Siamycin attenuates *fsr* quorum sensing mediated by gelatinase biosynthesis-activating pheromone in *Enterococcus faecalis*

Running title: Siamycin attenuates *E. faecalis fsr* quorum sensing

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ABSTRACT

The expression of two Enterococcus faecalis virulence-related proteases, gelatinase (GelE) and serine protease (SprE), is positively regulated by a quorum sensing system encoded by the fsr gene cluster. In this system, E. faecalis secretes an autoinducing peptide, gelatinase biosynthesis-activating pheromone (GBAP), which triggers the FsrC-FsrA two component regulatory system controlling the expression of two transcripts, fsrBDC and gelE-sprE. In the present study, we screened for inhibitors of the fsr quorum sensing from actinomycetes metabolites. E. faecalis was cultured with each tested actinomycetes culture supernatant and the productions of gelatinase and GBAP were tested for the first and second screenings, respectively. Culture supernatant of Streptomyces sp. Y33-1 showed the most potent inhibitory effect on both gelatinase and GBAP productions without inhibiting E. faecalis cell growth. The inhibitor in the culture supernatant was identified as a known peptide antibiotic, siamycin I. Siamycin I inhibited both gelatinase and GBAP productions at submicromolar concentrations, and inhibited E. faecalis cell growth at concentrations above micromolar concentrations. Quantitative analysis of fsrBDC and gelE-sprE transcripts revealed that siamycin I suppressed the expression of both transcripts at the sublethal concentration. Siamycin I attenuated gelatinase production even when an overdose of GBAP was exogenously added to the culture. These results suggested that siamycin I inhibited the GBAP-signaling via FsrC-FsrA two-component regulatory system in a noncompetitive manner. The sublethal concentrations of siamycin I also attenuated biofilm formation. Siamycin would offer a novel means of treating enterococcal infections.

INTRODUCTION

Enterococcus faecalis is a gram-positive intestinal bacterium commensal of human and other animals but sometimes causes opportunistic infections, including urinary tract, bloodstream
and wound infections, endoophthalmitis and endocarditis (22). Notably, in the past two decades, nosocomial infections caused by multiple antibiotic resistant or vancomycin resistant *E. faecalis* have become a serious clinical problem (6, 33, 36, 49).

Besides cytolysin, which is lethal by itself for a broad range of prokaryotic and eukaryotic cells (10), several virulence-related factors have been described in *E. faecalis*, including aggregation substance (Agg), enterococcal surface protein (Esp) and two extracellular proteases, gelatinase (GelE) and serine protease (SprE) (35, 59). These factors have been thought to act synergistically to enhance virulence by facilitating colonization, translocation and biofilm formation (8, 16, 23, 28, 34, 35, 52, 60, 62, 65). GelE and SprE are encoded in an operon, *gelE-sprE*, whose expression is positively regulated by a quorum sensing system encoded for by the *fsr* locus (45, 46). Several *in vivo* studies have shown contribution of the *fsr* system to virulence by using an animal or nematode models (17, 19, 37, 46, 53).

The *fsr* locus is comprised of four genes, designated *fsrA, fsrB, fsrC* and *fsrD* (38, 40, 45, 46). In this system, a cyclic peptide, gelatinase biosynthesis-activating pheromone (GBAP) acts as an autoinducer (38, 39). It was proposed that the prepropeptide of GBAP is translated from *fsrD* and then processed and cyclized by FsrB, resulting in mature form of GBAP (40). When the concentration of GBAP accumulated outside cells reaches a threshold level that is around one nanomolar, it triggers the two component regulatory system consisting of a histidine kinase (FsrC) and a response regulator (FsrA). The activated FsrA induces the expression of *fsrBDC* transcript, which is involved in an autoregulatory circuit resulting in a boost of GBAP signaling, and eventually induces the expression of *gelE-sprE* transcription.

