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Lasso peptides svuceucin and siamycin I have anti-virulence activity and restore vancomycin effectiveness in vancomycin-resistant *Enterococcus* sp. and *Staphylococcus aureus*

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Abstract

Antibiotic resistance is a major threat to human health and new drugs are urgently needed. Ideally, these drugs should have several cellular targets in pathogens, decreasing the risk of resistance development. We show here that two natural ribosomally-synthesized lasso peptides (LP), svuceucin and siamycin I, (i) abolish bacterial virulence of pathogenic enterococci, (ii) restore vancomycin clinical susceptibility of vancomycin-resistant (VR) enterococci *in vitro* and in a surrogate animal model, and (iii) re-sensitize VR *Staphylococcus aureus*. Mode of action (MoA) analyses showed that they do so by inhibiting the histidine kinases (HKs) FsrC and VanS controlling these phenotypes. Strains resistant to the vancomycin/LP combination were difficult to obtain, and were still fully susceptible to the anti-virulence effect of the LPs, highlighting the advantage of multiple targets. Together with the highly sought-after MoA as HK inhibitors, such properties make these lasso peptides promising candidates for the development of next generation antibiotics.

Significance statement

New drugs and treatment strategies are urgently needed to counter antibiotic resistance and one promising strategy is molecules that combine multiple activities, as the risk of simultaneous development of resistance to all activities is less likely. The peptides svuceucin and siamycin I interfere with bacterial virulence and reverse vancomycin resistance in pathogenic bacteria by inhibiting the histidine kinases (HKs) which regulate the corresponding phenotypes. As HKs are key bacterial regulators controlling a wide range of bacterial behaviors, they represent an attractive drug target. The combined activities of these peptides, their ability to inhibit HKs, their stability and their customizability make them valuable drug candidates.

Introduction

The alarming increase of infections caused by multidrug-resistant (MDR) bacteria is a main threat for human health worldwide¹. The World Health Organization (WHO) published a list of the MDR bacteria

for which new antibiotics are urgently needed. On this list, vancomycin-resistant enterococci (VRE) as well as methicillin-resistant *Staphylococcus aureus* (MRSA) are classified as “high priority”. These organisms are leading causes of hospital-acquired infections in high-income countries, and a high prevalence of MDR isolates among these strains renders clinical treatments difficult. Vancomycin resistant *Staphylococcus aureus* strains (VRSA) were also reported^{2,3}.

Enterococcus sp. and *S. aureus* are commensal Gram-positive bacteria^{2,4}. *Enterococcus* species are common colonizers of the gastrointestinal tract in humans and other animals⁴. Pathogenic *Enterococcus* species, mainly *E. faecium* and *E. faecalis*, can cross the intestinal barrier and cause bacteremia, endocarditis, and urinary tract infections in immunocompromised patients⁵. *S. aureus* is a commensal of human nasal mucosa. This species is a leading cause of bacterial infections in healthcare and community settings. Skin infections and bloodstream infections occur when mucosal barriers are damaged or owing to invasive medical devices⁶.

Both pathogenic enterococci and *S. aureus* pose a therapeutic challenge due to their ability to form biofilms at the infection sites in addition to their frequent resistance to antibiotics^{6,7}. Enterococcal proteases such as the gelatinase GelE and the serine protease SprE are associated with biofilm formation during endocarditis⁵. In *E. faecalis*, biofilm formation is regulated by the Fsr quorum sensing system. The response regulator FsrA and the HK FsrC form a two-component regulatory system (TCS), which is responsible for the quorum sensing-dependent activation of biofilm formation through the activation of *gelE* and *sprE* expression^{8,9}.

Vancomycin is the antibiotic of choice to treat enterococci and *S. aureus* infections. Vancomycin inhibits peptidoglycan biosynthesis by binding to N-acetylglucosamine-N-acetylmuramic acid-pentapeptide ending with D-alanyl-D-alanine (D-Ala-D-Ala) dipeptide¹⁰. Vancomycin resistance occurs following the acquisition of *van* genes, resulting in the synthesis of modified peptidoglycan precursors. The substitution of the last D-Ala of the pentapeptide by D-lactate (D-Lac) greatly lowers the affinity of vancomycin to its target¹¹. Several types of *van* clusters have been described. However, VanA- and VanB-types are the most relevant in the clinic¹². In VanA-type resistance, the D-Lac dehydrogenase VanH converts pyruvate to D-Lac, whereas the ligase VanA forms the dimer D-Ala-D-Lac and the dipeptidase VanX hydrolyses the endogenous D-Ala-D-Ala dimers. The corresponding enzymes in VanB-type resistance are VanH_B, VanB, and VanX_B, respectively¹¹. The *van* gene cluster is regulated by a TCS consisting of the HK VanS (named VanS_B in VanB-type resistance) and the response regulator VanR (named VanR_B in VanB-type resistance). In the presence of vancomycin, the kinase VanS undergoes autophosphorylation, which subsequently leads to the phosphorylation of VanR, triggering its dimerization and increasing its affinity for the promoter located upstream of *vanHAX*. In non-inducing conditions, the phosphatase activity of VanS keeps VanR in a dephosphorylated state, preventing the expression of the *van* operon¹³.

The therapeutic efficiency of the few approved antimicrobial agents active against vancomycin-resistant bacteria¹⁴ seems to be temporary, since resistance to these new drugs has already been described. Therefore, as pointed out by the WHO, new antibiotics or alternative approaches are urgently needed to combat these Gram-positive pathogens with critical clinical importance. The lack of identification of new drugs with novel molecular scaffolds and uncommon mechanisms of action has renewed interest in the investigation of alternative strategies. Ideally, the next generation antimicrobials would interfere with bacterial virulence without inhibiting bacterial growth, which is expected to lower the probability of resistance development because of reduced selective pressure^{15,16}. Another promising approach consists in interfering with antibiotic resistance mechanisms to restore the original sensitivity¹⁷. Such molecules could be used in synergy with conventional antibiotics¹⁸.

TCSs represent the majority of signaling pathways in bacteria and they control a wide range of behaviors crucial for bacterial adaptation, including virulence, biofilm formation, and antibiotic resistance. Most HKs contain a variable N-terminal sensor domain and a conserved C-terminal

cytoplasmic kinase domain¹⁹. The kinase domain comprises a catalytic ATP binding (CA) and a dimerization/histidine phosphorylation domain. The conservation of the kinase domain allows for the potential design of broad-spectrum antimicrobial agents^{20,21}. The toxicity risk of such molecules for humans is likely low, given the fact that HKs are absent in mammals. Pathogenic bacteria often contain a significant number of HKs. For instance, 17 HKs have been identified in the core genome of *S. aureus* strains²². This represents multiple possibilities to interfere with bacterial fitness, virulence, and antibiotic resistance, features expected for next generation antibiotics¹⁶.

We report herein that the lasso peptides sviceucin²³ and siamycin I²⁴ display both anti-virulence and resistance-breaking activity in *Enterococcus* sp. and *S. aureus* through the inhibition of corresponding HKs. They are thus very promising candidates for the development of next generation antimicrobials. Lasso peptides belong to the family of ribosomally synthesized and post-translationally modified peptide, natural products produced by prokaryotes. They display a mechanically interlocked structure composed of an N-terminal macrolactam ring and a C-terminal tail that is held firmly within the ring^{25,26}. This compact and rigid scaffold makes them highly stable and excellent protein ligands, leading to enzyme inhibition or receptor antagonism. Sviceucin and siamycin I are among the few representatives of the class I lasso peptides (C1LP), characterized by the presence of two disulfide bonds (Fig. 1a and Fig. S1). They show distinct growth inhibition activity of Gram-positive bacteria: sviceucin is a weak antibacterial whereas siamycin I displays a good activity, notably towards *Enterococcus* sp. and *S. aureus*. In *S. aureus*, siamycin I was shown to bind to lipid II, hence blocking the biosynthesis of peptidoglycan, which is supposed to be the basis of its antibacterial activity²⁷. Moreover, sviceucin and siamycin I are known to attenuate the TCS FsrAC-mediated quorum sensing in *Enterococcus*^{23,28}, with direct inhibition of the HK FsrC by siamycin I^{29,30}. This study extends the activity of these C1LPs to interfere with other TCS-controlled processes, i.e. resistance to vancomycin. Through detailed microbiological, genetic, and biochemical analyses, the molecular target of restoring clinical susceptibility to vancomycin in VRE and VRSA is firmly established to be the HK VanS.

Results

Sviveucin and siamycin I attenuate the virulence of *E. faecalis*

It has been shown that siamycin I binds *in vitro* to the HK FsrC of *E. faecalis* and inhibits its ATPase activity³⁰. Therefore, we wondered if siamycin I and the related C1LP sviveucin (Fig. 1a) decreased virulence of *E. faecalis*. Subinhibitory concentrations of siamycin I (2 μ M) and sviveucin (10 μ M) were used throughout all experiments of this work. At these concentrations, the peptides decreased the growth rate by about 50 % compared to that in the absence of the lasso peptides (Fig. 1b). We first monitored the expression of the Fsr regulated *gelE* gene by RT-qPCR. The peptides were solubilized in a 1:1 water:methanol formulation, and the formulation was used as the control. Both peptides led to a downregulation of expression of *gelE* (Fig. 1c), which is consistent with the previously reported reduction of gelatinase (GelE) activity by sviveucin²³.

Galleria mellonella larvae were then used as a model of *E. faecalis* infection to assess the anti-virulence activity of siamycin I and sviveucin (Fig. 1d). The *E. faecalis* strain V583 was first pre-treated with either C1LP or the formulation and subsequently injected into the larvae. Formulation-treated V583 was highly virulent for the larvae, with an average mortality of 95 % at 24h post-injection. By contrast, bacteria pre-treated with sviveucin or siamycin I were significantly less virulent for the larvae, with mortality rates of 13 % and 10 %, respectively. Similar effects on virulence have been obtained using sviveucin for *E. faecalis* strains MMH594 and OG1RF (Fig. S2).

