusion bodies were resuspended in binding buffer containing 20 mM imidazole and 8 M urea and sonicated to dissolve. Following centrifugation to remove the remaining insoluble precipitates, the dissolved protein in 8 M urea was purified through IMAC by washing with gradient dilution of urea concentration and finally eluting with binding buffer containing 2 M urea and 500 mM imidazole.

His$_6$-SuiA was mainly purified from inclusion bodies. The urea containing crude His$_6$-SuiA was subjected to a reverse-phase HPLC system with a C_4 semipreparative column (Dalian Elite, China) using a water-acetonitrile solvent system. The standard HPLC method consisted of a 15% to 30% acetonitrile gradient for 10 min followed by 30% to 65% acetonitrile gradient for 30 min at a flow rate of 1 ml/min. Peptides were detected by their absorbance at 220 and 280 nm. The purified peptides

were lyophilized and dissolved in double-distilled water (ddH_2O) for further experiments. $SuiT_{159}$-His_6 and His_6-SuiR were purified from the supernatant with the methods described above. The protein concentration was quantified by the use of a standard bicinchoninic acid (BCA) assay kit (Thermo Scientific). Purified proteins were identified by the use of 16.5% Tricine–SDS-PAGE (26).

***In vitro* reconstitution of $SuiT_{159}$-His_6.** Purified His_6-SuiA precursor peptides and their analogues were incubated with $SuiT_{159}$-His_6 under conditions of 50 mM Na_2HPO_4, 50 mM Na_2SO_4 (pH 7.4), and 1 mM DTT at 25°C for 3 h. $SuiT_{159}$-His_6 was then removed by heating at 60°C for 10 min and subsequent centrifugation at 12,000 × *g* for 10 min. Supernatant containing suicin was prepared for analysis by the use of a C_{18} ZipTip (Millipore, Germany) or purified by reverse-phase high-pressure liquid chromatography (RP-HPLC) using a C_{18} analysis column (Shimadzu, Japan). Purified suicin was lyophilized to dissolve in ddH_2O and identified by 16.5% Tricine–SDS-PAGE.

Peptide modification and MS analysis. Suicin and its mutants were subjected to treatment for chemical modification with NEM, which reacts with free thiols on Cys and results in a 125-Da increase per thiol. The NEM reaction was performed by incubation of purified sample with 1 mM TCEP and 10 mM NEM (pH 7.5) in an ice bath for 30 min. After the reaction, the samples were applied to a C_{18} ZipTip and detected by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) using a model 4700 Proteomics Analyzer mass spectrometer (Applied Biosystems). Mass spectra were obtained in the positive reflectron mode for peptides smaller than 5 kDa and linear mode for peptides larger than 5 kDa. A CHCA (α-cyano-4-hydroxycinnamic acid) matrix was prepared by dissolving 5 mg CHCA in 1 ml of 50:50 acetonitrile/water containing 0.1% trifluoroacetic acid (TFA).

Antimicrobial assay. Antimicrobial spectra of suicin were determined by an agar diffusion assay using the indicator strains shown (see Table 4) as described previously (27). Briefly, 25 μl of 10 μg/ml suicin was applied to 5-mm-diameter wells lawned with the indicator strain. Indicator strains were inoculated under appropriate conditions, and diameters of inhibition zone were determined by the use of a caliper. The MIC was determined for the sensitive strain *M. flavus* NCIB 8166 by a microdilution method using a 96-well plate as described by Levengood et al. (28).

Determination of membrane potential. Membrane potential disruption was monitored by DiBAC4(3), which is a sensitive slow-response membrane potential probe that measures potential-dependent changes by accompanied fluorescence changes (29). *M. flavus* NCIB8166 was grown in S1 medium at 30°C to an OD_{600} of ca. 0.6 to 0.8 followed by centrifugation (4°C and 5,000 × *g* for 5 min) to collect viable bacterial cells. Cells were then washed and resuspended in 5 mM HEPES buffer (5 mM HEPES, 0.4% glucose, 2.5 mM $MgSO_4$, pH 7.8) to an OD_{600} of ca. 0.05 to 0.1. A 120-μl volume of the cell suspension was added to the wells of a 96-well microplate. DiBAC4(3) was added to achieve a final concentration of 1 μM and incubated at room temperature for 4 min before suicin or nisin Z was added. Then, 30 μl of suicin (25× or 50× MIC) was mixed in and the mixture was incubated for another 10 min. Nisin Z (50× MIC) was used as a positive control. Fluorescence was monitored by the use of a microplate reader (BioTek) at excitation and emission wavelengths of 493 and 516 nm, respectively.

EMSA. An electrophoretic mobility shift assay (EMSA) was performed to determine if SuiR could bind to the promoter of *suiA*. A 293-bp promoter region of *suiA* (P_{suiA}) was amplified with primers, and the 5′ primer was labeled with biotin. Biotin-P_{suiA} was purified from 1% agarose gel. Then, increasing concentrations of purified His_6-SuiR ranging from 0 to 200 μg/ml were incubated with biotin-P_{suiA} at 20°C for 20 min. For a competition assay, a mixture that included a 100-fold excess of unlabeled P_{suiA} (U-P_{suiA}) was used. After the incubation, samples were applied to 4.5% nondenaturing polyacrylamide gel with 80 V for 1 to 1.5 h. Biotin-P_{suiA} was transferred to a nylon membrane, and streptavidin-horseradish peroxidase (HRP) conjugates were used to specifically bind biotin. Followed by X-ray film exposure, retarded biotin-labeled probes were visualized.

Activation of GFP by SuiK-SuiR. The assay was modified from an assay described previously (24). Briefly, pMG36e-derived plasmids containing *suiKR* or their mutants were transformed into *L. lactis* NZ9000 by the use of an electroporation method as described by Holo and Nes (30). Transformants were incubated in GM17 at 30°C to an OD_{600} of ca. 0.4 to 0.5, and then suicin was added in concentrations ranging from 10 to 400 ng/ml for induction if needed. After induction was performed for 4 h, viable cells were collected to determine green fluorescent protein (GFP) fluorescence with a Zeiss Axio Imager A1 microscope.