Quorum sensing has been recently proposed as a new target for antimicrobial drug therapy (42, 48, 56). This kind of compound, which would attenuate virulence without bactericidal or bacteriostatic activity, is called “antipathogenic”. For example, macrolides such as azithromycin, which inhibit *N*-acylhomoserine lactone-mediated quorum sensing but do not
inhibit the growth of *Pseudomonas aeruginosa*, are known to efficiently decrease symptoms of
cystic fibrosis (CF) and diffuse panbronchiolitis (DPB) (57, 58). Furthermore, a number of
other studies have already revealed inhibitors targeting *N*-acylhomoserine lactone-mediated
quorum sensing of gram-negative bacteria (26, 43, 50, 54, 55). In the case of gram-positive
pathogens, quorum sensing inhibitors have been investigated with staphylococci, which have a
well-known regulatory system designated *agr* (32, 41). The *agr* system is mediated by a cyclic
peptide pheromone as is the enterococcal *fsr* system and positively regulates expression of
some virulence factors via a regulatory RNA molecule called RNA-III. Lyon et al. has
successfully attempted rational design of a peptide antagonist of the *agr* pheromone (31, 32).
An RNA-III inhibiting peptide (RIP) found in culture filtrates of some staphylococcal strains is
also expected to be an anti-staphylococcal agent (1, 4, 9, 13, 21, 63).

In the present study, we screened inhibitors for the *E. faecalis* *fsr* quorum sensing from
actinomycetes culture supernatants, because actinomycetes are rich sources of biologically
active compounds. To our knowledge, this is the first screening study to target natural
compounds for quorum sensing inhibitor against gram-positive pathogen.

**MATERIALS AND METHODS**

*E. faecalis* strains, media and culture condition. *E. faecalis* OG1RF was used as a standard
gelatinase-positive strain in this study (15). *E. faecalis* OU510 was a clinical isolate with the
*fsrB* mutation resulting in the lack of GBAP production and was used as an indicator strain for
GBAP assay because gelatinase production of this strain completely depends on exogenously
added GBAP (40). *E. faecalis* OU510B was OU510 carrying *fsrB* translationally fused to
pNZ8048 *Nco* I site (29). This strain was used for the screening of *fsr* quorum sensing inhibitors
because its high activity of gelatinase and GBAP. Except for liquid chromatography/mass
spectrometry (LC/MS) experiment and biofilm formation assay, an overnight culture of *E.*
faecalis was inoculated into Todd-Hewitt Broth (THB; Oxoid, Hampshire, UK) to an OD660 of 0.01 and was then cultivated at 37°C with gentle shaking. For LC/MS experiment, E. faecalis was cultivated in a chemically defined medium (CDM) established for Lactobacillus plantarum (27). CDM overnight culture (0.5 ml) of E. faecalis OG1RF was inoculated into 10 ml of fresh CDM and was grown at 37°C for 7 h with gentle shaking.

**Isolation and culture of actinomycetes.** Soil samples were collected from 33 different places in Kyushu, Japan. One hundred milligrams of the soil samples were suspended in 1 ml of phosphate-buffered saline (PBS) by 10-sec vortexing, 30-sec sonication and 15-min gentle mixing at 37°C in eppendorf tubes. Then, 100 µl of the suspensions were mixed with 900 µl of SDS-YE solution [0.05% SDS (wt/vol), 6% yeast extract (wt/vol), 5 mM PBS] and incubated at 40°C for 20 min with gentle mixing. Serial dilutions of the suspension were spread onto humic acid-vitamin agar medium (24), Bennet agar medium [peptone, 2% (wt/vol); yeast extract, 0.1% (wt/vol); meat extract, 0.1% (wt/vol); glucose, 1% (wt/vol), cycloheximide, 50 mg/L, agar, 1.5% (wt/vol); pH 7.2], and yeast-starch agar medium [yeast extract, 0.2% (wt/vol); soluble starch, 1% (wt/vol); glucose 1% (wt/vol), cycloheximide, 50 mg/L, agar, 1.5% (wt/vol); pH 7.2]. These agar plates were incubated at 30°C for one to two weeks. As a result, 179 actinomycetes strains were isolated in total. For the preparation of culture supernatant used for the screening, actinomycetes isolates were inoculated into three different liquid media (5 ml) of Bennett, yeast-starch, and humic acid-vitamin and were grown aerobically at 30°C for 3 days, 7 days, or 14 days.

For the production of siamycin I, Streptomyces sp. Y33-1 was precultured in 10 ml of Bennett liquid medium at 30°C for one week. One milliliter of the preculture was inoculated into 100 ml of same medium in a flat-bottom flask. In total, ten flasks were prepared for 1-L culture. The culture was grown aerobically at 30°C for one week.

**Assay for gelatinase and GBAP.** Gelatinase activity was measured by using azocoll
(Calbiochem, San Diego, Calif.) as a substrate for gelatinase according to the protocol described previously (38). Briefly, 25 µl of E. faecalis culture supernatant was added to 0.5 ml of azocoll suspension, incubated for 4 h with constant mixing (170 rpm), centrifuged at 20,000 x g for 5 min and then OD$_{540}$ of the supernatant was measured.

