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Development of a Peptide Antagonist against *fsr* Quorum Sensing of *Enterococcus faecalis*

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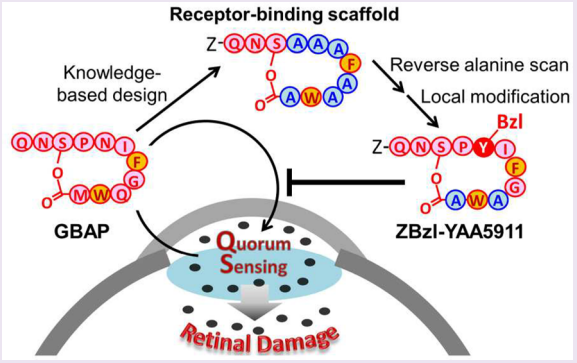
Supporting Information

ABSTRACT: *Enterococcus faecalis fsr* quorum sensing (QS) involves an 11-residue cyclic peptide named gelatinase biosynthesis-activating pheromone (GBAP) that autoinduces two pathogenicity-related extracellular proteases in a cell density-dependent fashion. To identify anti-pathogenic agents that target *fsr* QS signaling, peptide antagonists of GBAP were created by our unique drug design approach based on reverse alanine scanning. First of all, a receptor-binding scaffold (RBS), [Ala^{4,5,6,8,9,11}]Z-GBAP, was created, in which all amino acids within the ring region of GBAP, except for two essential aromatic residues, were substituted to alanine. Next, the substituted alanine residues were changed back to the original amino acid one by one, permitting selection of those peptide combinations exhibiting increased antagonist activity. After three cycles of this reverse alanine scan, [Ala^{5,9,11}]Z-GBAP was obtained as a maximally reverted peptide (MRP) holding the strongest antagonist activity. Then, the fifth residue in MRP, which is one of the critical residues to determine agonist/antagonist activity, was further modified by substituting with different types of amino acids including unnatural amino acids. As a result, [Tyr(Bzl)^{5}, Ala^{9,11}]Z-GBAP, named ZBzl-YAA5911, showed the strongest antagonist activity [IC_{50} = 26.2 nM and K_d against GBAP receptor (FsrC) = 39.4 nM]. *In vivo* efficacy of this peptide was assessed with an aphakic rabbit endophthalmitis model. ZBzl-YAA5911 suppressed the translocation of *E. faecalis* from the aqueous humor into the vitreous cavity by more than 1 order of magnitude and significantly reduced retinal damage. We propose that ZBzl-YAA5911 or its derivatives would be useful as anti-infective agents to attenuate virulence expression in this opportunistic pathogen.



The quorum sensing (QS) systems are cell density-dependent regulatory networks that orchestrate the communal behavior of unicellular microorganisms.[1] Many pathogenic bacteria employ QS to coordinate the expression of genes involved in their pathogenicity. Under the current status of increasing antibiotic-resistant bacteria, QS inhibitors have attracted interest as anti-pathogenic agents that attenuate virulence rather than killing bacteria.[1,2] Especially, synthetic inhibitors targeting molecules involved in specific cell−cell signaling within pathogens would be considerably valuable because of their pathogen specificity without selecting for drug resistance among the general population of resident flora.

Enterococci are commensal bacteria of the normal intestinal flora, oral cavity, and female genital tract of both humans and animals. They are facultative anaerobes that can survive and grow in various environments and occasionally cause various types of opportunistic infections, e.g., bacteremia, endocarditis, urinary tract infections, post-treatment endodontic infections, and endopthalmitis.[3,4] With the ever increasing incidences of antibiotic resistance reported among enterococci, including resistances to "last-line" vancomycin and to more recently developed therapies such as daptomycin, the enterococci remain nosocomial pathogens of significant concern and are challenging to treat in the clinic.[5−8]

Among enterococci, *Enterococcus faecalis* accounts for around 80% of all enterococci infections.[9] Gelatinase (GelE) is recognized as a virulence factor that degrades a broad range of host proteinaceous substrates.[10−15] There are also some reports showing its involvement in biofilm formation.[10,16,17] Indeed, *in vivo* and *in vitro* models have long established the