RESULTS

Identification of genes that might be involved in biosynthesis of a putative lantibiotic suicin. In three epidemic-causing and genome-sequenced *S. suis* serotype 2 (SS2) strains, 98HAH33 (accession number CP000408), 05ZYH33 (accession number CP000407), and SC84 (accession number FM252031), *salK* and *salR*, members of a two-component system which encode a histidine kinase and a response regulator, respectively, were identified on a unique 89K PAI. In *S. suis* SC84, SalK (YP_003024863) and SalR (YP_003024862) were named after SalK-SalR from *S. salivarius* because of their 26.0% and 41.0% identity with their counterparts from *S. salivarius*, respectively (31). Further analysis indicated that the SalK-SalR exhibited 34.3% and 55.3% identity with BovK-BovR from *S. bovis*, respectively. Like BovK-BovR, SalK in *S. suis* was presumed to be an eight-transmembrane kinase and SalR was a cytoplasmic regulator composed of an N-terminal signal receiver domain and a C-terminal HTH (helix-turn-helix) DNA binding domain. Both BovK-BovR of *S. bovis* and SalK-SalR of *S. salivarius* were demonstrated to be responsible for autoregulation of lantibiotic biosynthesis (24, 31). Thus, we speculated that SalK-SalR in *S. suis* serotype 2 might be involved in lantibiotic production.

Based on the sequence analysis of the genes neighboring *salK* and *salR*, one putative *lan*-resembling locus containing 9 genes, which was designated *sui*, was revealed in the aforementioned SS2 strains (Fig. 1A). *salK-salR* was therefore renamed *suiK-suiR* here. The other 7 open reading frames (ORFs) were designated *suiA*, *suiM*, *suiT*, *suiF*, *suiE*, *suiG*, and *suiI*, respectively. Therefore, the potential lantibiotic was termed suicin. In these three *sui* loci, the putative *suiA* precursor gene and *suiI* immunity gene were omitted from annotation in 98HAH33 and 05ZYH33 whereas only *suiI* was omitted from SC84 in the NCBI database due to the small size or nonconserved nature of those three genes. Several interruptions or mutations existed in each gene cluster. The *suiM* gene was found to be disrupted by a 7.9-kb integron that most probably originated from *E. faecalis* (22), while another nucleotide deletion led to a frameshift of *suiM* in *S. suis* 98HAH33. In addition, there was one nucleotide deletion in *suiG* of *S. suis* 98HAH33 and one each in *suiE*, *suiG*, and *suiK* of 05ZYH33, leading to frameshifts of these genes. The disruption of *suiK* in strain 05ZYH33 had led to the absence of three N-terminal transmembrane domains. The deduced *sui* locus displayed organizational similarity to the bovicin HJ50 gene cluster (Fig. 1B), suggesting its primary function of suicin biosynthesis. The bovicin HJ50 gene cluster was resequenced and reannotated here on the basis of the finding that *bovT* was actually one gene instead of two separate genes (*bovT* and *bovE*) as first annotated (32). In addition, *orf1* and *orf2* were reannotated as *bovE* and *bovG*, and *bovI*, located downstream of *bovR*, was unveiled.

The suicin gene cluster in *S. suis* SC84 was comparatively complete and was assayed by alignment with bovicin HJ50 gene cluster (Table 1). *suiA* encoded a 57-aa precursor peptide, SuiA,