For the screening of fsr quorum sensing inhibitors, 4 ml of fresh THB medium was mixed with 1 ml of culture supernatant of tested actinomycetes and was inoculated into an overnight culture (30 µl) of E. faecalis OU510B. After 5-h incubation at 37°C, the culture supernatant was collected by centrifugation at 6,500 x g for 5 min. For the first screening to examine the inhibition of gelatinase production, 25 µl of the OU510B culture supernatant was directly subjected to the measurement of gelatinase activity. For the second screening to examine the inhibition of GBAP production, 2 ml of the OU510B culture supernatant was applied onto a Sep-Pak Plus C18 cartridge column (100 mg; Waters, Milford, Mass.), washed with 2 ml of 20% (vol/vol) acetonitrile containing 0.1% (vol/vol) trifluoroacetic acid (TFA), and was then eluted by 1 ml of 40% acetonitrile containing 0.1% TFA. The eluate was dried up by a Speed-vac concentrator, redissolved in 5 ml of fresh medium, and was then inoculated by 30 µl of E. faecalis OU510 overnight culture. After 5-h incubation at 37°C, gelatinase activity was measured with 25 µl of the OU510 culture supernatant as described above and the gelatinase activity was taken as GBAP activity.

GBAP was also measured by LC/MS. CDM culture supernatant (10 ml) was loaded into a Sep-Pak Plus C18 cartridge column (360 mg; Waters), washed with 10 ml of 10% acetonitrile containing 0.1% TFA and was then eluted by 5 ml of 60% acetonitrile containing 0.1% TFA. The eluate was evaporated to dryness and then redissolved in 200 µl of 10% acetonitrile containing 0.1% TFA, and 80 µl of this solution was injected to LC/MS (LC: Agilent HP1100, column: Agilent Zorbax Eclipse XDB-C18, 2.1 x 50 mm, MS: JEOL Accutof T100LC, Tokyo, Japan). The column was eluted at a flow rate of 0.2 ml/min at 30°C with a linear gradient of
acetonitrile (20 to 40% in 20 min after 5 min of 20%) in 0.05% TFA aqueous solution. The eluates were directly loaded into the electrospray ionization-time of flight mass spectrometer. Mass analyses were performed under the following conditions: positive polarity, capillary temperature 260°C, needle voltage of 2.0 kV, orifice voltage of 70 V, and ring lens voltage of 10 V. After scanning for molecular ions derived from column eluates in the \( m/z \) range of 100 to 2,000, extracted ion chromatograms were plotted with detector counts at \( m/z \) ranging from 1303.5 to 1304.2 which covers a protonated molecular ion of GBAP. GBAP was detected at 19 min.

**Purification of siamycin I.** The culture supernatant of *Streptomyces* sp. Y33-1 (850 ml) was collected by centrifugation at 6,520 x \( g \) and applied to an Amberlite XAD-7 column (50 cc; Sigma, St. Louis, Mo.). The column was eluted by 300 ml of methanol. The eluate was evaporated to dryness, redissolved in 50 ml of Milli-Q water and was then applied to Sep-Pak Vac C18 cartridge column (35 cc, 10 g; Waters). After washing with 150 ml of 35% acetonitrile containing 0.1% TFA, the column was eluted with 50 ml of 50% acetonitrile containing 0.1% TFA. The eluate was evaporated, lyophilized and then redissolved in 50 ml of 10% acetonitrile containing 0.1% TFA. The solution was divided into five aliquots and each was applied to reverse-phase HPLC (Inertsil ODS-3, 20 x 150 mm; GL Sciences Inc., Tokyo, Japan). The column was developed by a gradient of 10 to 80% acetonitrile in 0.1% TFA for 35 min at 10 ml/min. The active fractions were pooled, lyophilized, and then rechromatographed in the same column by a gradient of 40 to 65% acetonitrile in 0.1% TFA for 35 min. Finally, 2.8 mg of siamycin I was purified.