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crucial involvement of gelatinase in enterococcal infections.[14,18−20] Notably, it was proven that GelE plays a major role in the pathogenesis of *E. faecalis*-induced postoperative endophthalmitis by using an aphakic rabbit model.[14]

The expression of GelE and indeed another pathogenicity-related extracellular protease, SprE is controlled by a QS system termed *fsr*.[21−23] In the *fsr* system, a cyclic peptide named gelatinase biosynthesis-activating pheromone (GBAP) (Figure 1) autoinduces the expression of the *gelE-sprE* operon via the

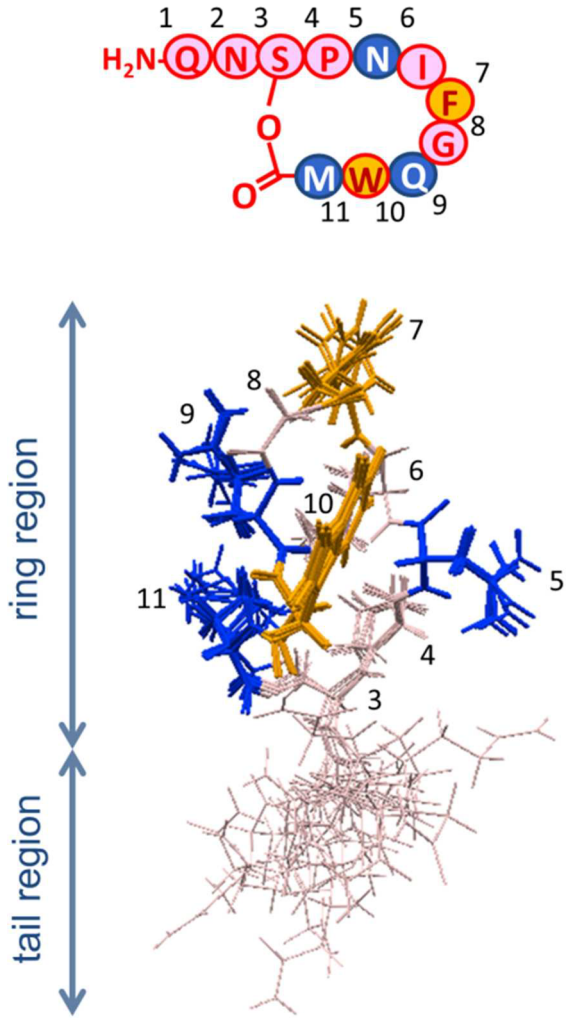

Figure 1. Structure of GBAP. Primary structure of GBAP (top) was determined in our previous study.[22−24] The tertiary structure (bottom) represents the 10 lowest-energy structures determined by energy minimization following solution state NMR spectroscopy analysis.[39] Orange indicates two aromatic residues, Phe[7] and Trp[10], which play essential roles for receptor binding as well as antagonist activity. Blue indicates the residues critical to determine agonist/antagonist activity, indicated by the reverse alanine scan in this study. Stereo view of the GBAP tertiary structure is shown in Supplementary Figure 1.

FsrC-FsrA two-component regulatory system.[22,24−26] Interference of this GBAP-mediated QS is a compelling approach since it is expected to suppress the expression of the two pathogenicity-related proteases but not to kill the bacteria. This anti-pathogenic strategy may resolve the impasse in the development of antibiotic chemotherapy for enterococci that display high level intrinsic resistance to conventional antibiotics.

To identify *fsr* QS inhibitors, we have previously screened microbial secondary metabolites and successfully identified siamycin I and ambuic acid as specific inhibitors of the FsrC-FsrA two-component regulatory system and GBAP biosynthesis, respectively.[27−29] In addition, we attempted to design a GBAP antagonist as it is thought to offer completely specific inhibitor against *fsr* QS through the blockage of receptor activation. However, no antagonist was successfully generated as described below.