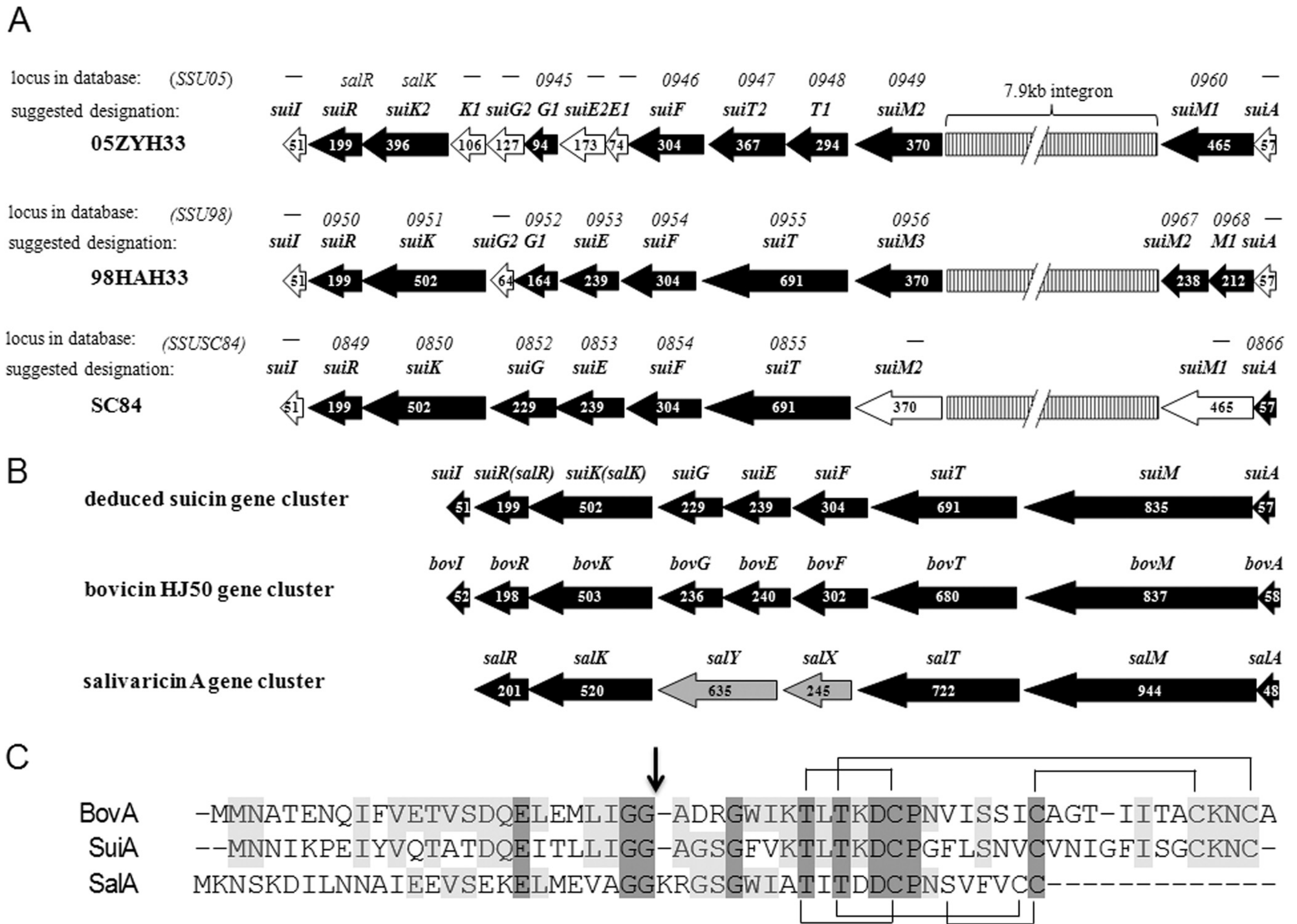

FIG 1 Schematic representation of *sui* loci and comparison of SuiA with BovA and SalA. (A) Interrupted *sui* loci in three virulent *S. suis* serotype 2 strains—*S. suis* 05ZYH33, 98HAH12, and SC84. Black horizontal arrows indicate genes annotated in the NCBI database, while white arrows represent omitted but reannotated genes. Numbers in the arrows indicate the numbers of amino acids contained in the proteins. Above each locus, the upper designations represent gene loci in each genomes and the lower designations represent recommended gene redesignations. (B) Comparison of deduced complete suicin gene cluster with bovicin HJ50 and salivaricin gene clusters. (C) Comparison of precursor peptide of SuiA with that of BovA and SalA. The vertical arrow indicates the GG cleavage site, and cross-linkages indicate the bridge patterns.

consisting of a 24-aa leader peptide and 33-aa core peptide. SuiA exhibited characteristics of type AII lantibiotics, including a conserved Glu-8 and a double-glycine motif (GG) in the leader peptide and conserved dehydratable Thr residues and ring-forming Cys residues in the core peptide (Fig. 1C). SuiA and BovA shared 49.2% identity, and their core peptides shared 50.0% identity. Despite being disrupted, the putative complete SuiM shared 40.0% identity with the bovicin HJ50 modification enzyme BovM. SuiT was an ABC transporter protein with an N-terminal C39 cysteine protease domain that was postulated to be dedicated to lantibiotic leader peptide processing. It exhibited 41.4% identity with BovT. Downstream of *suiT*, there were three genes, *suiFEG*, encoding components of ABC transporters that shared 61.5%, 32.5%, and 38.9% identity with BovFEG, respectively, indicating their roles in immunity. Situated downstream of *suiK*-*suiR*, *suiI* encoded a 51-aa protein that showed 38.5% identity with BovI or TepI, which acted as a self-immunity protein from *S. thermophilus* SBT 1277 with respect to the lantibiotic thermophilin 1277, which is identical to bovicin HJ50 in terms of primary sequence as well as structure (33, 34). Thus, SuiI was speculated to exert an immunity function.

TABLE 1 Reannotation and suggested designation of genes in *sui* locus of *S. suis* SC84

Locus tag	Redesignation	Length(s) (aa)	Best hit (% identity/ % similarity)	Functionality
SSUSC84-0866	SuiA	57	BovA (49.2/66.1)	Precursor suicin
–[b]	$SuiM_1$-$SuiM_2$	465/370	BovM (40.0/53.4)[a]	Modification
SSUSC84-0855	SuiT	691	BovT(41.4/59.5)	Export
SSUSC84-0854	SuiF	304	BovF(61.5/71.7)	Immunity
SSUSC84-0853	SuiE	239	BovE (32.5/56.7)	Immunity
SSUSC84-0852	SuiG	229	BovG (38.9/56.9)	Immunity
salK	SuiK	502	BovK (34.3/52.7)	Histidine kinase
salR	SuiR	199	BovR (55.3/70.4)	Response regulator
–	SuiI	51	BovI (38.5/50.0)	Immunity

[a] The putative intact SuiM was used for alignment with BovM.
[b] –, no annotation in the genome of *S. suis* SC84.

We amplified the *sui* locus from the genomic DNA of SS2 strain *S. suis* Habb, which was isolated from an STSS patient in Jiangsu, China, and had virulence similar to that of sequenced SS2