**NMR experiment.** NMR spectra were measured on a Varian Unity INOVA600 spectrometer at 10°C and 40°C. Sample solution was prepared at a concentration of 2 mM in 50% (vol/vol) \( \text{CD}_3\text{OD} \) and 50% (vol/vol) \( \text{H}_2\text{O} \). Chemical shifts were referenced to the water resonance (4.92 and 4.63 ppm at 10 and 40°C, respectively). Standard \( ^1\text{H}-^1\text{H} \) homonuclear NMR methods were
used to obtain a series of 2D spectra of double quantum filtered correlation spectroscopy (DQF-COSY), total correlation spectroscopy (TOCSY) [mixing time = 45 ms], and nuclear Overhauser enhancement spectroscopy [mixing time = 300 ms].

**Effect of siamycin I on NisK-NisR two-component regulatory system.** *Escherichia coli* lacZ gene was cloned into Neo I and Sph I sites of pNZ8048 (29) and the resultant plasmid named pNZ8048lacZ was introduced into *E. faecalis* OG1RF together with pNZ9530 carrying nisRK. The resultant strain, OG1RF( pNZ8048lacZ+pNZ9530) was cultured in THB medium containing 3 nM of nisin A and various concentrations of siamycin I for 5 h at 37°C. The nisin-induced β-galactosidase activity was measured and expressed as Miller unit according to the method described in the web-site (http://rothlab.ucdavis.edu/protocols/beta-galactosidase-3.html).

**Real-time quantitative reverse-transcription-PCR (RT-PCR).** An overnight culture (30 µl) of *E. faecalis* OG1RF was inoculated into 5 ml THB medium with or without 1 µM of siamycin I and grown for 4 h. Cells were harvested by centrifugation at 8,500 x g for 5 min. Total RNA was extracted from the harvested cells according to the method described in a previous paper (38) and finally dissolved in 50 µl of diethyl pyrocarbonate (DEPC)-treated water to yield about 10 µg RNA/µl. Five microliters of this solution were treated by 1 µl of DNase (Nippon gene, Tokyo, Japan) in a total reaction volume of 50 µl according to the manufacturer’s instruction. After 15-min incubation at 37°C, 5 µl of stop solution was added to the reaction solution and was then incubated at 70°C for 10 min. Transcription level of 16S rRNA, fsrBDC and gelE-sprE were quantified by one-step real-time RT-PCR. The RT-PCR was done in duplicate for each RNA sample with one step SYBR RT-PCR kit (Takara, Kyoto, Japan). For quantification of fsrBDC or gelE-sprE, 1 µl of the DNase-treated RNA solution was used as a template in 17 µl of total reaction mixture containing 0.2 µM of primers, fsrBF1 (5’-TGGATCAGGAAGATCAATCAGG-3’) and fsrBR1
(5’-GTACGACGTATAACAATAAAGGTTTCG-3’), or gelEF
(5’-AGTGAACGCTACAGATGGAAC-3’) and gelER
(5’-CGTTCCGTGTAAAGCAATTCC-3’), respectively. For 16S rRNA, the DNase-treated RNA solution was diluted 100-times and 1 µl was used as a template in 17 µl of total reaction mixture containing 10 µM of primers, Enc-F-rt (5’-CCCTTATTGTTAGTGCCATCATT-3’)
and Enc-FR-rt (5’-ACTCGTTGTACTTCCCATTGT-3’) (51). PCR reaction, real-time monitoring of amplified product and comparative quantitation were carried out in a real-time PCR system (Mx3000; Stratagene, La Jolla, Calif.) with MxProTM software Ver. 3.00. Relative expression level was calculated from standard curve constructed with serial dilutions of the RNA sample prepared from the culture without siamycin I.

**Biofilm formation assay.** Assays were performed by using the protocol of Seno et al. (52) with minor modifications. Briefly, *E. faecalis* OG1RF that had been grown overnight was diluted 1:100 in 200 µl of tryptic soy broth supplemented with 0.25% (wt/vol) glucose and inoculated into wells of a sterile flat-bottomed 96-well polystyrene microtiter plate (Corning Inc., Corning, N.Y.). Siamycin I was dissolved in methanol (10 µg/µl) and added to the medium at final concentrations of 0.125, 0.25, 0.5 or 1 µM. After 24 h of static incubation at 37°C, plates were processed, stained with 0.3% crystal violet for 45 min, and rinsed with distilled water. The bound dye was solubilized in ethanol-acetic acid (95:5, vol/vol), and optical density at 570 nm using a microplate reader (model 680; Bio-Rad Japan, Tokyo, Japan) was determined. The effect of methanol was examined in the absence of siamycin I and it was confirmed that methanol did not show significant effect on the biofilm formation in the concentration range used in this experiment. Each assay was performed in quadruplicate on two occasions.