Staphylococci also possess a cyclic peptide-mediated QS system termed *agr*.[30−32] *agr* QS coordinates the expression of a series of virulence factors, e.g., exotoxins or proteases. There are four different autoinducing peptides (AIPs) classified into three subgroups, each of which interferes with the activities of the other types as antagonists.[31,33] On the basis of the cross reactivity among these different AIP groups, rational drug design has been successful to create effective antagonists.[32,34−37]

Unlike the staphylococcal AIPs, no variants of GBAP have been found, suggesting no QS interference among enterococcal strains.[38] This fact necessitates a new approach for *de novo* design of a novel GBAP antagonist. Thus far, we have performed alanine scanning in which alanine was substituted for each residue of GBAP one by one.[39] However, no alanine substitutes showed detectable antagonist activity, though some significantly abolished agonist activity, suggesting significant challenges might be expected for generating antagonists using the GBAP sequence as template. This prompted us to initiate a novel and unique approach based on reverse-alanine scanning, as summarized in Figure 2. In this strategy, instead of starting with agonist, a receptor-binding scaffold (RBS), [Ala^{4,5,6,8,9,11}]Z-GBAP, holding a minimum structure necessary for receptor binding was used as the starting point. Antagonist activity was

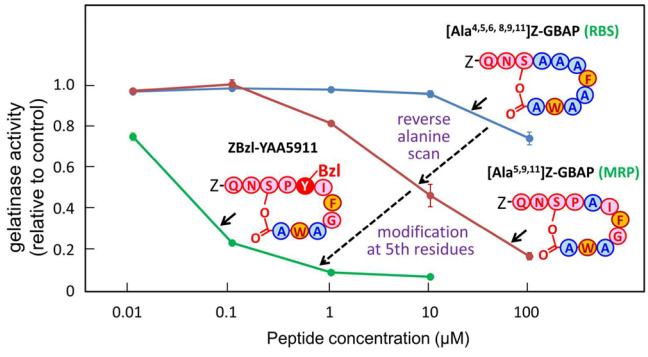

Figure 2. Scheme and the result of the development of GBAP antagonists. First, [Ala^{4,5,6,8,9,11}]Z-GBAP was designed as a receptor binding scaffold (RBS) holding two aromatic amino acids (orange residue) essential for receptor binding ability. Then, antagonist activity was enhanced by the reverse alanine scan approach in which the substituted alanine residues (blue color) were changed back to original amino acid one by one. The resultant peptide, [Ala^{5,9,11}]Z-GBAP, called maximally reverted peptide (MRP), was then modified at fifth residue to further enhance its activity. Finally, a potent antagonist, [Tyr(Bzl)^{5},Ala^{9,11}]Z-GBAP, named ZBzl-YAA5911, was obtained. Dose−response data for antagonist activity of these peptides were obtained by quantifying gelatinase activity of *E. faecalis* OG1RF cultured with each peptide. The graph was plotted with the ratio to the control cultured without peptide. The reduction in gelatinase activity represents antagonist activity. The experiment was done in more than duplicate and average ± standard deviation was plotted. Abbreviation: Z, benzyloxycarbonyl; Bzl, benzyl.

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then enhanced step-by-step by reverse alanine scanning and consequently [Ala^{5,9,11}]Z-GBAP was established as a maximally reverted peptide (MRP) that most closely resembled GBAP and held strongest antagonist activity. We then further modified MRP to increase the antagonist activity still further until finally generating an antagonist named ZBzl-YAA5911 with K_d to FsrC comparable with that of GBAP. Finally, we demonstrated efficacy of ZBzl-YAA5911 for the protection of *E. faecalis*-mediated endophthalmitis in an *in vivo* rabbit model.

RESULTS AND DISCUSSION

Design of RBS. Our previous study on the structure–activity relationship of GBAP had indicated that the ring region with rigid conformation is crucial for agonist activity, whereas the tail region with flexible structure is not critical for the agonist activity (Figure 1).[39] Especially, two aromatic residues in the ring region, Phe^7 and Trp^{10}, are essential for receptor binding as well as agonist activity. On the basis of this knowledge, we designed a RBS, in which the two essential aromatic residues, Phe^7 and Trp^{10}, were present and remained unchanged, while the rest of the residues in the ring region were substituted to alanine. The designed RBS was benzyloxycarbonylated at the amino terminus; the benzyloxycarbonyl (Z) group has been previously used as a protecting group during the synthesis of cyclic peptides of GBAP and its derivatives, and it has been shown that there is no significant influence of this amino-terminal modification on GBAP activity.[24] The designed peptide, [Ala^{4,5,6,8,9,11}]Z-GBAP, showed a weak but significant inhibitory effect on gelatinase expression (24.9 ± 3.2% inhibition at 100 μM (Figure 2), suggesting that it has a receptor-binding ability but not receptor-activation activity, namely, it can be used as a RBS for the following reverse alanine scan.