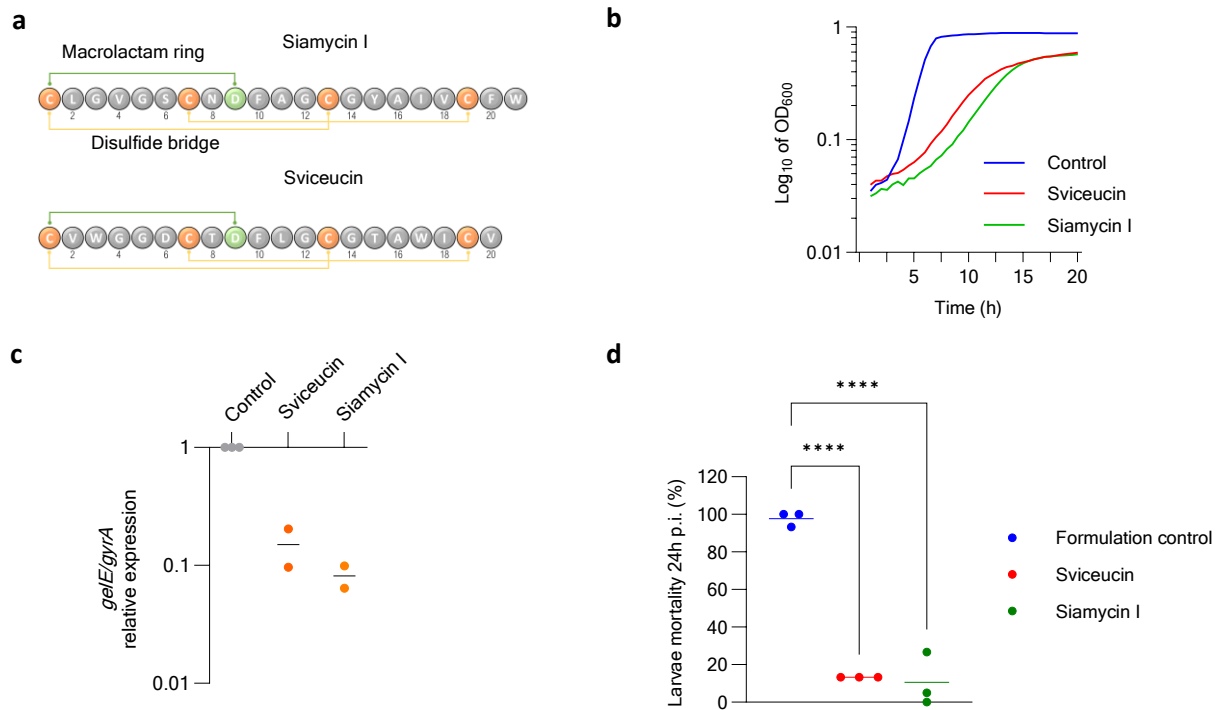


Figure 1. Anti-virulence activity of sviceucin and siamycin I.

a. Peptide sequences of sviceucin and siamycin I. The macrolactam ring is shown in green. The disulfide bonds are shown in yellow.

b. Effect of sviceucin and siamycin I on the growth of *E. faecalis* V583.

c. Effect of sviceucin and siamycin I on the expression of *gelE*, determined by RT-qPCR.

d. Mortality of *Galleria mellonella* larvae 24 h post-infection (p.i.) with *E. faecalis* V583, pre-treated with either siamycin I, sviceucin or formulation. **** $p < 0.0001$ (Bonferroni's multiple comparisons test). Data are averages of three biological replicates.

Svceucin and siamycin I restore vancomycin susceptibility

Since sviceucin and siamycin I inhibit signal transduction of the FsrAC TCS *in vivo*, we next wondered if the lasso peptides would also target other TCSs. In *E. faecalis*, the VanRS TCS regulates resistance to vancomycin, an antibiotic of clinical importance. The minimum inhibitory concentrations (MICs) of this antibiotic in combination with the lasso peptides were thus determined for clinically relevant *Enterococcus* and *S. aureus* strains in our in-house collection.

Most interestingly, both lasso peptides re-sensitized VanB-type *E. faecalis* strains to vancomycin whereas only siamycin I was active to restore susceptibility of VanA strains to this glycopeptide (clinical and laboratory standards institute (CLSI) breakpoint $> 4 \mu\text{g/mL}$). The MIC of vancomycin in strain V583 decreased from 32 to 2 $\mu\text{g/mL}$ in the presence of either one of the two peptides (Table 1), becoming as sensitive as an isogenic strain with a deleted *vanB* gene (V583 ΔvanB). This suggests that the effects of the peptides are *van*-operon type dependent. The same activity spectrum of the lasso peptides related to the *van*-operon type was also observed in VRSA strains. Svceucin re-sensitized only the VanB-type resistant strain, while siamycin I synergized with vancomycin in both VanA- and VanB-type VRSA strains (Table 1). Additionally, we tested the MIC for vancomycin of a vancomycin-intermediate *S. aureus* (VISA) strain in combination with the lasso peptides. VISA strains do not harbor a *van* operon.

The low level of vancomycin resistance (MICs 4 to 8 $\mu\text{g}/\text{mL}$) is due to spontaneous mutations in genes predominantly involved in cell envelope biosynthesis³¹. In the presence of either LP, no reduction of the MIC of vancomycin was observed, further suggesting that the action of these peptides is *van*-operon dependent. Considering the relevance of a vancomycin resistance-breaking activity, we subsequently focused our efforts on (i) providing an in-host proof of the efficacy and (ii) deciphering the underlying MoA of the lasso peptides.

We used *Galleria mellonella* insect larvae to investigate the efficacy of the vancomycin/C1LP combinatorial treatment (Fig. 2). As sviceucin and siamycin I are hydrophobic peptides^{23,32}, they were first solubilized in a nanoemulsion previously shown to improve the solubility and the bioavailability of lipophilic drugs³³. A nanoemulsion with a sufficient drug payload is necessary for the treatment of infected larvae, and this was only obtained for siamycin I, for which it contained 400 $\mu\text{g}/\text{mL}$ of the peptide. Larvae were infected with the wild-type strain *E. faecalis* V583, the isogenic vancomycin-sensitive mutant V583 ΔvanB , or an equivalent volume of saline solution. At $t_{90\text{min}}$ and $t_{24\text{h}}$ post-infection (p.i), the larvae were treated with the nanoemulsion alone (referred to as formulation), the nanoemulsion loaded with siamycin I (3.92 $\mu\text{g}/\text{larvae}$; about 18 mg/kg), vancomycin (1 $\mu\text{g}/\text{larvae}$; about 4.6 mg/kg) alone, or a combination of both. Larvae survival was assessed, and statistical analysis was performed at the endpoint $t_{96\text{h}}$. The formulation and siamycin I-loaded nanoemulsions did not exhibit toxicity towards the larvae (Fig. 2). 95 % of infected larvae with the WT strain and treated with the formulation were killed 24 h p.i. Siamycin I treatment significantly delayed the killing of the larvae at the beginning of the experiment (p value = 0.0486 at 48 h p.i), although most larvae were also dead by the end of the experiment. This attenuation of the killing rate is likely related to the anti-virulence activity of the peptide. Larvae infected with the WT strain and treated with the combined treatment resulted in a significant improvement of survival in comparison to the condition where larvae were treated with either vancomycin or siamycin I alone. Moreover, the combination therapy resulted in a survival rate similar to that of larvae infected with the V583 ΔvanB strain and treated with vancomycin. We therefore provide a proof-of-concept for the potential use of siamycin I as an adjunctive therapy for the treatment of vancomycin-resistant *E. faecalis* in an infected host.

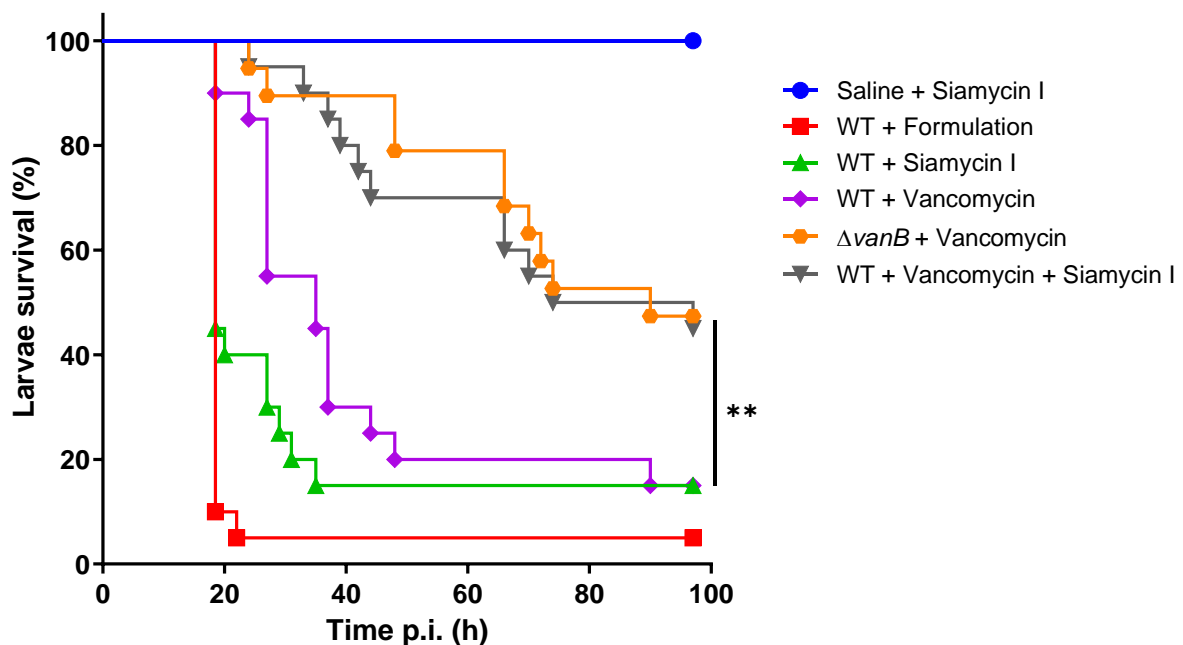


Figure 2. Siamycin I reverse vancomycin resistance. Survival plot of *G. mellonella* larvae infected with 10^6 CFU of the WT strain, the isogenic ΔvanB mutant, or injected with saline solution. Larvae were then treated at $t_{90\text{min}}$ and $t_{24\text{h}}$ with the formulation, siamycin I, vancomycin or the combination of both. ** p < 0.001 (Logrank test).