**RESULTS**

**Screening of inhibitors targeting fsr quorum sensing from actinomycetes culture**
supernatants. One hundred and seventy-nine actinomycetes strains were isolated from several soil samples in Kyushu, Japan and subjected to screening. In the first screening, *E. faecalis* was grown with culture supernatant of each isolate and gelatinase production was examined. Twenty-two strains showed an inhibitory effect on gelatinase production without growth inhibition. In the second screening of these 22 strains, the inhibitory effect of culture supernatant on GBAP production was examined and three strains significantly inhibited GBAP production. Among them, *Streptomyces* sp. Y33-1, which showed the most potent inhibitory effect on GBAP production, was selected for further study.

The inhibitor in Y33-1 culture supernatant was purified and subjected to structural analysis. Ultraviolet absorption spectrum of this compound showed a typical profile of peptidic compound containing tryptophan residue, which exhibited a shoulder around 220 nm and a peak at 280 nm. Mass spectrometry suggested the molecular mass of this compound to be 2164, which is identical to those of siamycin I (14, 61), NP-06 (7) and RP-71955 (18). These compounds had been previously screened with anti-HIV activity and are known to have antimicrobial activity against Gram-positive bacteria as well (7, 18, 30, 61). Their structures were found to be tricyclic peptides consisting of 21 amino acids. An identical structure has been reported for siamycin I and NP-06. RP-71955 differs from the others only at residue No.4 and No.17. A series of $^1$H-$^1$H 2D-NMR analyses, DQF cosy, TOCSY, and NOE, allowed assignment of almost all protons in the inhibitor of Y33-1 (Table 1). The indicated chemical shifts were mostly identical to those of NP-06 (7) and the chemical shifts of Val-4 and Ile-17 protons were clearly different from those of Val-17 and Ile-4 protons of RP-71955 (18). These differences in chemical shift agreed with the data reported in the previous study comparing NP-06 with RP-71955 (7). As a result, the inhibitor of Y33-1 was identified as siamycin I and was henceforth referred to as siamycin I.

**Effect of siamycin I on growth and gelatinase production in *E. faecalis***. Siamycin I
slightly inhibited the growth of *E. faecalis* at 1 µM (80% growth 5 h after inoculation) and completely inhibited it at 5 µM (no growth 12 h after inoculation). This growth inhibitory activity coincides with the data previously reported (61). Figure 2 shows the effect of various concentrations of siamycin I on gelatinase production of *E. faecalis* OG1RF. Siamycin I slightly inhibited gelatinase production at 10 nM and strongly inhibited it at 1 µM, in which IC$_{50}$ was around 100 nM.

Figure 3 shows the time course of cell growth, pH and gelatinase activity of *E. faecalis* culture with or without 1 µM siamycin I. The inhibition of gelatinase production was observed to the same extent until the end of this time course experiment (7h, nearly stationary phase). This indicated that the siamycin I did not delay the onset of gelatinase production but constitutively inhibited the production. As shown by the dotted lines in Fig. 3, the pH of culture supernatant was decreased during the cell growth, which was due to lactate fermentation by *E. faecalis* cells. The pH decrease was slightly slower in the presence of siamycin I as well as cell growth, while gelatinase production was greatly reduced by the addition of siamycin I. It is likely that the inhibition of gelatinase production by the sublethal concentration of siamycin I is due to more than the pleiotropic effect on general metabolism.

**Inhibitory effect of siamycin I on GBAP production and GBAP response of *E. faecalis*.**

The effect of siamycin I on GBAP production was examined by LC/MS (Fig. 4). Siamycin I showed slightly inhibited GBAP production at 10 nM and abolished its production at 1 µM. Taken together, siamyicin I attenuates GBAP production as well as gelatinase production at sublethal concentrations. These results suggested the following two possibilities: 1) siamycin I inhibits the FsrB function for GBAP biosynthesis; 2) siamycin I inhibits the GBAP signal transduction via FsrC-FsrA two component regulatory system. In order to clarify this point, the effect of siamycin I on GBAP responsiveness of *E. faecalis* cells was examined by using *E. faecalis* OU510 which has amber mutation on fsrB resulting in the loss of GBAP production. In
this strain, induction by exogenous GBAP is necessary for gelatinase production. In this experiment, 100 nM of GBAP (dose ten-fold higher than that in late-log phase culture) and various concentration of siamycin I was added to early-log phase culture at the same time. The culture was continued for an extra 3 h and then the activity of gelatinase in the culture supernatant was measured. As shown in Fig. 5, siamycin I inhibited the GBAP-induced gelatinase production at concentrations higher than 100 nM. This suggested that the site of action of siamycin I is not on GBAP biosynthesis but on the GBAP signal transduction.