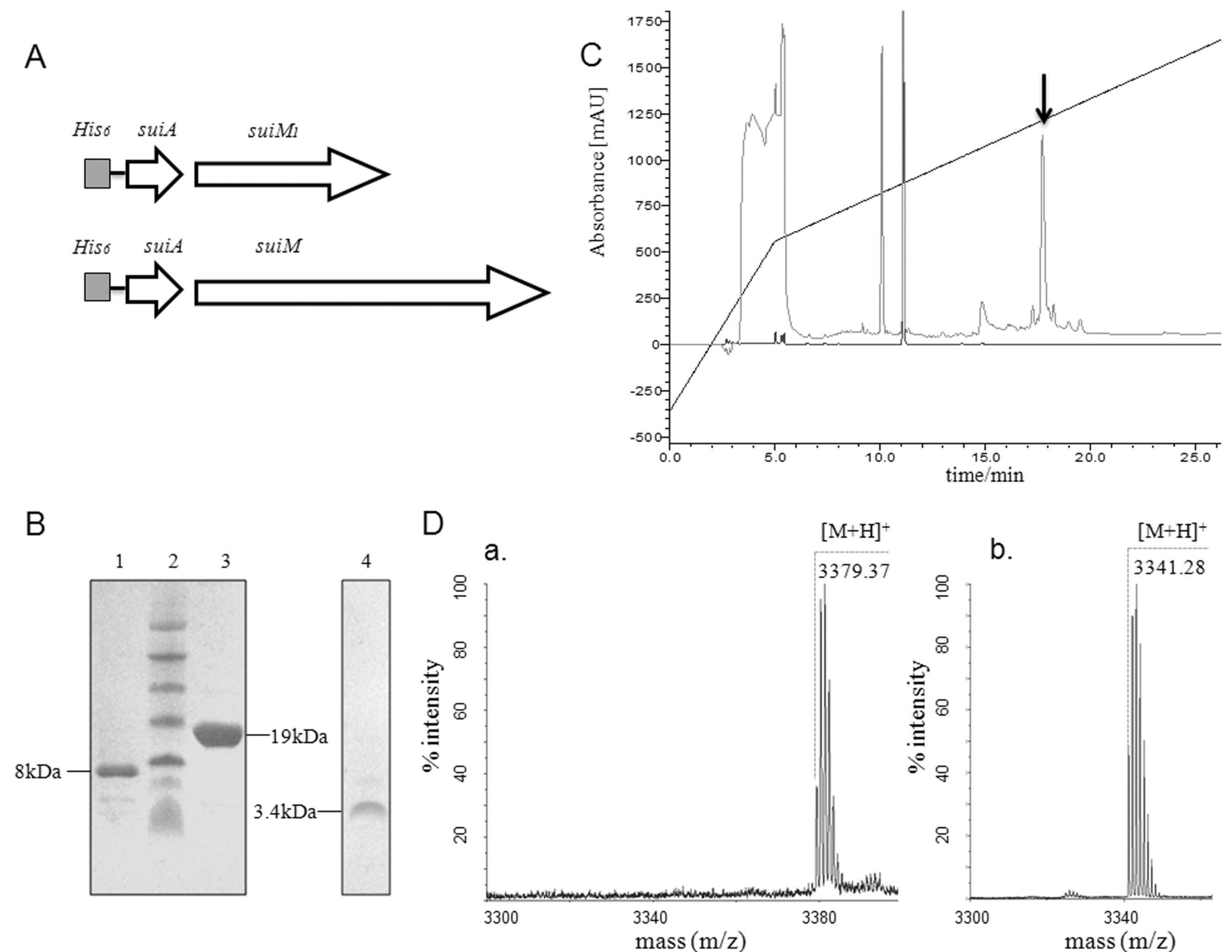

FIG 2 *In vitro* biosynthesis of suicin. (A) Schematic representation of coexpression combinations of precursor genes and modification genes. (B) Tricine-SDS-PAGE analysis: lane 1, His_6-mSuiA; lane 2, protein marker; lane 3, $SuiT_{159}$-His_6; lane 4, suicin. (C) C_{18} RP-HPLC purification of suicin. The black arrow indicates the peak of suicin. mAU, milli-absorbance units. (D) MALDI-TOF MS analysis of unmodified SuiA core peptide (a) and suicin (b).

strains (20). The *sui* locus was sequenced and turned out to be identical to that in *S. suis* SC84.

Biosynthesis of functional suicin in *E. coli*. To obtain suicin, a semi-*in vitro* biosynthesis (SIVB) strategy was introduced (35), consisting of an *in vivo* modification via coexpression of *suiA* with *suiM* and an *in vitro* digestion via the peptidase domain of SuiT.

The two separate parts of $suiM_1$ and $suiM_2$ were linked to reconstitute the function of SuiM (Fig. 2A). Whether modified or not, His_6-SuiA was mostly expressed in inclusion bodies (data not shown) and was subsequently purified by IMAC and C_4 RP-HPLC (Fig. 2B). Mass spectrometry analysis revealed that the molecular mass of protonated His_6-SuiA was 8,149.7 Da when SuiA was coexpressed with complete SuiM, resulting in a deviation of 39.5 Da from the theoretical mass of 8,189.2 Da. This indicated that SuiM modified SuiA and that two dehydrations had happened (Table 2). However, the mass of His_6-SuiA coexpressed with only $SuiM_1$ differed by 3.4 Da from the calculated mass, indicating that no dehydration had happened. The 3.4-Da difference might have been due to inaccurate detection of the linear mode in MS analysis of masses higher than 5 kDa or to spontaneous formation of disulfide bridges. The results presented above demonstrated that reconstituted complete SuiM, other than $SuiM_1$, can fulfill its original function *in vivo* to modify the SuiA substrate.

Incubation of modified precursor peptide His_6-mSuiA with C39 peptidase domain $SuiT_{159}$-His_6 released the mature lantibiotic suicin. Suicin was purified through C_{18} RP-HPLC with a retention time of 17.8 min (Fig. 2C). MS analysis of suicin showed an $[M+H]^+$ of 3,341.28 Da (Fig. 2Db), which was a 38.35-Da decrease from the theoretical mass of core peptide of SuiA of

TABLE 2 Modification of SuiA by $SuiM_1$ and SuiM

Precursor	LanM	Calculated molecular mass (Da)	Molecular mass by MS (Da)	Molecular mass Δ (Da)	No. of dehydrations
His_6-SuiA	$SuiM_1$	8,189.2	8,185.8	3.4	0
His_6-SuiA	SuiM	8,189.2	8,149.7	39.5	2

TABLE 3 NEM modification of suicin and its mutants

Suicin designation	Calculated molecular mass (Da)	Molecular mass by MS	PTM[a]	NEM addition (Da)	Molecular mass Δ (Da)[b]	No. of NEM
WT	3,379.63	3,341.28	$2H_2O$ + S-S	3,593.55	252.27	2
T8A/T10A	3,319.61	3,319.44	–[c]	3,819.87	500.43	4
C13A	3,347.66	3,309.47	$2H_2O$	3,561.55	250.08	2
C21A	3,347.66	3,311.52	$2H_2O$	3,436.58	125.06	1
C30A	3,347.66	3,311.53	$2H_2O$	3,436.60	125.07	1
C33A	3,347.66	3,309.52	$2H_2O$	3,561.64	250.12	2
T10A/C33A	3,317.65	3,299.64	$1H_2O$	3,549.69	250.05	2

[a] PTM, posttranslational modification.
[b] Data represent the mass difference after NEM addition.
[c] –, no modification.

3,379.63 Da. This indicated that the cleavage site was right after a pair of glycine residues (Gly-2 and Gly-1), and the 38.35-Da decrease was thought to correspond to two dehydrations and one disulfide bridge formation. The His_6-SuiA coexpressed with $SuiM_1$ was also digested by $SuiT_{159}$-His_6, and the product exhibited an $[M+H]^+$ of 3,379.37 Da (Fig. 2Da), which was in good agreement with theoretical mass of the core peptide of SuiA. This further indicated that His_6-SuiA was unmodified by $SuiM_1$.