Mutations in VanS_B lead to resistance to the vancomycin /lasso peptide combinatorial treatment

To gain further insights into the molecular targets of these lasso peptides to reverse vancomycin resistance, resistant clone isolation followed by whole genome sequencing was performed. *E. faecalis* V583, a VanB strain, was subjected to selective pressure in the presence of vancomycin combined with either lasso peptide. Four resistant mutants were isolated when bacteria were plated in the presence of a lethal concentration of combined vancomycin (32 µg/mL) and sviveucin, occurring with an overall frequency of resistance of $\sim 10^{-8}$. By contrast, no resistant mutants could be obtained with lethal concentrations of combined vancomycin (32 µg/mL) and siamycin I. However, by performing serial passages in the presence of 10 µg/mL vancomycin and increasing sublethal concentrations of siamycin I up to a lethal concentration of 5 µM, we were able to isolate five resistant mutants. Three mutants identified from the vancomycin/sviveucin combination have mutations in the locus encoding the F1 ATP synthase, including substitutions in the *atpA* and *atpF* genes and an altered RBS upstream the *atpD* gene. Compared to the wild-type, these mutants were more resistant to vancomycin alone and the vancomycin/sviveucin combination, but still susceptible to the vancomycin/siamycin I combination (Table S1). Four mutants derived from the vancomycin/siamycin I combination have either point mutations or frameshift in the *ccpA* gene encoding the catabolite control protein A. These mutants were resistant to the effects of both C1LPs in combination with vancomycin (Table S1). Interestingly, two mutants, named Svic1 and SiaA and isolated respectively from vancomycin/sviveucin and vancomycin/siamycin I combinations, have a direct link to vancomycin resistance. Genome sequencing revealed that both have point mutations in the ATPase CA domain of VanS_B, leading to a S402I and a D398E substitution, respectively (Fig. 3a-c). Growth of these mutants was very similar to that of the wild-type strain. Both Svic1 and SiaA exhibited a 4-fold increase of the MIC of vancomycin, reaching 128 µg/mL regardless of the presence of sviveucin (Table 2). In the case of the combination of vancomycin/siamycin I, the SiaA mutant showed partial resistance while the Svic1 mutant remained sensitive. To provide evidence of the causal effect of S402I and D398E substitutions in VanS_B on resistance to the lasso peptides, we introduced both mutations individually into a wild-type V583 background. The resistance profiles of V583 *vanS_B^{S402I}* and V583 *vanS_B^{D398E}* were similar to the mutants Svic1 and SiaA, respectively, indicating that the resistance phenotypes of Svic1 and SiaA are due to the identified mutations in VanS_B. Additionally, deletion of *vanB* from the resistant mutant Svic1 led to loss of vancomycin resistance, arguing that the increase of the MIC of vancomycin in Svic1 and SiaA mutants occurs exclusively through *van* resistance genes. Our results strongly support that the histidine kinases VanS and VanS_B are direct targets of sviveucin and siamycin I.

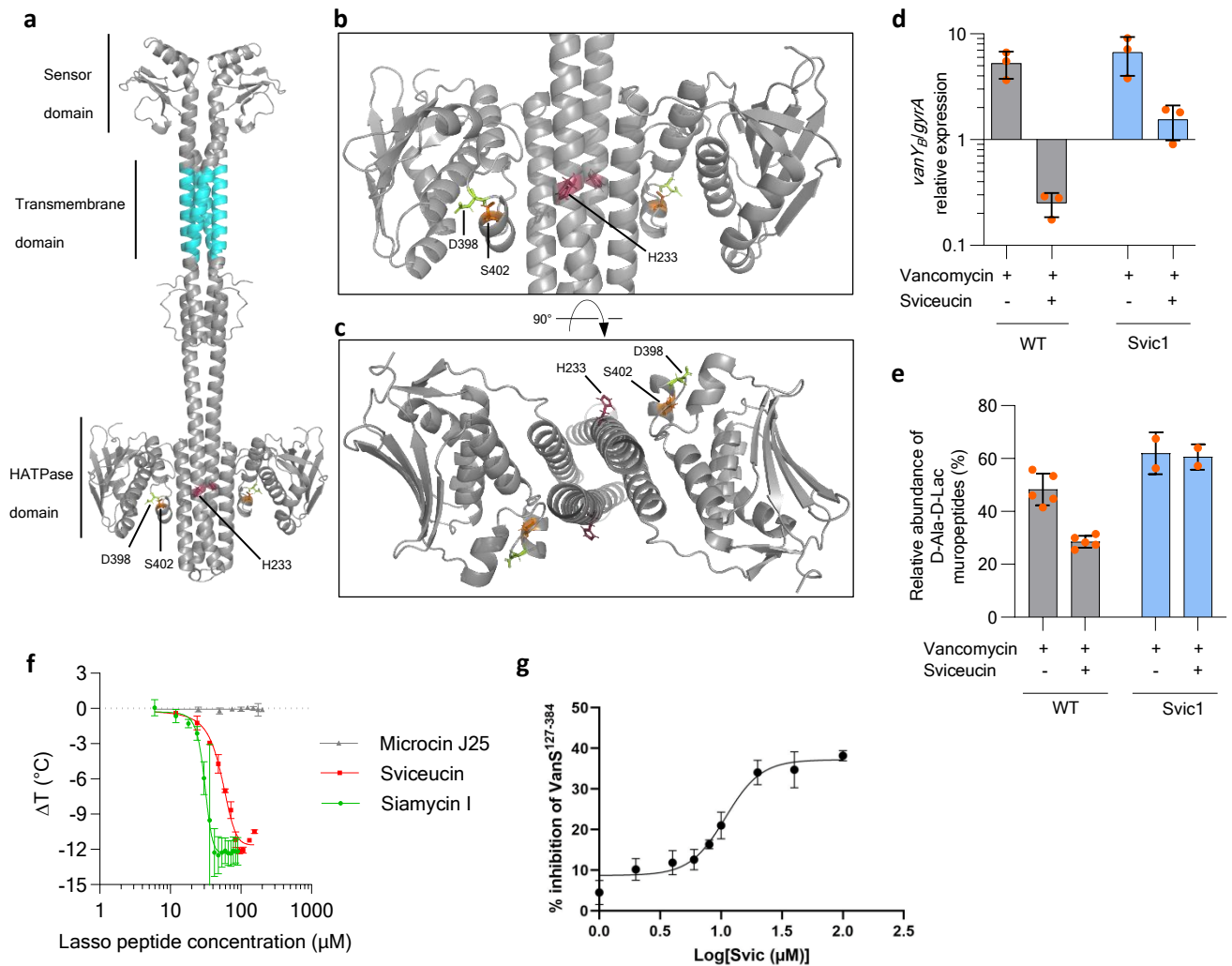


Figure 3. Characterization of VanS/VanS_B as the molecular target of sviceucin and siamycin I.

a-c. Model of the full-length VanS_B dimer generated using AlphaFold 1. D398E and S402I mutations and the conserved H233 are mapped on the structure (a). A zoom into the H-ATPase domain (b) and top view of the H-ATPase domain (c), are shown.

d. Relative expression of *vanY_B* in presence of vancomycin (1 μg/mL) or vancomycin and sviceucin in *E. faecalis* V583 and its mutant Svic1.

e. Relative abundance of D-Ala-D-Lac mucopeptide in presence of vancomycin (2 μg/mL) or vancomycin and sviceucin in *E. faecalis* V583 and its mutant Svic1.

f. Differential scanning fluorimetry analysis of VanS-lasso peptides interactions. ΔT of VanS¹²⁷⁻³⁸⁴ in presence of increasing concentrations of sviceucin, siamycin I or microcin J25.

g. Dose-response curve of VanS¹²⁷⁻³⁸⁴ inhibition by sviceucin (IC₅₀ 10.8 ± 1.5 μM, nonlinear regression fitting R² = 0.93).

Sviceucin leads to a decrease in *van* operon expression

Based on the above results, sviceucin and siamycin I are expected to reduce or block the expression of the *van* operon upon vancomycin induction in the wild-type strain V583 but not in the Svic1 and SiaA mutants. To decipher this part of the mechanism, we chose to focus on sviceucin and the sviceucin-resistant mutant Svic1. The expression of *vanY_B* was monitored by RT-qPCR when bacteria were cultured with vancomycin (1 μg/mL) alone or in combination with sviceucin. As expected, *vanY_B* expression was strongly induced by vancomycin alone in both wild-type and Svic1 strains (Fig. 3d). In the presence of sviceucin, *vanY_B* expression was reduced by a factor of 21 in the wild-type strain upon

vancomycin induction, suggesting that sviceucin interferes with the signal transduction of VanR_BS_B. By contrast, sviceucin led to only a 4-fold reduction of *vanY_B* expression in the Svic1 strain. The *vanY_B* expression level in the Svic1 mutant was 6.2 times higher than in the wild-type strain. This difference may explain the sviceucin-resistant phenotype of the Svic1, mutant in which, conversely to the wild-type, a sufficient expression level of *van* genes is reached to allow synthesis of modified peptidoglycan precursors in order to counter the action of vancomycin.

Sviceucin leads to a decrease in the percentage of muropeptides ending in D-Ala/D-Lac

To verify the latter hypothesis, we investigated the effect of sviceucin on peptidoglycan biosynthesis in the presence of vancomycin by quantifying the muropeptides ending in D-Ala-D-Ala and D-Ala-D-Lac in the wild-type and Svic1 strains by liquid chromatography coupled to mass spectrometry (LCMS) (Fig. 3e). When induced with vancomycin alone (2 µg/mL), the percentage of muropeptides D-Ala-D-Lac reached 48.3 % and 62.0 % in the wild-type and the Svic1 mutant, respectively, which is consistent with the increased MIC of vancomycin in Svic1. Importantly, the presence of sviceucin resulted in a decrease of the percentage of muropeptides D-Ala-D-Lac (28.5 %) in the wild-type strain, while that of the Svic1 mutant remained unchanged. Taken together, our data confirm that sviceucin breaks *van* operon-mediated vancomycin resistance by affecting the signal transduction of VanRS, leading to a decreased expression of the *van* operon and, subsequently, a decreased incorporation of the modified muropeptides D-Ala-D-Lac into the peptidoglycan. This should also hold true for siamycin I.

Sviceucin and siamycin I directly interact with the Van histidine kinase

Given that the resistant mutants harbor mutations located in the ATPase domain of VanS_B, this suggests that the peptides directly interact with this HK. This hypothesis was tested via genetic and biochemical experiments. First, a system allowing VanS-independent constitutive expression of the *van* operon was generated. It has been shown that the conserved aspartate residue D53 at the N-terminal receiver domain of VanR_B is the phosphate acceptor following phosphorylation by P-VanS_B^{34,35}, and that substituting the conserved aspartate by a glutamate residue mimics a phosphorylated response regulator (P-VanR_B)³⁶. We introduced a copy of a *vanR_B* variant gene encoding the phosphomimetic modification D53E into the chromosome of a vancomycin-sensitive V583Δ*vanS_B* background strain (V583 Δ*vanS_B* *malT::vanR^{D53E}*). In this strain, the MIC of vancomycin (32 µg/mL) was similar to that of the wild-type strain, indicating that the VanS_B-independent constitutive expression of the *van* operon was successfully elicited (Table 3). In contrast to the wild-type strain, sviceucin and siamycin I did not suppress the resistance to vancomycin, suggesting that the lasso peptides act upstream of VanR_B.