Reverse Alanine Scan Based on RBS. To enhance the antagonist activity of the RBS, each alanine residue in the ring region of [Ala^{4,5,6,8,9,11}]Z-GBAP was exchanged to the original amino acid of GBAP one by one and measured for agonist/antagonist activity (Figure 3). In the first cycle of the reverse alanine scanning, the Gly^8 revertant, [Ala^{4,5,6,9,11}]Z-GBAP, slightly increased the antagonist activity, while other revertants decreased the antagonist activity; especially, the Gln^9 revertant turned to be a strong agonist. Therefore, the Gly^8 revertant, [Ala^{4,5,6,9,11}]Z-GBAP, was subjected to a second round of reverse alanine scanning. As a result, Pro^4, Ile^6, and Gln^9 revertants increased the antagonist activity, while Asn^5 and Met^{11} revertants turned to be agonists. The strongest revertant, [Ala^{5,6,9,11}]Z-GBAP (Pro^4 revertant), was subjected to the third round. As a result, only an Ile^6 revertant, [Ala^{5,9,11}]Z-GBAP, increased the antagonist activity and was chosen for the fourth round. As a result of the fourth stage, all revertants dramatically decreased the antagonist activity, suggesting that the remaining fifth, ninth, and 11th residues should not be the original amino acids for maintaining the antagonist activity, and the reverse alanine scanning was hence terminated. After all, [Ala^{5,9,11}]Z-GBAP was obtained as a maximally reverted peptide (MRP) with the strongest activity, IC_{50} = 8.7 ± 2.3 μM, as shown in Figure 2.

Local Modification of MRP. The reverse alanine scanning approach adopted above suggested that the fifth, ninth, and 11th residues (originally, Asn, Gln, and Met, respectively) are critical for determining agonist/antagonist activity of the cyclic peptide. On the other hand, ordinary alanine scan in our previous study indicated that Gln^9 and Met^{11} can be replaced by alanine without loss of agonist activity, whereas Asn^5 cannot.[39] Taken together, Asn^5 was suggested in both studies to be a key residue for receptor activation. Indeed, our preliminary experiments with Asn^5 and Gln^9 suggested that Asn^5 has more potential for the enhancement of antagonist activity (Supplementary Figure 2). Therefore, the chemical preference at the fifth position for promoting the highest antagonist activity was determined. The GyrA mini-intein system was used to introduce a series of amino acids to the fifth residue position of [Cys^3, Ala^{5,9,11}]GBAP that has a level of antagonist activity similar to that of [Ala^{5,9,11}]Z-GBAP. As shown in Supplementary Figure 3, substitutions using Tyr, Met, Thr, His, Ile, or Phe showed a potent antagonist activity, suggesting that bulky amino acids are preferred for antagonist activity but that hydrophobicity and aromaticity are not determining factors. To further increase antagonist activity, the fifth residue of [Ala^{5,9,11}]Z-GBAP was exchanged to several bulky amino acids including unnatural amino acids (Figure 4). As a result, [Tyr(Bzl)^5,Ala^{9,11}]Z-GBAP, named ZBzl-YAA5911, which has two benzene rings in the side chain, was obtained as the strongest antagonist with IC_{50} = 26.2 ± 1.6 nM (Figures 2 and 4).