Ring topology elucidation of suicin. To dissect the ring topology of suicin, a series of suicin mutants were generated and subjected to NEM treatment and MS analysis (Table 3). The absence of dehydration in T8A/T10A demonstrated that the two dehydrations actually happened at Thr8 and Thr10. To dissect the bridge pattern, each Cys was mutated to Ala and the mutants were subjected to NEM treatment for conjugating free thiols. MS analysis showed that no Cys mutation had an influence on dehydration. However, C13A and C33A mutants had two NEM adducts, while C21A and C30A mutants had one. This indicated that Cys13 and Cys33 were involved in thioether ring formation, while Cys21 and Cys30 formed a disulfide bridge. This also indicated that mutant C13A has no influence on the thioether formation of Cys33 and vice versa. Furthermore, addition of two NEM molecules to mutant T10A/C33A was presumed to be attributable to Cys21 and Cys30. This indicated that the thioether bridges were formed by Thr10 and Cys33 as well as Thr8 and Cys13. Thus, the bridge pattern of suicin was dissected and found to contain two thioether bridges (rings A and B) and one disulfide bridge (ring C). Therefore, suicin is similar to bovicin HJ50 in structure (Fig. 3).

Suicin displayed antimicrobial activities against various Gram-positive pathogens. Suicin was tested against the series of Gram-positive and -negative strains listed in Table 4. Suicin exhibited inhibitory activity against some Gram-positive strains from bacilli, micrococci, lactobacilli, lactococci, streptococci, leuconostoc, and enterococci. In particular, suicin showed inhibition of *E. pernyi*, which is the causative agent of empty-gut disease of tussah, and of vancomycin-resistant *E. faecalis* V583, which has been clinically associated with urinary tract infection, bacteremia, and infective endocarditis (36). As expected, suicin exhibited no activity against Gram-negative strains. *M. flavus* NCIB8166 was the most sensitive strain and was used as an indicator for MIC determinations. Results showed that suicin inhibited growth of *M. flavus* NCIB8166 at concentrations as low as 0.195 μg/ml, which is its MIC for *M. flavus* NCIB8166. As a positive control, nisin Z exhibited better potency, with a MIC of 0.0325 μg/ml.

To elucidate whether thioether ring or disulfide bridge formation is required for the antimicrobial activity of suicin, a series of

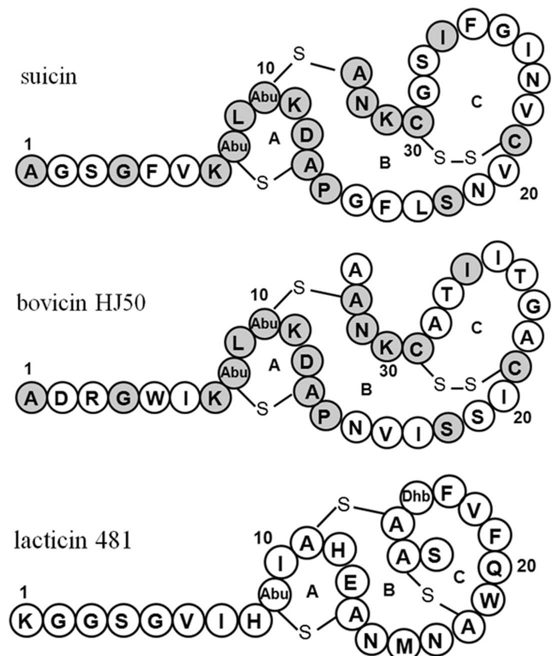

FIG 3 Structural comparison of suicin, bovicin HJ50, and lacticin 481. Dark gray circles indicate identical residues in suicin and bovicin HJ50.

Cys mutants of suicin were purified and tested for inhibitory activity against *M. flavus* NCIB8166. As shown in Fig. 4A, while bovicin HJ50 and wild-type suicin markedly inhibited the growth of *M. flavus* NCIB8166, disruption of any thioether ring and/or disulfide bridge in suicin totally abolished its bactericidal activity

TABLE 4 Antimicrobial spectrum of suicin

Indicator strain	No. of strains tested	Zone of inhibition (mm)[a]
Bacillus cereus	3	–
Bacillus brevis AS1.1165	1	7
Bacillus subtilis 168	1	8
Bacillus thuringiensis 1.1014	1	–
Enterococcus faecalis V583	1	8
Enterococcus durans 1.2023	1	6
Enterococcus pernyi 1.1010	1	8
Lactobacillus acidophilus 100-33	1	6
Lactobacillus curvatus LTH1174	1	9
Lactobacillus casei	3	–
Lactobacillus delbrueckii 8909	1	–
Lactococcus lactis MG1363	1	7
Leuconostoc dextranicum 181	1	11
Micrococcus flavus NCIB8166	1	23
Staphylococcus aureus 26122	1	–
Staphylococcus epidermidis 1.1229	1	–
Streptococcus bovis	2	–
Streptococcus mutans 1.2499	1	–
Streptococcus gordonii 1.2496	1	6
Escherichia coli DH5α	1	–
Pseudomonas aeruginosa	1	–
Shigella dysenteriae	1	–
Shigella flexneri	1	–

[a] Suicin (25 μl of 10 μg/ml) was applied to each indicator strain. The values stand for the diameter of the inhibition zone. –, no inhibition zone.