To further support this, we investigated the origins of the different activity spectra of sviceucin and siamycin I between VanA- and VanB-type vancomycin resistance. We first replaced *vanS_B* by *vanS* in the VanB-type strain *E. faecalis* V583. VanS-VanR_B strain was sensitive to vancomycin (MIC = 2 µg/mL), probably because of an impaired signal transduction between VanS and VanR_B. To bypass this issue, we replaced the *vanR_BS_B-P_{vanY_B}* fragment by the *vanRS-P_{vanH}* fragment from a VanA-type operon, resulting in a VanA-type transduction system fused to VanB-type resistance genes. In this strain, the phenotype of a VanA-type strain was restored, meaning resistance to sviceucin while remaining sensitive to siamycin I (table 4). Furthermore, the replacement of the *vanB* gene encoding the D-Ala:D-Lac ligase by its VanA-type homolog *vanA* did not affect the sensitivity of the strain towards sviceucin, confirming that the activity spectrum of the peptides is linked to the TCS and not the resistance genes.

Taken together, these results confirm that svuceucin and siamycin I interfere with the signal transduction of the TCS at the level of the HK VanS_B/VanS.

To provide evidence for a direct interaction, the cytoplasmic domains of the HKs were recombinantly produced in *E. coli*. Despite numerous efforts, only the C-terminal His₆-tagged VanA-type VanS¹²⁷⁻³⁸⁴ could be obtained as a soluble protein. The peptide-protein interactions were analyzed by Differential Scanning Fluorimetry (DSF). The affinities of siamycin I, svuceucin, and an unrelated lasso peptide microcin J25³⁷ for VanS¹²⁷⁻³⁸⁴ were determined by monitoring the changes of the melting temperature (T_m) of the polypeptide (Fig. 3f). While microcin J25 did not affect the T_m of VanS¹²⁷⁻³⁸⁴, svuceucin and siamycin I caused a dose-dependent reduction of its thermal stability, with an equilibrium dissociation constant (K_d) of 52±1.6 μM and 38±1.0 μM, respectively. We therefore conclude that svuceucin and siamycin I specifically interact with the VanA-type HK VanS. Next, we investigated whether this interaction leads to inhibition of the autophosphorylation activity of the HK, using a continuous assay based on the detection of ADP³⁸. Indeed, svuceucin inhibited VanS¹²⁷⁻³⁸⁴ with IC₅₀ of 10.9±1.5 μM (Fig 3g). By contrast, microcin J25 had no effect on VanS¹²⁷⁻³⁸⁴ (Fig. S3). Unfortunately, we were unable to determine the IC₅₀ for siamycin I since the peptide made the enzymes precipitate under our assay conditions. Given that the CA domains in HKs are highly conserved²¹ and that svuceucin and siamycin I share the same 3D structure (Fig. S1), it is likely that these C1LPs also target the VanB-type kinase VanS_B.

Collectively, these data confirm that the molecular basis of restoring vancomycin sensitivity by these C1LPs is the direct inhibition of Van HKs. It is worth noting that svuceucin and siamycin I have distinct spectra towards VanA- or VanB-type vancomycin resistance (Table 1), which could, at least in part, be due to the higher affinity of siamycin I for VanS.

Discussion

Bacteria use TCSs to control a wide variety of physiological responses, including virulence, biofilm formation, competence, and antibiotic resistance, notably in the context of host infection. Interference with these processes likely does not impact bacterial viability, hence reducing the risk of resistance appearance, which is imposed by the selection pressure. Therefore, targeting TCSs represents a promising strategy to combat the antibioresistance crisis. To our knowledge, svuceucin and siamycin I are the only natural products known to target both virulence and vancomycin resistance in major Gram-positive pathogens via inhibition of related HKs. Previous reports and our study show that the C1LPs interfere with FsrC in *E. faecalis*, leading to an attenuation of virulence in a *G. mellonella* infection model. The anti-virulence effect can be extrapolated to *S. aureus* because one of its master virulence regulator, AgrC, is a close homolog of FsrC³⁹. This work also establishes that C1LPs inhibit directly VanS, which is the molecular basis of reverting vancomycin resistance. The effectiveness of combined vancomycin/C1LP treatments to restore clinical susceptibility of VRE and VRSA to vancomycin has been proved in the *G. mellonella* infection model. Such an elegant dual mechanism of C1LPs to kill two birds with one stone stands at an immense advantage, as having multiple clinically-relevant targets further decreases the risk of simultaneous developments of resistance to all actions of these molecules. In this regard, siamycin I seems particularly interesting. Siamycin I has another direct target, lipid II, an essential precursor for cell wall biosynthesis in Gram-positive bacteria, and the inhibition of Lipid II led to compromised cell growth²⁷.

Breaking antibiotic resistance is a validated therapeutic strategy, as exemplified by the clinical use of β-lactam antibiotic and β-lactamase inhibitor combination⁴⁰. Similar approaches should also be successful to potentiate the use of vancomycin. Being the first-line antibiotic for the treatment of MRSA, β-lactam resistant enterococci, and *Clostridioides difficile* infections^{2,3,41,42}, vancomycin encounters resistance frequently in enterococci, especially *E. faecium*, but is hitherto rare for MRSA isolates. In *C. difficile*, vancomycin resistance genes are present and have been associated with elevated vancomycin MICs⁴³. High-level vancomycin resistance of *C. difficile* would have dramatic

clinical consequences. Therefore, drugs that counteract the vancomycin resistance mechanism would be immediately applicable for VRE infections, but could also be used as precautionary drugs for the treatment of other resistant strains, such as *C. difficile*. Previous studies reported several molecules disrupting the TCS VanSR^{14,44}, including a synthetic antimicrobial peptide⁴⁵. However, their molecular mechanisms remained elusive as no direct evidence has been provided that they directly inhibit the TCS VanSR.

To understand the molecular details of how the C1LPs suppress vancomycin resistance, *E. faecalis* mutants resistant to vancomycin in the presence of either svieceucin or siamycin I were isolated. The only overlap between the two combinations were point mutations in VanS. Genetic and biochemical characterization showed C1LPs interact directly with VanS and inhibit its autophosphorylation activity. This leads to blockage of *van* gene expression and subsequent reduction of the incorporation of the modified pentapeptides into the peptidoglycan. Among other spontaneous resistant mutants, mutations have been mapped in the (F₁F₀) H⁺ ATPase (F-ATPase) operon or in the *ccpA* gene, but none of these mutants were shared by the two different combinations. The F-ATPase mutants were specifically resistant to svieceucin, whereas *ccpA* mutants were resistant to both C1LPs. In both cases, introduction of the corresponding mutation in a wild-type background conferred vancomycin resistance in the presence of the C1LPs, although effects on general fitness are expected for such mutants. The F-ATPase functions in lactic acid bacteria, including *E. faecalis*, as a proton pump to maintain intracellular pH homeostasis⁴⁶. In *S. aureus*, ATP synthase mutants show altered growth kinetics and biofilm formation, and are more susceptible to antimicrobial peptides^{47,48}. By comparison, CcpA is a global regulator of genes encoding activities for the transport and catabolism of secondary substrates in Gram-positive bacteria⁴⁹. Knock-outs of *ccpA* have pleiotropic effects on the physiology of enterococci and staphylococci, including reduced biofilm formation, antibiotic resistance, and virulence^{50,51}. Although not directly linked to the *van* operon, these mutations could provide insights into the interplay between vancomycin resistance and bacterial physiology, which in turn could inspire novel resistance-breaker strategies⁴⁰. Remarkably, these mutants are all still fully susceptible to the anti-virulence activity of the C1LPs, as shown by the *G. mellonella* infection assay (Fig. S4), supporting strongly that developments of resistance to one action of the C1LPs do not induce resistance to other activities of the C1LPs.

Siamycin I and svieceucin have a high structural homology²³ but differ considerably in several aspects. For both anti-virulence and anti-vancomycin resistance activities, svieceucin requires an effective concentration 5-fold higher than that of siamycin I. Moreover, siamycin I acts on VanA- and VanB-type vancomycin resistant strains, whereas svieceucin is only active on VanB-type strains. Surprisingly, *in vitro* characterization using the soluble cytoplasmic domain of VanS showed that siamycin I only has a slight higher affinity (2-fold) for VanS. The discrepancy of *in vitro* assays and the whole-cell phenotyping could be attributed to: (i) a truncated cytoplasmic domain may not reflect the functioning of a full-length HK, which involves the sensor domain and interactions with the response regulator; (ii) transport into the cytoplasm and subsequent availability are different for these peptides; (iii) binding of siamycin I to lipid II, a membrane-bound precursor for cell wall synthesis, may somehow favor the interaction between this peptide and VanS.

In conclusion, this study identifies the C1LPs as promising HK inhibitors for the development of next generation antibiotics. Their highly stable structure and their capacity to target several HKs, which confers them dual anti-virulence and anti-vancomycin resistance properties, are particularly advantageous. They can be envisioned to be used as an adjuvant in combination with vancomycin to break high-level resistance to this antibiotic. The C1LPs reported here thus represent a blueprint for engineering new HK inhibitors for the development of next generation antibiotics.

Material and methods

Strains, plasmids, and growth conditions

Bacterial strains used in this study are listed in Table S2. *Enterococcus* strains were cultured in M17 medium⁵² supplemented with glucose 0.5 % (GM17) at 37 °C without shaking. *Staphylococcus* strains were cultured in MH medium⁵³ at 37 °C with shaking at 120 rpm. *Escherichia coli* strains were cultured in LB medium⁵⁴ at 37 °C or 30 °C with shaking at 120 rpm. Growth was assessed by monitoring the optical density at 600 nm (OD₆₀₀) and performed in 96-well microplates (CytoOne) in a spectrophotometer (Tecan Infinite® M Nano.) with agitation for *S. aureus* strains and without agitation for *Enterococcus* strains. Media were supplemented with chloramphenicol at 10 or 20 µg/mL (Thermo Fisher Scientific, USA), kanamycin at 15 µg/mL (Merck, USA) or tetracycline at 10 µg/mL (Serva, Germany) when necessary for mutant constructions.