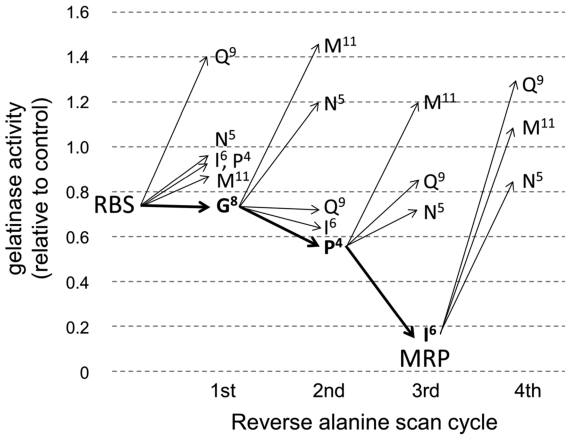

Figure 3. Reverse alanine scan from [Ala^{4,5,6,8,9,11}]Z-GBAP as RBS. The indicated residues, corresponding to the original amino acids in GBAP, were individually brought back into the template peptide. Revertant showing the most enhanced antagonist activity was chosen in each cycle (bold letter). After three cycles of this scan, [Ala^{5,9,11}]Z-GBAP was obtained as a maximally reverted peptide (MRP) that could not gain antagonist activity in further reverse scan. The agonist/antagonist activity of each revertant peptide was examined by comparing gelatinase activity in *E. faecalis* OG1RF cultured with 100 μM of each peptide to the control cultured without synthetic peptide. The values higher and lower than 1.0 represent agonist and antagonist activities, respectively. The experiments were done in more than duplicate, and the averages were plotted in this graph.

***In Vitro* Antagonist Activity of ZBzl-YAA5911.** To confirm that ZBzl-YAA5911 is competitively bound to GBAP receptor, FsrC, the following two experiments were performed. First of all, a fluorescence-labeled GBAP (FITC-GBAP) was incubated with FsrC-expressing bacterial cells in the presence or absence of ZBzl-YAA5911. Expectedly, as shown in Figure 5A, ZBzl-YAA5911 inhibited the binding of FITC-GBAP to FsrC, suggesting the competitive binding of ZBzl-YAA5911 to FsrC. To address the stoichiometry of this antagonism of ZBzl-YAA5911, Schild-plot analysis was performed. In the assay, a mutant strain, *E. faecalis* OU510, which lacks GBAP production but is sensitive to GBAP, was used as an indicator strain instead

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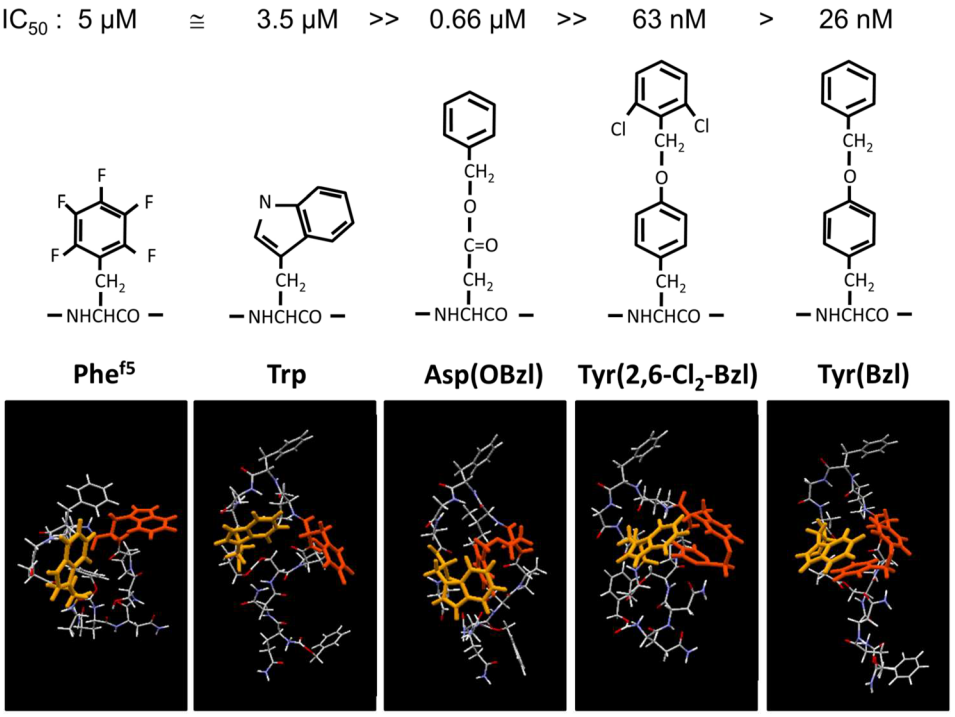