The inhibitory mode of siamycin I was examined by measuring the GBAP-induced gelatinase production in the presence of 1 µM of siamycin I and various concentrations of GBAP. As shown in Fig. 6, even in the presence of 1 mM GBAP (ca. 1000 titer), 1 µM of siamycin I inhibited the GBAP-induced gelatinase production. This suggested that siamycin I is not a competitive inhibitor of GBAP.

**Effect of siamycin I on NisK-NisR two-component regulatory system heterologously expressed in *E. faecalis*.** To address the specificity of inhibitory effect of siamycin I, NisK-NisR two-component regulatory system originated from *Lactococcus lactis* (29) was heterologously expressed in *E. faecalis* and the effect of siamycin I on the NisK-NisR signal transduction was examined. In the reporter strain, the lacZ gene was cloned under the nisin-inducible nisA promoter controlled by the NisR-NisK two-component regulatory system. This reporter strain was cultured with 3 nM of nisin A and various concentrations of siamycin I and the nisin-induced β-galactosidase activity was measured. As shown in Fig. 7, the induction of β-galactosidase was partially reduced at 400 nM of siamycin I and mostly abolished at 600 nM of siamycin I. This result indicated that siamycin I inhibited the NisK-NisR two-component regulatory system as well as the FsrC-FsrA system. However, siamycin I did not affect the induction of β-galactosidase at 100 nM and 200 nM whereas it clearly reduced gelatinase production at the same concentrations, suggesting the NisK-NisR two component regulatory
system was less sensitive to siamycin I than the FsrC-FsrA system.

**Effect of siamycin I on transcription of *fsrBDC* and *gelE-sprE***. The effect of siamycin I on the transcription of *fsrBDC* and *gelE-sprE* was examined by using real-time quantitative RT-PCR. As shown in Fig. 8, both transcriptions were strongly suppressed. The transcription of *gelE-sprE* was more strongly suppressed than that of *fsrBDC*. This result may reflect the signal transduction pathway in which *fsr* regulatory system positively regulates *gelE-sprE* expression.

**Effects of siamycin I on cell morphology and biofilm formation of *E. faecalis***. Effect of siamycin I on cell morphology was examined by microscopy. In the absence of siamycin I, cell chain length of *E. faecalis* was mostly between two to four. On the other hand, in the presence of 0.5 or 1.0 µM siamycin I, the cell chain length was clearly increased to around ten (data not shown).

The effect on biofilm formation of *E. faecalis* was also examined. As shown in Fig. 9, siamycin I slightly inhibited biofilm formation at 0.25 µM and the inhibitory effect was marked at the concentrations higher than 0.5 µM.

**DISCUSSION**

To target antipathogenic compounds against *E. faecalis*, we screened our actinomycetes collection for inhibitors of GBAP-mediated quorum sensing. Inhibitions of gelatinase production and GBAP production were assayed for the first and second screening, respectively. The two-step screening allowed for efficient screening for inhibitors targeting the GBAP-mediated quorum sensing. This screening system possibly detects two types of inhibitors; one targets FsrC-FsrA two component regulatory system and the other one targets FsrB, which is the biosynthetic enzyme of GBAP. These two can be distinguished by adding physiological concentrations of GBAP at the same time as the addition of tested sample. Unlike the case of siamycin I, if the inhibitor targets FsrB, gelatinase production can be recovered.
Recently, it has been demonstrated that FsrB belongs to AgrB protein family which is predicted to have a cysteine protease-like function (40, 47). Inhibitors targeting FsrB would be effective against some other gram-positive bacteria including staphylococci, which use cyclic peptide-mediated quorum sensing (32, 47).

As a result of screening, a known secondary metabolite of actinomycetes, siamycin I, was found as a potent inhibitor of GBAP-mediated quorum sensing. Three varieties of siamycin have been screened as anti-HIV compounds and found to be tricyclic peptides consisting of 21 amino acids. They differ from one another only at position 4 (Val or Ile) and 17 (Val or Ile), whose difference is unlikely to make much of a difference in terms of conformational and functional properties (12). Due to their structural similarity, the other siamycins may inhibit GBAP-mediated quorum sensing.