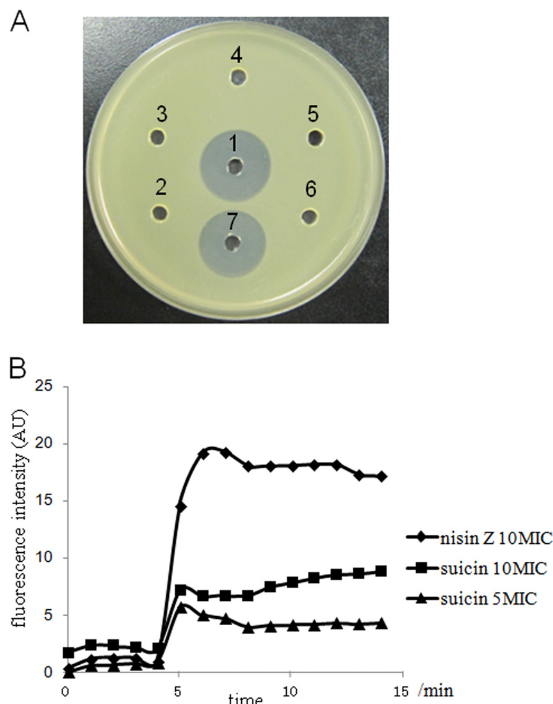

FIG 4 Growth inhibition and membrane potential disruption of *Micrococcus flavus* NCIB8166 by suicin. (A) Antimicrobial activity of suicin and its derivatives against *M. flavus* NCIB8166, with bovicin HJ50 as a positive control. To each hole, 25 μl of 5 μg/ml of each peptide was applied. 1, suicin; 2, unmodified core peptide of SuiA; 3, suicin C13A; 4, suicin C21A; 5, suicin C29A; 6, suicin C33A; 7, bovicin HJ50. (B) Effect of suicin and nisin on membrane potential of *M. flavus* NCIB8166. *M. flavus* NCIB8166 cells were treated with suicin or nisin Z with a time duration of 15 min, and fluorescence was recorded with excitation at 493 nm and emission at 516 nm. Diamond, nisin Z at 10-fold MIC (0.325 μg/ml); square, suicin at 10-fold MIC (1.95 μg/ml); triangle, suicin at 5-fold MIC (0.975 μg/ml).

against *M. flavus* NCIB8166, which was the most sensitive strain among all tested strains (Table 4). Therefore, both thioether rings and the disulfide bridge are essential for the function of suicin.

Suicin could disrupt the membrane potential of sensitive cells. It has been well established that lantibiotics, especially type A lantibiotics, primarily target and form pores in the cytoplasmic membrane of sensitive cells, thus resulting in disruption of the membrane potential and release of the intracellular components (4, 37). To determine whether suicin acts through disintegrating the membrane of sensitive cells, we monitored membrane potential changes of *M. flavus* NCIB8166 after application of suicin at 5- or 10-fold MIC. In the assay, we used a voltage-sensitive fluorescence dye, DiBAC4(3), to measure the membrane potentials. When the membrane potential is disrupted as a result of pore formation in the membrane, DiBAC4(3) enters the cell membrane and its fluorescence is enhanced (38). As shown in Fig. 4B, addition of suicin to the *M. flavus* NCIB8166 cells caused a robust increase of fluorescence in a concentration-dependent manner, indicating that the membrane potential of indicator cells was disrupted by suicin. This increase was also observed upon application of nisin (Fig. 4B), which is known to disrupt membrane potential with high potency (4). That the efficiency of suicin is lower than that of nisin in inducing fluorescence fluctuation might be due to the fact that the potency of suicin is lower than that of nisin in disrupting membrane potential. Therefore, forming pores in the membrane might be one important, if not the only, mode of action for suicin in inhibition of sensitive cell growth.

Virulence-associated SuiR could bind the *suiA* promoter. As a TCS in the deduced *sui* locus, SuiK-SuiR was supposed to be responsible for autoregulation of suicin biosynthesis, though suicin production might be abolished by disruptions of the suicin gene cluster. To confirm that supposition, EMSA was conducted to identify the binding ability of His_6-SuiR toward *suiA* promoter P_{suiA}. His_6-SuiR was purified to homogeneity and identified by Tricine-SDS-PAGE analysis (Fig. 5B). His_6-SuiR was then incubated with biotin-P_{suiA} and a 100-fold excess of unlabeled P_{suiA} (U-P_{suiA}). The transferability-retarded bands of biotin-P_{suiA}, which disappeared in the presence of excess unlabeled P_{suiA}, clearly indicated that His_6-SuiR could specifically bind P_{suiA} (Fig. 5C).

Sequence analysis of P_{suiA} indicated there were three inverted repeats (IR) located upstream of *suiA* (Fig. 5A). IR1 was located between a ribosome binding site (RBS) and a −10 box, and IR2 was located before a −35 box. IR3 was presumed to be the terminator of gene *SSUSC84_0868*. To determine whether IR1 or IR2 was the binding site of SuiR, EMSA analysis was conducted using the IR-deleted version of P_{suiA} (ΔIR). The mutations of ΔIR1 and ΔIR2 were performed via SLIM on the pMG36e-*suiKRPsuiAgfp* plasmid described later, and the corresponding probes were subsequently amplified with primers as used for biotin-P_{suiA}. The results showed that His_6-SuiR could bind P_{suiA} ΔIR1 but not P_{suiA} ΔIR2 (Fig. 5D and E). This indicated that SuiR binds to the AT-rich inverted repeat IR2.

The SuiK-SuiR TCS was competent to activate P_{suiA}. To determine if SuiK-SuiR could activate transcription of P_{suiA}, the SuiK-SuiR TCS was expressed in *L. lactis* NZ9000 and *gfp* was used as the reporter gene, whose expression is under the control of P_{suiA} (Fig. 5F). pMG36e-*suiKRPsuiAgfp* was transformed into *L. lactis* NZ9000, and green florescence was visualized, indicating GFP expression. However, GFP expression was independent of suicin induction (Fig. 5Ga and b), which might have been due to autophosphorylation of SuiK resulting from high-level expression of SuiK-SuiR driven by strong lactococcal promoter P32 (24). To see if SuiK was required for P_{suiA} activation, pMG36e-*suiRPsuiAgfp* was constructed and transformed into *L. lactis* NZ9000, which resulted in much lower GFP expression (Fig. 5Gc). This indicated that SuiK was necessary for SuiR phosphorylation to exert its full function, though other histidine kinases in *L. lactis* NZ9000 might have transferred a phosphoryl group to SuiR at a low level.