Figure 4. Chemical modification of side chain at the fifth residue on MRP. The modified chemical structures at the fifth residue of [Ala[5,9,11]]Z-GBAP are shown with antagonist activities. The antagonist activity was examined using *E. faecalis* OG1RF as a GBAP responder strain, and the IC_{50} was determined as described in Methods. The experiment was performed in more than duplicate, and the averages of the determined IC_{50}'s are shown. The molecular models of tertiary structure of these modified peptides (bottom) were constructed based on the NMR structure of GBAP by energy minimization using the AMBER99 force field (for detail, see Supporting Information). Orange and orange-red indicate Trp[10] and the modified fifth residue, respectively. Stereo view of the tertiary structure of ZBzl-YAA5911 is shown in Supplementary Figure 4.

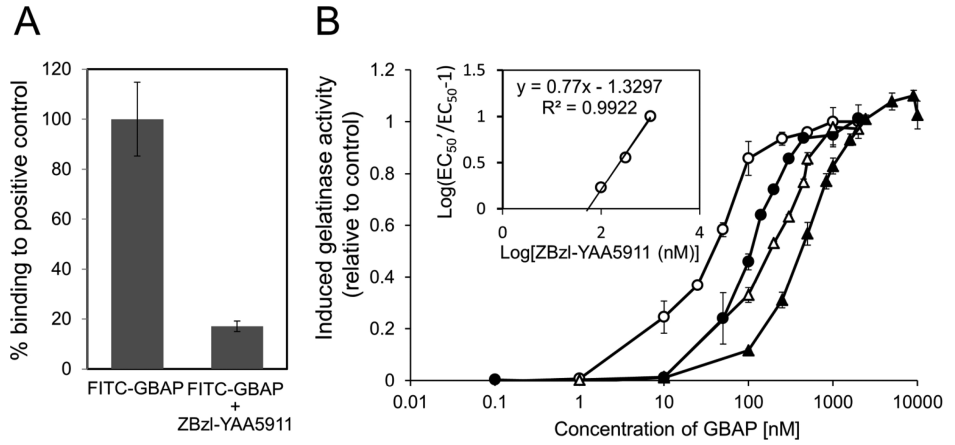

Figure 5. *In vitro* antagonist activity of ZBzl-YAA5911. (A) Inhibition of receptor binding of GBAP by ZBzl-YAA5911. The binding of GBAP to the receptor was measured by the fluorescence of FsrC-expressing *L. lactis* cells incubated with FITC-GBAP (1 μM) and ZBzl-YAA5911 (10 μM). For the positive control, the cells were incubated only with FITC-GBAP (1 μM). The experiment was done in duplicate, and the average ± standard deviation was plotted. (B) Schild-plot analysis of the inhibitory effect of ZBzl-YAA5911. The dose response of *E. faecalis* OU510 to GBAP was examined in the absence (○) or presence of 100 nM (●), 320 nM (△) or 1,000 nM (▲). The experiment was done in triplicate, and the average ± standard deviation was plotted. The Schild plot (Inset) was constructed with the values, $\log(EC_{50'}/EC_{50} - 1)$ and log(ZBzl-YAA5911), where $EC_{50'}$ and EC_{50} are the half maximal effective concentration of GBAP in the presence and absence of ZBzl-YAA5911, respectively.

of the wild-type OG1RF strain. Different concentrations of synthetic GBAP and ZBzl-YAA5911 were exogenously added to the indicator strain, and the induced gelatinase activity was measured (Figure 5B). The Schild plot showed a normal linear regression with a slope close to unity, suggesting competitive binding of ZBzl-YAA5911 to FsrC. The X-intercept indicated the dissociation constant (K_d) of ZBzl-YAA5911 was equal to 39.4 ± 4.3 nM, which was comparable with the EC_{50} of GBAP (33.5 ± 0.9 nM).