All plasmids and primers used in this study are listed in Tables S3 and S4, respectively. Plasmids were replicated in *E. coli* strains. Recombinant plasmids were constructed by *in vivo* recombination using DNA fragments with overlapping ends, as previously described⁵⁵. DNA fragments were obtained by PCR using Q5® High-Fidelity DNA Polymerase (New England BioLabs, USA). Plasmid extractions were performed with the NucleoSpin Plasmid kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. Purified plasmids were then electroporated into competent *E. faecalis* V583 cells with a Gene Pulser™ (2,25 kV, 200 Ω, 25 µF) (Biorad, USA). Mutations were introduced into the chromosome by double crossing-over of the plasmid as previously described⁵⁶. Mutants were controlled by amplification of the corresponding loci with GoTaq DNA Polymerase (Promega, USA) and sequencing.

Production and purification of C1LPs

The heterologous expression strain *Streptomyces coelicolor* M1146 harboring the P4H7 cosmid²³ and the native producing strain *Streptomyces* sp. SKH 2344⁵⁷ were used for the production of svieceucin and siamycin I, respectively. Both strains followed the same fermentation procedure. The *Streptomyces* strain was grown from a mycelium stock in 150 mL GYM medium⁵⁸ for 4 days at 30 °C with agitation. This pre-culture was used to inoculate a 3 L fresh GYM culture, which continued growth at 30 °C for 7 days. The cell pellets were collected and extracted by 400 mL MeOH at room temperature for 4 h. After solvent evaporation, the dried crude extract was resuspended in 3 mL 80 % acetonitrile and purified by semi-preparative HPLC on a Phenomenex C18 column (Luna C18 (2), 5 µm, 100 Å, 250 x 10 mm) at 3 mL/min with UV detection at 280 nm. Eluents used were A (H₂O with 0.1 % formic acid) and B (acetonitrile). For svieceucin purification, the gradient was a linear increase of 45 % to 60 % B in 10 min. For siamycin I purification, the gradient was a linear increase of 20 % to 70 % B in 30 min. The corresponding peak containing the peptide was collected. For peptide quantification, a predicted extinction coefficient at 280 nm of 11860 M⁻¹.cm⁻¹ for svieceucin and 7450 M⁻¹.cm⁻¹ for siamycin I were used.

Determination of minimal inhibitory concentration

The MIC of vancomycin was determined by the microdilution method described in the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2012). For enterococci, GM17 medium was used instead of MH medium since enterococci cannot degrade starch, the main carbohydrate of MH medium. The media were inoculated with 5.10⁵ CFU/mL. Subsequent 2-fold serial dilutions of the tested antibiotic were performed to obtain concentrations ranging from 0.5 to 512 µg/mL. Lasso peptides were then added to the wells at the desired concentration. The MIC is the lowest concentration that prevents visible *in vitro* growth.

Extraction of mucopeptides

Extraction of mucopeptides was performed as previously described⁵⁹ with modifications. Bacteria were cultured overnight in GM17 medium supplemented with 2 µg/mL of vancomycin (Merck, USA), 10 µM of svieceucin, or 2 µM of siamycin I. Bacteria were pelleted by centrifugation for 5 min at 10000 rpm and then resuspended in 1 mL of 0.1 M Tris-HCl (pH 6.8), 0.25 % SDS. Samples were boiled for 20 min

in a heating block (Bioblock Scientific, USA) and then centrifuged for 5 min at 10000 rpm. The pellets were washed twice with 1 mL of cold ultrapure water before sonication for 30 min (Branson 1800 Cleaner, USA). 500 μ L of DNase 15 μ g/mL and RNase 60 μ g/mL (Sigma-Aldrich, USA) were added to each sample then incubated at 37 °C for 60 min. Samples were then treated with trypsin at 5 μ g/mL (Promega, USA) and incubated at 37 °C for 2 h. Enzymes were inactivated by boiling the samples for 3 min and then washed once with ultrapure water. Pellets were resuspended in 12,5 mM sodium phosphate buffer (pH 5.5), then mucopeptides concentration was determined and standardized to an OD₅₇₈ of 3. Samples were then treated with mutanolysin (Sigma-Aldrich, USA) at 5000 U/mL and incubated at 37 °C, 150 rpm shaking, for 16 h. Samples were boiled for 3 min to inactivate the enzyme and then centrifuged. The supernatants containing the mucopeptides were collected and stored for LCMS analysis.

Quantification of mucopeptides by mass spectrometry

LCMS analysis was performed on an ultra-high performance LC system (Ultimate 3000 RSLC, Thermo Scientific, USA) coupled to a high-resolution electrospray ionization-quadrupole-time of flight mass spectrometer (MaXis II ETD, Bruker Daltonics, USA). The mucopeptide solution was diluted five times in MeOH before analysis. LC separation was performed on an Acclaim RSLC Polar Advantage II column (2.2 μ m, 2.1 x 100 mm, Thermo Scientific, USA) with a flow-rate of 0.3 mL/min using the following gradient (eluent A: water with 0.1 % formic acid (v/v); eluent B: acetonitrile): 2 % B for 3 min, followed by a linear increase to 15 % B over 7 min and a final increase to 100 % B in 1 min. The mass range of 250-1500 in positive mode was acquired. Mass data were analyzed by Data Analysis 4.4 (Bruker Daltonics, USA). For quantification, the *m/z* corresponding to singly- and doubly-charged species of monomer, as well as doubly- and triply-charged species of dimers of mucopeptides were manually searched. These were the most abundant masses observed under our experimental conditions. Subsequently, the areas of the peaks corresponding to the same mucopeptide in the extracted ion chromatograms were combined for calculation.

Analysis of gene expression by qPCR

For *gelE* gene expression measurements, bacteria were cultured in GM17 medium supplemented with the 1:1 water:methanol formulation or either sviveucin at 10 μ M, or siamycin I at 1 μ M. Cultures were incubated at 37 °C until reaching OD₆₀₀ of 0.5. For *vanY* gene expression measurements, bacteria were cultured in GM17 medium supplemented with either vancomycin at 1 μ g/mL or sviveucin at 10 μ M and vancomycin at 1 μ g/mL at 37 °C to induce the expression of *van* genes until reaching OD₆₀₀ of 0.5. Bacteria were recovered by centrifugation at 8000 rpm for 2 min. Once the supernatant was discarded, the pellets were stored at -80 °C. Pellets were resuspended in TE buffer or ML lysis buffer, depending on kit used, and transferred in Lysis Matrix B Tubes (MP Biomedicals™, USA) before cell lysis with a FastPrep-24™ 5G (2x40 s at 6 m/s, 5 minutes on ice in-between cycles) (MP Biomedicals™, USA). After bead-beating lysis, RNA extraction was performed using Direct-Zol™ RNA Miniprep kit (Zymo Research, USA) for *gelE* gene expression measurements or NucleoSpin[®] miRNA Miniprep kit (Macherey-Nagel, Germany) for *vanY* gene expression measurements, according to the manufacturers' instructions. Retrotranscription was performed using QuantiTect[®] Reverse transcription (QIAGEN, Germany). qPCR on cDNA was performed with GoTaq[®] qPCR (Promega, USA). The housekeeping gene *gyrA* was used as a reference for normalization. Data were analyzed with CFX Manager software (Biorad, USA).

Directed evolution and selection of resistant mutants

Resistant clones to sviveucin were selected by plating bacteria on GM17 agar medium supplemented with vancomycin at 32 μ g/mL and sviveucin at 10 μ M. Growth and MIC of vancomycin and sviveucin of the resistant clones were tested. The selection for siamycin I resistance was done by performing serial passages of bacteria in GM17 medium supplemented with vancomycin 10 μ g/mL and increasing concentrations of siamycin I from 0.5 to 5 μ M. Single nucleotide polymorphism (SNPs) analysis was performed on the sequenced gDNA of each selected resistant mutant.

The genomic DNA of the selected resistant mutants and the WT strain *E. faecalis* V583 were extracted using GenElute™ (Sigma-Aldrich, USA). Whole genome sequencing was then performed at the iGE3 genomics platform of the University of Geneva. The Illumina DNA prep kit was used for library preparation according to manufacturer's specifications, with 200 ng of genomic DNA as input. Library molarity and quality was assessed with the Qubit and TapeStation using a DNA High sensitivity chip (Agilent Technologies). Libraries were sequenced on a NovaSeq 6000 Illumina sequencer with a minimum of 15 M of paired-end 100 reads per sample. The sequencing quality control was done with FastQC. The reads were mapped with the BWA v.0.7.10⁶⁰ software to the NCBI *Enterococcus faecalis* V583 NC_004669.1 reference. After sorting and marking the PCR replicates with samtools⁶¹, the alignments were screened for variants with freebayes v.0.9.21, by specifying a haploid genome.

Nanoemulsions formulation process

The formulation of the nanoemulsions was based on the spontaneous nanoemulsification method previously described by Séguy *et al.*³³ and transposed to lasso peptides. The present nanoemulsions were developed from anhydrous mixtures composed of excipients already described in the European Pharmacopeia (World Health Organization, 2018) and already proven to be hemocompatible on human whole blood. Monodisperse nanoemulsions could be formulated at a drug payload of 400 µg/mL for siamycin I. At these concentrations, the drug added to the anhydrous mixture during the formulation process appeared to be completely dissolved. After the addition of the aqueous phase, a homogeneous colloidal suspension was observed. The nanoemulsions were characterized in terms of granulometric and physicochemical properties (Table S5). The hydrodynamic diameter, zeta potential, and PDI were not changed relative to the blank nanoemulsions, remaining lower than 0.25, indicating the presence of a monodispersed population.

***Galleria mellonella* infection and C1LP treatments**

Bacteria were collected from overnight cultures by centrifugation, washed three times with normal saline solution (NSS), then adjusted to an OD₆₀₀ of 0.3 corresponding to ~10⁸ CFU/mL. 10 µL of the pre-treated bacterial suspension were injected subcutaneously into in-house reared *Galleria mellonella* larvae using a syringe pump (KD scientific, USA) in order to inoculate 10⁶ CFU into each larva or the equivalent volume of NSS as a control. To assess the anti-virulence effect of the C1LPs, *E. faecalis* V583 was grown overnight in a medium supplemented with either 10 µM svicucin or 2 µM siamycin I. 15 larvae were used for each condition. Larvae were recorded as dead in absence of reactivity to stimuli at 24 hours p.i. To assess the C1LPs efficacy as treatment of *E. faecalis* infection, the strains V583 or V583 Δ vanB were grown without previous C1LP exposition. 20 larvae were used for each condition. At 1.5 and 24 hours p.i, larvae were injected with 4.76 mg of vancomycin per kilogram and/or 18 mg of siamycin I per kilogram, or the equivalent volume of nanoemulsion as a control. Larvae killing was then monitored between 1.5 and 96 h p.i. Data and statistical analysis were performed using the Kaplan-Meier R package and the logrank test, respectively.