To further confirm the role of SuiK-SuiR signal transduction in activating P_{suiA}, analysis of alignment of SuiK-SuiR with LanK-LanR showed that there exists one conserved His residue (His311) in SuiK and an Asp (Asp54) in SuiR (data not shown). Then, site-directed mutagenesis targeting conserved residues was performed and the function of mutant SuiK-SuiR was assessed. We first found that P_{suiA} activation was inhibited by both H311A in SuiK and D54A in SuiR (Fig. 5Gd and e). However, a conservative mutation (D54E) of SuiR had no effect on the signaling potency of SuiK-SuiR (Fig. 5Gf). Furthermore, when the binding site of SuiR on P_{suiA} (IR2) was deleted, P_{suiA} ΔIR2 could not be activated by SuiK-SuiR any more (Fig. 5Gh). Paradoxically, though IR1 of P_{suiA} was not involved in SuiR binding to P_{suiA} *in vitro* (Fig. 5E), its deletion significantly attenuated P_{suiA} activation by SuiR *in vivo* (compare Fig. 5Gg with Fig. 5Ga). This indicated that IR1 and IR2

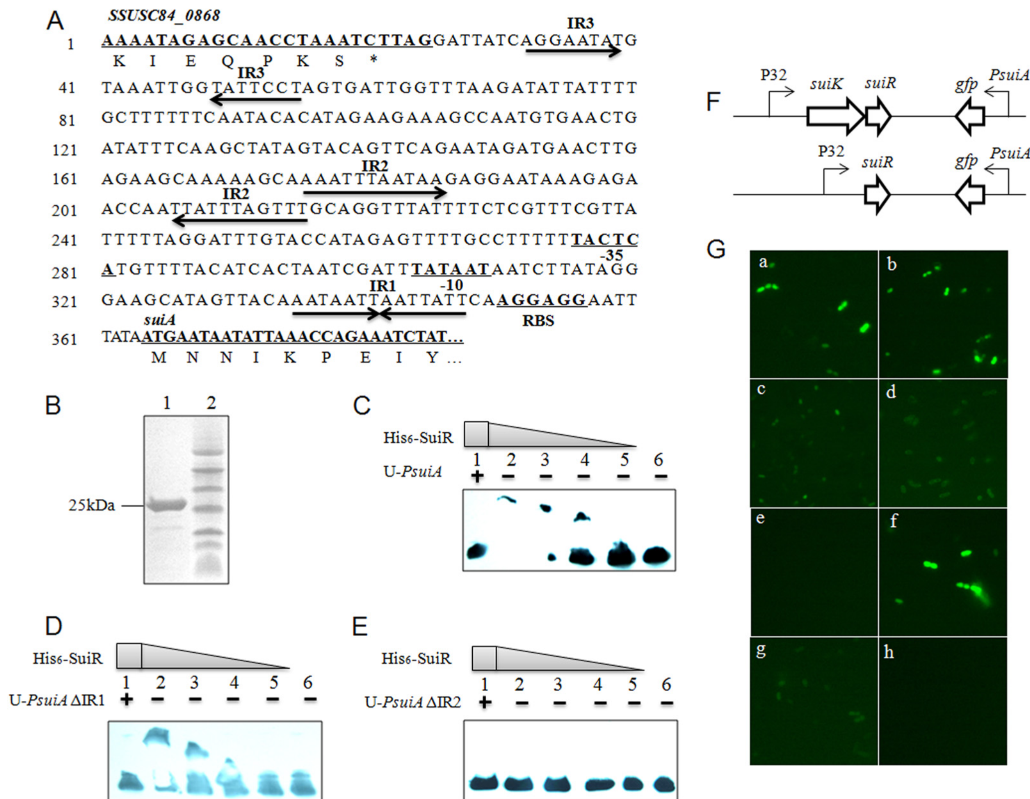

FIG 5 *In vitro* binding activity of His$_6$-SuiR and *in vivo* activation of P$_{suiA}$ by SuiK-SuiR. (A) Sequence analysis of *suiA* promoter P$_{suiA}$. (B) Purified His$_6$-SuiR analyzed by Tricine-SDS-PAGE. Lane 1, His$_6$-SuiR; lane 2, protein marker. (C) EMSA analysis of binding activity of His$_6$-SuiR toward P$_{suiA}$. Lane 1, biotin-labeled P$_{suiA}$ incubated with His6-SuiR and a 100-fold excess of unlabeled P$_{suiA}$ (U-P$_{suiA}$); lanes 2 to 5, gradient dilution of His$_6$-SuiR with biotin-labeled P$_{suiA}$; lane 6, biotin-labeled P$_{suiA}$ only. (D and E) EMSA analysis of binding activity toward P$_{suiA}$ ΔIR1 and ΔIR2, respectively. (F) Illustration of plasmids pMG36e-*suiKRPsuiAgfp* and pMG36e-*suiRPsuiAgfp*. (G) GFP visualization of *L. lactis* NZ9000 transformants with pMG36e-derived plasmids was examined with a fluorescence microscope. In the following, NZ-SuiK-SuiR and NZ-SuiR refer to *L. lactis* NZ9000 transformants of pMG36e-*suiKRPsuiAgfp* or pMG36e-*suiRPsuiAgfp*. a, NZ-SuiK-SuiR; b, NZ-SuiK-SuiR induced by 50 ng/ml suicin; c, NZ-SuiR; d, NZ-SuiK(H311A)-SuiR; e, NZ-SuiK-SuiR(D54A); f, NZ-SuiK-SuiR(D54E); g, NZ-SuiK-SuiR(P$_{suiA}$ΔIR1); h, NZ-SuiK-SuiR(P$_{suiA}$ΔIR2).

might play complementary roles in mediating binding of SuiR to P$_{suiA}$, though IR2 was more dominant.