The target molecule of siamycins in anti-HIV activity has been proposed to be HIV envelope glycoprotein gp41 because siamycins exhibit a strong inhibitory effect on syncytium formation and have a sequence similarity to a part of gp41 (7, 11, 18). However, unlike between siamycin and gp41, there is no sequence similarity between siamycin I and GBAP. The overdose of GBAP could not outcompete the inhibition by siamycin I. These suggested that the inhibitory mode is not caused by specific binding of siamycin I to the GBAP-binding site on FsrC. It has been reported that siamycins also inhibited the growth of gram-positive bacteria in micromolar concentrations but did not on gram-negative bacteria (61). Although no information about the antibacterial mode of action has been reported, the antibacterial property specific to gram-positive bacteria is well known in bacteriocins which are membrane-active peptides found from many gram-positive bacteria. As in the case of bacteriocins, siamycins may also be integrated into the cell membrane and inhibit the growth of gram-positive bacteria (25). At sublethal concentrations, siamycins may disturb receptor kinase function of FsrC by localizing in the cell membrane. It was speculated that this mode of action would be commonly effective...
on the other membrane kinases. As expected, the NisK-NisR two-component regulatory system heterologously expressed in *E. faecalis* was also inhibited by sublethal concentrations of siamycin I. However, the FsrC-FsrA two component regulatory system was highly sensitive compared to the NisK-NisR system, suggesting the possibility of specific interaction between siamycin I and FsrC.

At the sublethal concentrations, siamycin I inhibited GBAP and gelatinase production of *E. faecalis*, while it did not have great influence on the cell growth and acid production. Transcriptional analysis also indicated that sublethal concentrations of siamycin I inhibited the expression of *fsrBDC* and *gelE-sprE* while it slightly reduced the 16S rRNA expression. These results suggested that the inhibitory effect of sublethal concentration of siamycin I on *fsr* system is due to more than the pleiotropic effect on general metabolism. How specific siamycin I inhibits transcription would be aid in understanding the mode of action of siamycin. Nowadays, DNA microarray analysis is available (5) and provides us the information of genes suppressed by siamycin I. This kind of information would allow us to understand the precise mode of action of siamycin I, especially, whether siamycin I targeted only *fsr* regulatory system or other genes under a different regulatory system.

The effects of siamycin I on cell morphology and biofilm formation were also examined and it was found that siamycin I led to increased cell chain length and decreased biofilm formation at sublethal concentrations. It has been reported that a knockout of *gel* or *fsr* led to the same changes (23, 44, 64). This coincidence may suggest that the observed effects of siamycin were due to the inhibition of *fsr* quorum sensing associated with gelatinase induction. However, it should be noted that a number of factors other than gelatinase are involved in the biofilm formation of *E. faecalis* (52) and siamycin I may also influence some of those factors. Since pathogenic bacteria are often capable of surviving antibiotic treatment through encapsulation into biofilms, these kind of inhibitor are expected to efficiently eliminate biofilm/quorum...
sensing-associated infections, which would be synergistic with other antibiotics (2, 3, 20, 21).

Considering the fact that siamycin I has antibacterial activity at micromolar concentrations, siamycin I may have a potential to eliminate and inhibit biofilm-associated *E. faecalis* infection by themselves.

This work was an initial screening study to target GBAP-mediated quorum sensing. Larger scale screening on natural and/or synthetic compounds would provide more information on other compounds potentially antipathogenic against *E. faecalis* and perhaps other gram-positive pathogens.

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Figure legends

FIG. 1. Structure of the inhibitor of Y33-1 [siamycin I (14, 61) and NP-06 (7)].

FIG. 2. Effect of various concentrations of siamycin I on the cell growth and gelatinase production of *E. faecalis* OG1RF. *E. faecalis* OG1RF was grown for 5 h in the presence of indicated concentrations of siamycin I and then the cell density at OD$_{660}$ (open circles) and gelatinase activity at OD$_{540}$ (closed circles) in the culture supernatant were measured as described in Materials and Methods. The data are averages ± standard deviation of duplicate determinations.

FIG. 3. Time course of cell growth, pH and gelatinase activity of *E. faecalis* culture with or without siamycin I (1 µM). *E. faecalis* OU510B was inoculated to fresh medium with (closed symbols) or without (open symbols) siamycin I (1 µM) and was then cultured. Culture supernatant was collected every hour and the cell density at OD$_{660}$ (broken lines and triangle symbols), pH (dotted lines and diamond symbols) and gelatinase activity at OD$_{540}$ (solid lines and circle symbols) were measured. The data are averages ± standard deviation of duplicate determinations.