Proteins purification

1 L of Terrific medium⁶² equally dispatched in four 1 L baffled Erlenmeyer flasks was inoculated with an overnight culture of *E. coli* BL21DE3 possessing the plasmid pET29b(+) *vanS*¹²⁷⁻³⁸⁴ to a final of OD₆₀₀ 0.1 (Table S2). The culture was incubated at 37 °C with agitation at 180 rpm. Induction of protein expression was achieved by adding IPTG to a final concentration of 0.1 mM when the bacterial density reached an OD₆₀₀ of 0.5. The culture was then incubated for 20 h at 25 °C with agitation at 180 rpm. The cells were then washed twice in purification buffer (20 mM sodium phosphate buffer, 500 mM NaCl, 5 mM MgCl₂, 10 mM imidazole (pH 7.4)). Cells were treated with lysozyme at 1 mg/mL final concentration for 30 minutes at 4 °C and lysed by sonication with the Fisherbrand Model 120 Sonic Dismembrator (Fisher scientific, USA). The total fraction recovered was centrifuged at 10000 rpm at 4

°C for 30 to 45 min and only the supernatant, representing the soluble fraction, was recovered for purification.

Protein purification was carried out on a HisTrap™ FF Crude 5 mL column (Cytiva, USA) using the ÄKTA start device (Cytiva, USA), following the manufacturer's recommendations. The column was equilibrated with the purification buffer and then loaded with the soluble fraction. The protein of interest was washed and eluted with an imidazole gradient of 10 mM to 500 mM in purification buffer. The purified protein was desalted and concentrated on a Vivaspin® 6 30 kDa MWCO column (Cytiva, USA) according to the manufacturer's recommendations. Protein concentration was determined with the Pierce™ BCA Protein Assay Kit (Thermo Scientific, USA) according to the manufacturer's recommendations and then stored at -80 °C.

Differential scanning fluorimetry

All the experiments were performed in 96 Fast PCR Plates full skirt (Sarstedt, Germany) and in CFX96 Real-Time PCR system (BioRad, USA). Screening was conducted (Table S6) to determine the best buffer and ratios of protein and SYPRO® Orange Protein Gel Stain (Sigma-Aldrich, USA). For the experiments, each well contained 25 µL of 10 µM VanS solution, 1/450 X of SYPRO® Orange Protein Gel Stain and lasso peptides at various concentrations: siamycin I – 6 to 90 µM; sviceucin – 12 to 180 µM; microcin J25 – 25 to 200 µM. VanS, SYPRO® Orange and lasso peptides were diluted in 20 mM potassium phosphate buffer (pH 7.5, Table S6). Each condition was replicated three times per plate and each plate was replicated three times. CFX Manager was used to recover data from runs. The T_m of each well was determined on the Melt Curve panel with a negative peak type. T_m means were calculated for each condition before model analysis. ΔT was calculated from the subtraction of the T_m of the protein with the buffer alone and the T_m with the lasso peptides. All analyses were carried out as previously described⁶³.

Autophosphorylation assays for VanS

The phosphorylation assay followed reported procedure with some modifications³⁸. A standard reaction of 200 µL contained 50 mM Tris-HCl (pH 7.8), 100 mM NaCl, 2 µM purified C-His₆-VanS¹²⁷⁻³⁸⁴, 0-100 µM of respective lasso peptide, 5 mM ATP, 10 mM MgCl₂, 0.5 mM NADH, 2 mM phospho(enol)pyruvic acid (PEP), 18 U of L-lactate dehydrogenase (LDH, Roche, Switzerland) and 9 U of pyruvate kinase from rabbit muscle (PK, Sigma-Aldrich, USA). The peptide was pre-incubated with the kinase at room temperature for 10 min followed by the addition of PEP/LDH/PK and incubation at 37 °C for 3 min. The reaction was allowed to start by adding ATP and NADH, and monitored continuously at 340 nm at 37 °C by a microplate reader (POLARstar OMEGA, BMG Labtech, Germany). Each measurement was performed in triplicate, and at least three independent experiments were repeated. Nonlinear regression data fitting and IC₅₀ calculation were performed with GraphPad Prism 10.

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Tables

Table 1: Svceucin and siamycin I reverse vancomycin resistance. MICs of antibiotic alone, in combination with svceucin, or with siamycin I.

Strain	Resistance type	Vancomycin MIC (µg/mL)		
		Control	Svceucin	Siamycin I

<i>E. faecalis</i> V583	VanB	32	2	2
<i>E. faecalis</i> JH2-2 08048	VanB	64	2	<0.5
<i>E. faecalis</i> merz96	VanB	32	1	2
<i>S. aureus</i> T-SAR12 VRSA.B	VanB	64	2	2
<i>E. faecalis</i> 1 231 410	VanA	128	128	2
<i>E. faecalis</i> HIP11704	VanA	1024	512	<0.5
<i>E. faecium</i> 1 231 502	VanA	128	128	2
<i>E. faecium</i> 1 230 933	VanA	512	256	2
<i>S. aureus</i> T-SAR12 VRSA.A	VanA	>512	>512	2
<i>E. faecalis</i> V583 Δ vanB	van-	2	2	2
<i>S. aureus</i> 16038 (VISA)	van-	4	4	4

Table 2. MICs ($\mu\text{g}/\text{mL}$) of vancomycin of Svic1 and SiaA resistant mutants.

Strain	Control	Sviceucin	Siamycin I
V583	32	2	2
Svic1	128	128	2
Svic1 Δ vanB	2	2	2
V583 $vanS_B^{S402I}$	128	128	2
SiaA	128	128	32
V583 $vanS_B^{D398E}$	128	128	16

Table 3. Effect of the phosphomimetic modification VanR^{D53E} on the MICs ($\mu\text{g}/\text{mL}$) of vancomycin.

Strain	Control	Sviceucin	Siamycin I
V583	32	2	2
V583 Δ vanS _B	2	2	2
V583 Δ vanS _B <i>malT::vanR^{D53E}</i>	32	32	32

Table 4. Effect of the signal transduction system on the spectrum of activity of sviceucin and siamycin I on vancomycin MIC ($\mu\text{g}/\text{mL}$).

Strain	Control	Sviceucin	Siamycin I
V583	32	2	2
V583 Δ vanS _B - <i>vanR_B::vanS-vanR</i>	32	32	2
V583 Δ vanB:: <i>vanA</i>	32	2	2

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Supplementary data

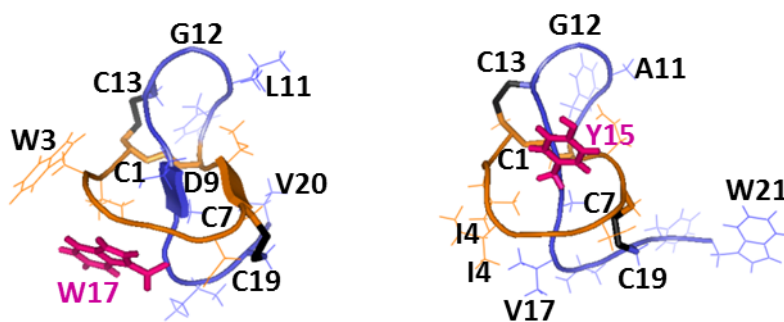


Figure S1. Three-dimensional structures of class I lasso peptides.

Left: svuceucin² (PDB: 2LS1); Right: RP-71955³ (PDB: 1RPC) which is a close homolog of siamycin I (primary sequence: CLGIGSCNDFAGCGYAVVCFW).

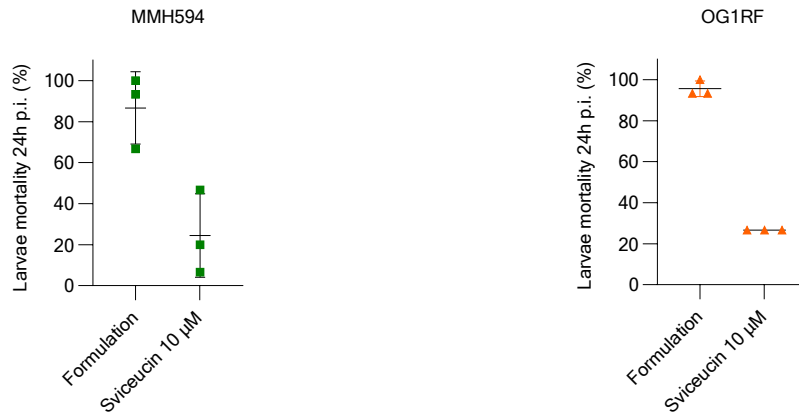


Figure S2. Survival of *Galleria mellonella* larvae infected with *E. faecalis* MMH594 or OG1RF treated with svuceucin.

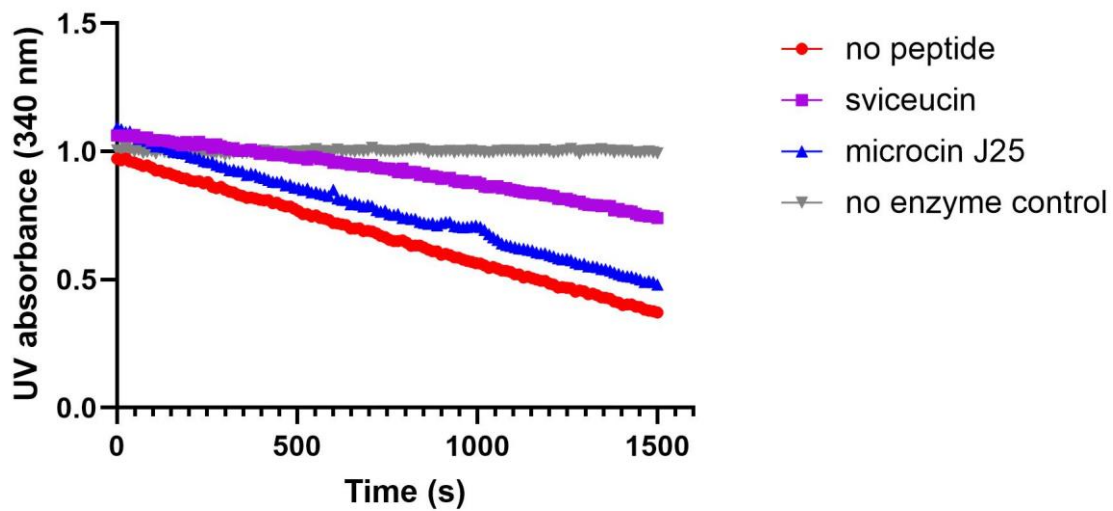


Figure S3. Autophosphorylation activity of VanS¹²²⁻³⁸⁴ in the presence of lasso peptides.