DISCUSSION

The wide existence of lantibiotic gene clusters in bacteria, especially streptococci, has been extensively acknowledged in previous research, and the quantity of known gene clusters has recently been increased by genomic mining (12). However, disrupted or unannotated genes of small size or nonconserved nature might sometimes hamper context-based prediction by the use of such tools as BAGEL3 (15). As we identified here, the *sui* loci were probably overlooked because of the existence of interruptions or frameshift mutations or omissions of annotation. Following the clue of SuiK-SuiR sharing similarity with the TCS for lantibiotic regulation and comparison of its neighboring *lan* relics with bovicin HJ50 and salivaricin gene clusters, a primarily identical but disrupted *sui* locus was unveiled and reannotated in three SS2 genomes. Indeed, *sui* locus was potentially wide distributed in Chinese *S. suis* isolates because of its location on the 89K PAI (39, 40). Notably, similar situations were also observed in cytolysin determinants in *E. faecalis* and the *sal* locus in *S. pyogenes* such that intact or disrupted versions were unveiled in different strains (31, 41, 42). This implied that remnant lantibiotic loci like *sui* might actually or even widely exist in bacterial genomes, which might be overlooked with traditional mining approaches. Hence, by a combination of *in silico* prediction and in-depth comparative analysis, here the hidden remnant *sui* loci were rediscovered.

As suicin might not be produced by those *sui* loci containing SS2 due to disruption of the *sui* loci, especially that of the crucial modification gene *suiM*, suicin was therefore biosynthesized and characterized successfully by reconstituting the intact SuiM in *E. coli* and by subsequent processing via SuiT$_{159}$ *in vitro*. As expected, SuiM$_1$ could not produce any dehydrated SuiA, supporting the idea that disruption of *lanM* might abolish lantibiotic production (32, 43), whereas reconstituted intact SuiM produced SuiA with two dehydrations, indicating that its modification function had been restored. SuiT$_{159}$, the peptidase domain of transporter SuiT, performed a digestion function at the double-Gly site to release mature suicin, which was confirmed by antimicrobial assay and MS analysis. Although authentic suicin was not produced, previous work has verified this strategy to obtain lantibiotics as genuine (35, 44). Suicin is, to date, the first lantibiotic originating from *S. suis* and showed remarkable inhibitory activity against various Gram-positive pathogens, implying a promising application as an antimicrobial agent. This work to obtain a new-to-nature lantibiotic, suicin, further exemplified the availability of applications to

revitalize and thus broaden the range of lantibiotic resources from cryptic and, especially, remnant gene clusters.

Structural elucidation indicated that suicin contained two methyllanthionine residues and one disulfide bridge, exhibiting an N-terminal linear structure and a C-terminal globular structure characteristic of type AII lantibiotics. However, different from canonical type AII lantibiotics such as lacticin 481 and salivaricin A, suicin showed structural similarity with bovicin HJ50 in containing a disulfide bridge other than a thioether bridge in ring C (Fig. 3). The disulfide bridge is rare in lantibiotics but was demonstrated here to be critical for suicin activity and has been previously underlined in bovicin HJ50 (35). A previous study showed that bovicin HJ50 could disrupt the membrane integrity of sensitive strain *M. flavus* NCIB8166, resulting in potassium efflux (45). Here, we found that suicin also disintegrated the cell membrane of *M. flavus* NCIB8166, leading to the disruption of membrane potential (Fig. 4B). Suicin has a ring A identical to that of bovicin HJ50, which contains a TxS/TxD/EC motif (the first x is in most cases a hydrophobic residue, the latter x is undefined) conserved among type AII lantibiotics (37). This motif has been shown to mediate binding of type AII lantibiotics to lipid II, an essential intermediate during peptidoglycan synthesis and subsequent cell wall formation (37). Therefore, further study is needed to elucidate whether suicin could display bactericidal activity by inhibiting cell wall synthesis. Interestingly, by using SuiA as a drive sequence, at least 16 SuiA-like putative lantibiotic precursor peptides have been found in the NCBI database. Except for known bovicin HJ50-identical lantibiotics such as thermophilin 1277 (33) and macedovicin (46), the others are distributed in *E. columbae*, in *Clostridium perfringens*, and especially in bacillus strains. By using the above-mentioned semi-*in vitro* biosynthesis strategy, three new lantibiotics have been biosynthesized and characterized from pathogenic *C. perfringens*, *B. cereus*, and *B. thuringiensis* which were structurally elucidated to be similar to suicin and bovicin HJ50, including the conserved disulfide bridge (data not shown). Thus, along with suicin, they were grouped into bovicin HJ50-like lantibiotics, which represented a novel disulfide-containing subgroup of type AII lantibiotics.

SuiK-SuiR has been demonstrated to be essential for virulence of SS2 by regulating expression of virulence-associated factors (21, 47). Here, we found that SuiK-SuiR was also involved in transcriptional activation of promoter of structural gene *suiA*, displaying conventional function of LanK-LanR. SuiK was supposed to transfer the phosphoryl group to SuiR, initiating its binding to the promoter region of *suiA* at an AT-rich inverted repeat and consequentially activating GFP expression in our experiments. Based on sequence alignment (data not shown), His311 of SuiK and Asp54 of SuiR are predicted to be the primary sites of phosphorylation. It was verified that either mutation disabled the signal transduction from SuiK to SuiR. However, this signaling appears to be independent of suicin application, as P_{suiA} was efficiently activated in the absence of suicin (Fig. 5Ga and b). We speculate that it might be due to high-level expression of SuiK driven by strong lactococcal promoter P32, which leads to autophosphorylation of SuiK and signal leakage (24). Yet, SuiR alone was able to activate P_{suiA} transcription, though at low efficiency, as was also observed with BovR and SpaR (24, 48). Interestingly, the genome of an *S. suis* serotype 9 strain isolated in China was recently sequenced and shown to contain an intact *sui* locus (*SSUD12_1302-SSUD12_1310*) nearly identical to the one unveiled here (40). Although it has not been tested for lantibiotic production, the operonic association of *suiK-suiR* with the intact *sui* locus might underscore its connection to suicin production.

In conclusion, from a remnant gene cluster in highly virulent *S. suis* serotype 2, a bioactive type AII lantibiotic suicin was biosynthesized and characterized to contain a rare disulfide bridge. Suicin displayed inhibitory activities exclusively against Gram-positive bacteria, including pathogenic streptococci and vancomycin-resistant enterococci, making it an alternative candidate for combating against bacterial pathogens. Further, we found that the virulence-associated SuiK-SuiR is involved in activating promoter of suicin precursor gene. Thus, we propose that *lan* loci, especially disrupted ones, might evolve to extended their functions to control virulence-associated factors in *S. suis* serotype 2.

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