FIG. 4. Effect of various concentrations of siamycin I on GBAP production of *E. faecalis* OG1RF. *E. faecalis* OG1RF were grown for 7 h in CDM containing the indicated concentrations of siamycin I. Then GBAP in each culture supernatant was detected by LC/MS. Extracted ion chromatograms were plotted with detector counts at m/z ranging from 1303.5 to 1304.2 which covers a protonated molecular ion of GBAP and normalized to the GBAP peak height in the absence of siamycin I (top chromatogram).
FIG. 5. Effect of various concentrations of siamycin I on gelatinase production in *E. faecalis* OU510 induced by 100 nM of GBAP. After 2 h of inoculation, 100 nM of synthetic GBAP and indicated amounts of siamycin I were added to the culture of *E. faecalis* OU510 and incubation of the culture was continued for another 3 h for the induction of gelatinase production. Then the cell density at OD\textsubscript{660} (open circles) and gelatinase activity at OD\textsubscript{540} (closed circles) in the culture supernatant were measured. The data are averages ± standard deviation of duplicate determinations.

FIG. 6. Effect of siamycin I (1 µM) on gelatinase production in *E. faecalis* OU510 induced by various concentrations of GBAP. *E. faecalis* OU510 was grown for 5 h in the presence of indicated concentrations of synthetic GBAP without (open circles) or with 1 µM siamycin I (closed circle). Then gelatinase activity in the culture supernatant was measured at OD\textsubscript{540} in duplicate and the average values were plotted.

FIG. 7. Effect of various concentrations of siamycin I on β-galactosidase induction and gelatinase production in *E. faecalis* OG1RF(pNZ8048\textit{lacZ} + pNZ9530). *E. faecalis* OG1RF(pNZ8048\textit{lacZ} + pNZ9530) was cultured for 5 h in the medium containing 3 nM of nisin A and the indicated concentrations of siamycin I. Cell growth (open circles), gelatinase activity (closed circles) and β-galactosidase activity (closed diamonds) of each culture was measured and expressed as OD\textsubscript{660}, OD\textsubscript{540} and Miller unit, respectively. The data are averages ± standard deviation of duplicate determinations.

FIG. 8. Effect of siamycin I (1 µM) on transcription of 16S rRNA, \textit{fsrBDC} and \textit{gelE-sprE} in *E. faecalis* OG1RF. *E. faecalis* OG1RF was grown for 4 h in the absence (control, grey bars) or
presence of 1 µM siamycin I (white bars). Total RNA was prepared from the grown cells and used as a template for one-step RT-PCR. Relative expression levels determined by the real-time PCR are illustrated as the ratio to the control. The culture was done in duplicate and RT-PCR was done in duplicate for each RNA sample. Standard deviations of the total quadruplicate determinations are indicated by the error bars.

FIG. 9. Effect of siamycin I on biofilm formation of *E. faecalis* OG1RF. Assays were performed by using tryptic soy broth supplemented with 0.25% glucose containing indicated concentrations of siamycin I and flat-bottomed 96-well polystyrene microtiter plates. After 24 h of incubation at 37°C, biofilm formation was quantified by measuring the optical density at 570 nm (OD$_{570}$) of crystal violet-stained biofilm. Each assay was performed in quadruplicate on two occasions, and the mean values and standard errors are shown.
Table 1. Observed proton chemical shifts of Y33-1 (siamycin I)

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<th>residue</th>
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<th>Hα&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Hβ</th>
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<sup>a</sup>The NMR data were obtained at 10ºC. <sup>b</sup>not detected. <sup>c</sup>M=methyl
gelatinase activity ($A_{540}$)

concentration of siamycin I (nM)

Cell density (OD$_{600}$)
gelatinase activity ($A_{540}$)

concentration of siamycin I (nM)

cell density ($OD_{620}$)
gelatinase activity ($A_{540}$) vs. concentration of GBAP (nM)
The graph shows the change in OD_570 with increasing concentration of siamycin I (µM). The OD values are indicated by error bars, suggesting variability in the measurements. The x-axis represents the concentration of siamycin I (µM) ranging from 0 to 1 µM, and the y-axis represents the OD_570 values ranging from 0 to 0.4.