A continuous assay with coupling enzymes was performed as previously described³⁸. Reaction rate was reflected by the consumption of NADH monitored by UV spectroscopy at 340 nm.

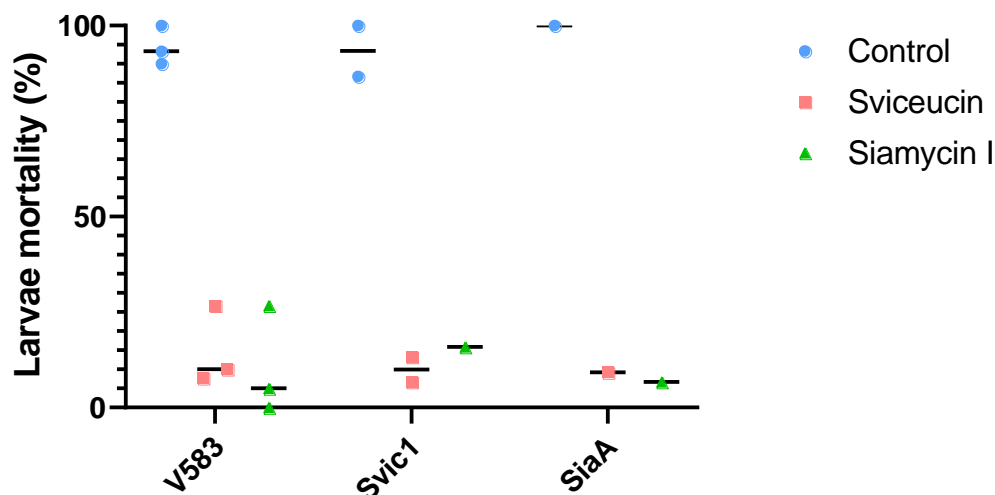


Figure S4. Anti-virulence activity of svceucin and siamycin I on V583, Svic1 and SiaA.

Mortality of *Galleria mellonella* larvae 24 h post-infection (p.i.) with *E. faecalis* V583, mutant Svic1 and mutant SiaA, pre-treated with either siamycin I (2 μ M), svceucin (10 μ M) or formulation (control).

Table S1. Characteristics of the spontaneous mutants resistant to lasso peptides and vancomycin combinations.

Clone no.	Isolation method	Position in v583 genome	V583 reference	Mutation	Mutations consequences	Vancomycin MIC (μ g/ml)	Vancomycin + svceucin MIC (μ g/ml)	Vancomycin + siamycin i MIC (μ g/ml)
V583	n.a.	n.a.	n.a.	n.a.	parental strain	32	2	2
Svic1	5*10 ⁸ plating on vancomycin (32 μ g/ml) and svceucin (10 μ M)	2216922	C	A	VanSB S402I	128	128	2
Svic2		2527311	C	A	non sense mutation in <i>atpA</i>	256	256	2
Svic3		2529182	C	A	non sense mutation in <i>atpF</i>	256	256	2
Svic4		2525799	C	T	<i>atpD</i> RBS modification	256	256	2
SiaA	serial passages	2216933	A	C	VanSB D398E	128	128	32
SiaB		1690453	T	A	CcpA D121V	64	32	32
SiaC		1690372	G	A	CcpA A148V	64	32	32
SiaD		1690550	C	G	CcpA A89P	64	32	32
SiaE		1690580	A	deletion	frameshift in <i>ccpA</i>	32	16	16

Table S2. Strains used for this study. DHR = Double Homologous Recombination, C = Conjugation, R = Resistant, S = Sensitive, V = Variable

Strain	Description	Origin
<i>E. faecalis</i>		
V583	Clinical isolate, PAI (esp ⁻ , cyl ⁻), van ^{RB}	Sahm <i>et al.</i> , 1989
V583 Δ vanB	DHR, van ^S	This study
V583 Svic1	Spontaneous mutant, van ^{RB} , svi ^R , vanS _B ^{S402I}	This study
V583 Svic1 Δ vanB	DHR, van ^S , vanS _B ^{S402I}	This study
V583 SiaA	Spontaneous mutant, van ^{RB} , svi ^R , sia ^R , vanS _B ^{D398E}	This study
V583 vanS _B ^{S402I}	DHR, van ^{RB} , svi ^R , vanS _B ^{S402I}	This study
V583 vanS _B ^{D398E}	DHR, van ^{RB} , svi ^R , sia ^R , vanS _B ^{D398E}	This study
V583 Δ vanS _B ::vanS	DHR, van ^S , Δ vanS _B ::vanS	This study
V583 Δ vanR _B S _B ::vanRS-P _{vanY}	DHR, van ^R , svi ^R , Δ vanR _B S _B ::vanRS-P _{vanY}	This study
V583 Δ vanB::vanA	DHR, van ^{RB} , Δ vanB::vanA	This study
V583 Δ vanS _B	DHR, van ^V , Δ vanS _B	This study
V583 Δ vanS _B malt::vanR ^{D53E}	DHR, van ^{RB} , (svi ^R , sia ^R) Δ vanS _B , malt::vanR ^{D53E}	This study
MMH594	Clinical isolate, PAI+, van ^S	Shankar <i>et al.</i> , 2002
OG1RF	Human isolate, PAI-, van ^S	Dunny <i>et al.</i> , 1978
JH2-2 08048	C, van ^{RB} , <i>E. faecium</i> 08048 van ^{RB} plasmid	Ladjouzi <i>et al.</i> , 2013
Merz96	Clinical isolate, van ^{RB}	Harrington <i>et al.</i> , 2004
1 231 410	Clinical isolate, van ^R	Broad Institute
HIP11704	Clinical isolate, Van ^R	Broad Institute
<i>E. faecium</i>		
1 231 502	Clinical isolate, van ^R	Broad Institute
1 230 933	Clinical isolate, van ^R	Broad Institute
<i>S. aureus</i>		
T-SAR12 VRSA.B	C, van ^{RB} , <i>E. faecium</i> 08048 van ^{RB} plasmid	This study
16038	Clinical isolate, VISA	Caen University Hospital
<i>E. coli</i>		

Top10F'	F ['] {lacIq Tn10 (TetR)} mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL endA1 nupG	ThermoFisher Scientific
EC1000	Δ(araA-leu)7697, *araD139+B/r, Δ(codB-lacI)3, galK16, galE15(GalS), λ-, e14-, relA1, rpsL150(strR), spoT1, mcrB1	Leenhouts <i>et al.</i> , 1996
NEB5α™	fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17	New England BioLabs®
BI21(DE3)	F ⁻ ompT hsdSB (rB-mB-) gal dcm (DE3)	ThermoFisher Scientific

Table S3. Plasmids used for this study.

Plasmid	Description	Origin
pEBM2	oriT, <i>repA</i> TS, lac promoter, lac operator, <i>lacZ</i> , <i>thyA</i> *, Cm ^R For <i>E. coli</i> : replicative For <i>E. faecalis</i> : replicative at 30°C and integrative at 37°C	Lab collection
pEBM2 Δ <i>vanS_B</i>	pEBM2-(<i>vanR_B</i> - <i>VanY_B</i>)	This study
pEBM2 Δ <i>vanS_B::vanS</i>	pEBM2-(<i>vanR_B</i> - <i>VanS</i> - <i>VanY_B</i>)	This study
pEBM2 Δ <i>vanR_BS_B::vanRS-P_{vanH}</i>	pEBM2-(<i>vanR_B</i> upstream- <i>vanR-vanS-P_{vanH}</i> - <i>vanY_B</i>)	This study
pEBM2 <i>malt::P_{hup}-vanR^{D53E}</i>	pEBM2-(start <i>malt-P_{hup}-vanR^{D53E}</i> -end <i>malt</i>)	This study
pAS222	Tet ^R , Amp ^R , <i>repA</i> -pG+ <i>host4</i> For <i>E. coli</i> : replicative For <i>E. faecalis</i> : replicative at 30°C and integrative at 37°C	Jönsson <i>et al.</i> , 2009
PAS222 <i>vanS_B^{S402I}</i>	pAS222-(<i>vanR_B</i> - <i>vanS_B^{S402I}</i> - <i>vanY_B</i>)	This study
PAS222 <i>vanS_B^{D398E}</i>	pAS222-(<i>vanR_B</i> - <i>vanS_B^{D398E}</i> - <i>vanY_B</i>)	This study
pWS3	pG ⁺ <i>host9</i> derivative, spectinomycin ^R , erythromycin ^S	Zhang <i>et al.</i> , 2011

	Suicide vector in <i>E. faecalis</i>	
pWS3 $\Delta vanB$	pWS3-(<i>vanHB-vanXB</i>)	This study
pWS3 $\Delta vanB::vanA$	pWS3-(<i>vanHB-vanA-vanXB</i>)	This study
pET29b(+)	Kan ^R , T7lac, His-tag, S-tag, cleavage of tags by thrombin	(Merck ?)
pET29b(+) <i>vanS</i> ₁₂₇₋₃₈₄	Heterologous production in <i>E. coli</i>	This study
pET29b(+) <i>croS</i> ₁₃₃₋₃₉₃	Heterologous production in <i>E. coli</i>	This study

Table S4. Primers used for this study.