***In Vivo* Evaluation of Anti-infection Efficacy of ZBzl-YAA5911 Using an Aphakic Rabbit Endophthalmitis Model.** It has been reported using an aphakic rabbit endophthalmitis model that *fsr*-controlled gelatinase expression is involved in the translocation of *E. faecalis* from the aqueous humor to the vitreous body, which consequently leads to

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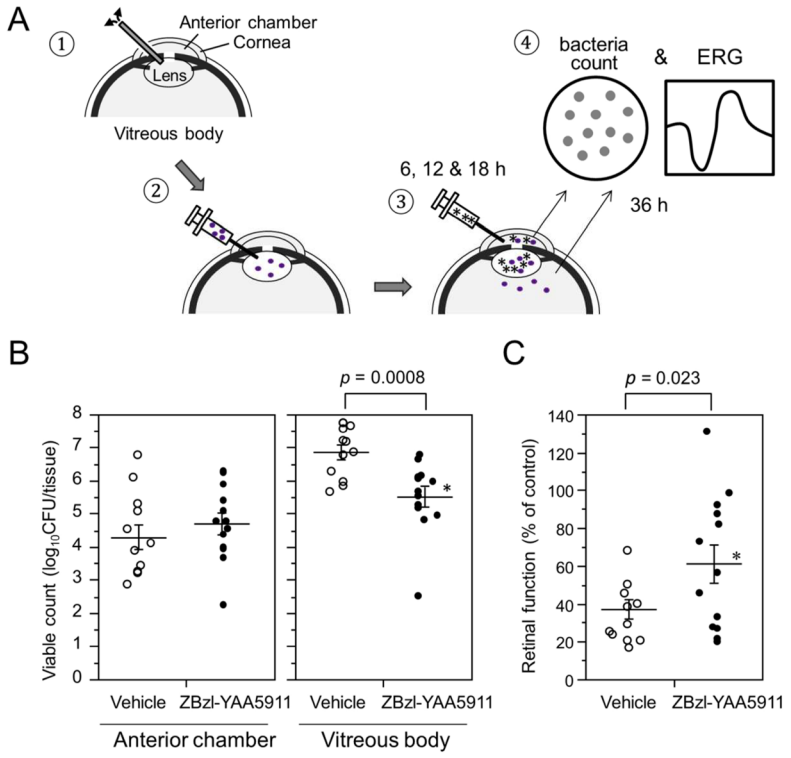

Figure 6. Evaluation of ZBzl-YAA5911 in an aphakic rabbit endophthalmitis model. Experimental procedure is illustrated in panel A. ① Lensectomy was performed on the rabbit's left eye by phacoemulsification. ② The eyes were inoculated from a lens capsular bag by injecting 0.05 mL of *E. faecalis* OG1S strain (5.49 $\log_{10}$ CFU mL^{-1}). ③ Rabbits were treated with intracameral injection of 0.05 mL of ZBzl-YAA5911 (10^{-4} M) or vehicle (2% DMSO/saline) at 6, 12, and 18 h after bacterial inoculation. ④ At 36 h, bacteria were recovered from the anterior chamber and the vitreous body and counted (B). In addition, retinal function was evaluated by electroretinography (ERG) (C). Percent retinal function was defined as the ratio of the B-wave amplitude of the infected eye to the B-wave amplitude of the contralateral control eye. Data are means ± SEM (n = 12–13).