Primers	Sequence	Description	Used in
OCG248	ATCAAGCTTATCGATACCGT	pEBM2 amplification ; pEBM2-upstream <i>vanR_B</i>	pEBM2 $\Delta vanS_B$; pEBM2 $\Delta vanS_B::vanS$; pEBM2 $\Delta vanR_B S_B::vanRS$
OCG249	ATCGAATTCCTGCAGC	pEBM2 amplification	- <i>P_{vanH}</i> ; pEBM2 <i>malt::P_{hup}</i> - <i>vanR^{D53E}</i>
OCG250	GGCGATCCCCGGGCTGCAGGAATTCGATTCGATCGATA CGAATTCTACTTGT	pEBM2- <i>vanR_B</i>	pEBM2 $\Delta vanS_B$
OCG244	GGTCAAAATCATTTAAGTTTTAAATATTACATTATAAT GATTCCTCCAA	<i>vanR_B-vanY_B</i>	
OCG104	CATTATAATGTAAATATTTAAACTTAAATGATTTTGAC C	<i>vanR_B-vanY_B</i>	
OCG251	CTCGAGGTCGACGGTATCGATAAGCTTGATTTGTATT CGGCGACTTT	<i>vanY_B</i> -pEBM2	
M13F	GTAAAACGACGGCCAG	Control	pEBM2 $\Delta vanS_B$; pEBM2 $\Delta vanS_B::vanS$
M13R	CAGGAAACAGCTATGAC	Control	
OCG242	AGGTTACCGATTGGAGGAATCATTATAATGTATATCGT TGCAATTGTTGT	<i>vanR_B-vanS</i>	pEBM2 $\Delta vanS_B::vanS$
OCG243	GGTCAAAATCATTTAAGTTTTAAATATTAGGACCTCC TTTTATCAACCA	<i>vanS-vanY_B</i>	
OHB37	TTGGTTGATAAAAGGAGGTCCTAAATATTTAAACTT AAATGATTTTGAC	pEBM2 $\Delta vanS_B$ amplification	

OCG106	GCAACGATATACATTATAATGATTCTCCAATCGGTAA CC			
OCG110	CGCGGTCGACGCACCAGATAATGATAATCCGAACCTG G	Control		
OCG111	CGCGGCGGCCCGCGGAAGCGCTGTGTCAATCTTTT TCTG	Control		
OHB29	GCGCTATCCAATGTCATATTG	Control		
OHB30	AATCCAGCCAGAACAAAACG	Control		
OBB003	TATGACTTTGACGGTGATGGCA	Control		
OBB004	GCTGGTACGATTGTTATTATGGCAA	Control		
OHB27	AAACGCCGCTGCATACAGTGAG	Control	pEBM2 $\Delta vanS_B::vanS$; pEBM2 $\Delta vanR_B S_B::vanRS$ $-P_{vanH}$	
OHB28	CGCTGGAAGCTCTACCCTAAAC	Control		
OBB056	GGACACAGACGAGCGGAAC	Control		
OBB057	AACTCGGTGGGAGTAAGGGA	Control		
OBB058	AGGGTAGAGCTTCCAGCGAT	Control		
OBB059	GTTTAAGGTCTTTGCCGCCAG	Control		
OSD5	CAGTCACGACGTTGTA AACGACGGC	Control		
OSD6	AACAATTTACACAGGAAACAGCTATGACCATGA	Control		
OBB041	GCTTTGAAACTACAGGAAACTACAGACTGAGGGCT TCATTATACAGGAA	Upstream $vanR_B-vanR$		pEBM2 $\Delta vanR_B S_B::vanRS$ $-P_{vanH}$
OBB016	CACATTGGAATGATAGTTGCTTTTTTCCATAATTAAGA CCAACCCTTTCT	$vanS-vanY_B$		
OBB036	GGCGGATCCCCGGGCTGCAGGAATTCGATGACTGA GGATGGGAATGTAT	pEBM2 upstream $vanR_B$ amplification		
OBB037	CTCGAGGTCGACGGTATCGATAAGCTTGATCAGTCTG TAGTTTCCCTGTA			
OBB039	GCTTTGAAACTACAGGAAACTACAGACTGATGGAA AAAAGCAACTATCA	Upstream $vanR_B-vanY_B$		
OBB018	CTCGAGGTCGACGGTATCGATAAGCTTGATTGGTTGT ATAACGCACAATC	$vanY_B$ -pEBM2		
OBB038	CAGTCTGTAGTTTCCCTGTAGT	pEBM2 upstream $vanR_B-vanY_B$ amplification ;		

		upstream <i>vanR_B-vanY_B</i>	
OBB040	ATGGAAAAAAGCAACTATCATTCC	pEBM2 upstream <i>vanR_B-vanY_B</i> <i>amplification</i>	
OBB042	GTATCTCTTATAGAGGAGGTGAAATGATCCATGTCGA TACGAATTCTACT	pEBM2- ΔP_{tuf} - <i>vanR^{D53E}</i> <i>amplification</i>	pEBM2 <i>malT::vanR^{D53E}</i>
OBB043	GAATATCTAAAAAAGTATGGTAGAATGCATGTTCTT GTTAACAATTCAA		
OBB044	TGCATTCTACCATACTTTTTTTAGAT	pEBM2- <i>P_{hup}</i>	
OBB045	GGATCATTTACCTCCTCTA	<i>P_{hup}-vanR^{D53E}</i>	
OCG224	GTAATGCCCGCAGCGGG	Control	
OCG231	CCATTTCTCCAGGTGTTGCG	Control	
OBB055	GAGTGGTCCAAAAACAGACAGCAA	Control	
OCG199	TATTCTTGAGATTATGCTGCCCGGTATGAATG	Control	
OCG225	GCGTTTGGGTCGCTATGGGAC	Control	
OSD5	CAGTCACGACGTTGTAAAACGACGGC	Control	
OSD6	AACAATTTACACAGGAAACAGCTATGACCATGA	Control	
OHB31	TTCAATTCGCTCATAATTAATCC	pAS222 <i>amplification</i>	pAS222 <i>vanS_B^{D398E}</i> ; pAS222 <i>vanS_B^{S402I}</i>
OHB32	AAAGGGGATTTTATGCGTGG		
OHB35	CCCTCGAGGTCCACGCATAAAATCCCCTTTATATCAA AATCTGCGGGCG	pAS222- upstream <i>vanS_B³⁹⁸</i>	
OHB36	GTTGTAAAGACTCTCGCGAGTTTATCAGGATCGGAC AGGACAATTTGTC	Upstream <i>vanS_B³⁹⁸-vanS</i> C1	
OHB37	TTGGTTGATAAAAGGAGGTCCTAAATTTAAACTT AAATGATTTGAC	<i>vanS</i> C1- downstream <i>vanS_B⁴⁰²</i>	
OHB38	GAAAAAGGATTAATTATGAGCGAATTGAATGTCTCAT TGGAAGCAGGAAC	Downstream <i>vanS_B⁴⁰²-</i> pAS222	
OCG218	CGGTGATGGCAGCACAGTCC	Control	
OHB30	AATCCAGCCAGAACAAAACG	Control	

OCG70	AAAGGGGTGACCCGTATGGAAAATGGTTTTTTGTTT TTAG	<i>vanA</i> - downstream <i>vanB</i>	
OCG71	AGTCATGCAACCGTGACAATCAGCAGATTC	Downstream <i>vanB</i>	
OCG72	CGCGGTCGACCCTGACAGAGCAGCTACTTGACCTTC	Downstream <i>vanB</i>	pWS3 Δ <i>vanB</i> ; pWS3 Δ <i>vanB</i> :: <i>vanA</i>
OCG73	CGCGGAATTCGGTACCGGGGAAATAATAGATTGCAA GC	Upstream <i>vanB</i>	
OCG92	TCCATACGGGGCTGTAAACTCCTTTCAAAGTTAAGAC AAT	Upstream <i>vanB</i>	pWS3 Δ <i>vanB</i>
OCG93	AGTTTACAGCCCCGTATGGAAAATGGTTTTTTGTTTT TAG	Downstream <i>vanB</i>	
2297L	TAGCGGAGAATCCAGAGGAA	<i>vanY_B</i>	<i>vanY_B</i> , RT-qPCR
2297R	GGGGTTCTGCCTGTTCACTA		
1818L	TAGGACAATCAGGTGCGTTG	<i>gelE</i>	<i>gelE</i> , RT-qPCR
1818R	CACTCTGAGTATCCGCACCA		
GyrAL	GATGGGGAAATCAGGGATTC	<i>gyrA</i>	<i>gyrA</i> , RT-qPCR
GyrAR	TCTTTTCCATTCGGCATTTC		

Table S5. Physicochemical characteristics of the uncharged and C1LP containing nanoemulsions.

	Z-average diameter (nm)	PDI	Zeta potential (mV)	Vancomycin MIC on <i>E. faecalis</i> V583
Blank NEs	54 ± 2	0.21 ± 0.02	-16 ± 4	32
Sviceucine-NEs	52 ± 1	0.17 ± 0.03	-15 ± 5	4
Siamycin-NEs	51 ± 1	0.17 ± 0.01	-17 ± 1	4

Table S6. Buffers screened for DSF.

Buffers	Final concentration
Tris pH 7.5	Tris 50 mM
	Glycerol 15 %
Tris NaCl pH 7.5	Tris 50 mM

	Glycerol 15 %
	NaCl 150 mM
Tris MgCl ₂ pH 7.5	Tris 50 mM
	Glycerol 15 %
	MgCl ₂ 5 mM
Tris NaCl/MgCl ₂ pH 7.5	Tris 50 mM
	Glycerol 15 %
	NaCl 150 mM
	MgCl ₂ 5 mM
Potassium phosphate pH 7.5	Potassium phosphate 20 mM
	Glycerol 10 %
Potassium phosphate NaCl pH 7.5	Potassium phosphate 20 mM
	Glycerol 10 %
	NaCl 500 mM
Potassium phosphate MgCl ₂ pH 7.5	Potassium phosphate 20 mM
	Glycerol 10 %
	MgCl ₂ 5 mM
Potassium phosphate NaCl/MgCl ₂ pH 7.5	Potassium phosphate 20 mM
	Glycerol 10 %
	NaCl 500 mM
	MgCl ₂ 5 mM
Sodium phosphate pH 7.5	Sodium phosphate 20 mM
	Glycerol 10 %
Sodium phosphate NaCl pH 7.5	Sodium phosphate 20 mM

	Glycerol 10 %
	NaCl 100 mM
Sodium Citrate pH 7.5	Sodium citrate 50 mM
	Glycerol 10 %
HEPES pH 7.5	HEPES 50 mM
	Glycérol 10 %
HEPES NaCl pH 7.5	HEPES 50 mM
	Glycérol 10 %
	NaCl 100 mM
HEPES MgCl ₂ pH 7.5	HEPES 50 mM
	Glycérol 10 %
	MgCl ₂ 5 mM
HEPES NaCl/MgCl ₂ pH 7.5	HEPES 50 mM
	Glycérol 10 %
	NaCl 100 mM
	MgCl ₂ 5 mM
PIPES pH 7.5	PIPES 50 mM
	Glycerol 10 %
MOPS pH 7.5	MOPS 50 mM
	Glycerol 10 %
MOPS NaCl pH 7.5	MOPS 50 mM
	Glycerol 10 %
	NaCl 10 mM
MOPS MgCl ₂ pH 7.5	MOPS 50 mM

	Glycerol 10%
	MgCl ₂ 5 mM
MOPS NaCl/MgCl ₂ pH 7.5	MOPS 50 mM
	Glycerol 10 %
	NaCl 10 mM
	MgCl ₂ 5 mM