serious retinal damage.[14] To examine the efficacy of ZBzl-YAA5911 for reducing or abolishing endophthalmitis, ZBzl-YAA5911 was intracamerally injected following inoculation of a gelatinase-positive *E. faecalis* strain, and then viable counts of bacteria from aqueous and vitreous humors were determined at 36 h (Figure 6). The bacterial count in the vitreous compartment was significantly reduced in the ZBzl-YAA5911-treated group compared to the vehicle group (p = 0.0008; their means differ more than one order in magnitude), whereas those in the anterior chamber showed approximately the same level for both the vehicle and ZBzl-YAA5911 groups (Figure 6B). This indicated that ZBzl-YAA5911 inhibited the translocation of *E. faecalis* from the aqueous humor into the vitreous cavity without direct bacteriocidal effect. Also, efficacy of ZBzl-YAA5911 against endophthalmitis was assessed by electroretinography (ERG) analysis to monitor retinal responsiveness to light stimulus. The damage in retinal function by enterococcal endophthalmitis was significantly reduced in the ZBzl-YAA5911-treated group compared to the control group (p < 0.05) (Figure 6C). On the other hand, ZBzl-YAA5911 itself did not reduce retinal function at any concentrations tested up to 10^{-4} M, suggesting no toxicity of this peptide in the practical concentrations used in this model (Supplementary Figure 5).

Significance. Despite the lack of any known naturally occurring antagonist of enterococcal QS, such as that described for the staphylococcal *agr* system, and the fact that previous studies identified no single alanine substitutes or tail-region-truncates in GBAP to exhibit antagonist activity, in this study, we have succeeded in creating a potent GBAP antagonist, ZBzl-YAA5911. A key to success was to take our unique strategy starting from RBS followed by the reverse alanine scanning approach adopted/developed in this study. It shed light on the three residues critical for determining agonist/antagonist activity and prompted us to focus on the fifth residue position as a key for modulating antagonist activity.

During the reverse alanine scan, except for the three critical residues, the fourth, sixth and eighth residues could be reverted to each corresponding original amino acid, proline, isoleucine, and glycine, respectively, with slight increase in the antagonist activity. Pro^4 and Gly^8 seem to play a role in forming the ring conformation, as they often locate to the corner of the β-turn in the peptide chain. Ile^6 is also suspected to be involved in the maintenance of ring conformation since the side chain of Ile^6 is oriented inside the ring according to the tertiary structure indicated by the solution NMR analysis of GBAP [39] (Figure 1 and Supplementary Figure 1). On the other hand, the side chains of the fifth, ninth, and 11th residues were oriented outside the ring in the NMR structure as well as Phe^7 and Trp^{10}, suggesting that these residues seem to be involved in intermolecular interaction rather than intramolecular interaction, which may associate with receptor activation.

Local modification at fifth residue of MRP finally led to the potent antagonist, ZBzl-YAA5911. Structure–activity relationship of the series of modified peptides indicated that the distal aromatic group at the fifth residues is crucial for the nanomolar level activity (Figure 4). Computer simulation of the modified peptides showed a very interesting aspect in these benzyl antagonists, which is like a π–π interaction between the distal aromatic group at fifth residue and the indol ring of Trp^{10} (Figure 4, bottom, and Supplementary Figure 4). This

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noncovalent bridge that seems to fix the ring conformation may play a crucial role in the block of receptor activation.

While enterococcal endophthalmitis is one of the most severe sight-threatening complications of cataract surgery,[7,40−42] several studies have since demonstrated that *fsr* QS-regulated protease expression is significantly associated with the translocation of bacteria from the anterior chamber to the vitreous chamber, which in turn is a key event in the progression of postoperative endophthalmitis and severe retinal damage.[14,20,43] Nowadays, fluoroquinolones and vancomycin are commonly used for prevention and treatment for endophthalmitis, respectively. However, enterococci resistant to these antibiotics have frequently appeared not only in the clinical setting but also in the natural environment, which is a matter of considerable concern for ophtalomatologists. This study demonstrated the efficacy of ZBzl-YAA5911 to block the *E. faecalis* translocation without killing bacteria and rescue the destruction of retinal function. Anti-pathogenic drugs, which may be led by ZBzl-YAA5911 to be more effective and substantial, would open the way for new chemotherapy for endophthalmitis.

The recent and ever-increasing availability of bacterial genome sequence data has revealed the widespread presence of *agr*-type QS systems in Gram-positive bacteria, mostly in low %GC *Firmicutes*.[44] Notably, the presence of genes encoding AgrB homologues suggests the possibility of using thiolactone or lactone as QS autoinducer. Some recent studies have indicated that Gram-positive pathogens, e.g., *Listeria monocytegenes* and *Clostridium perfringens*, rely on *agr*-type QS to control the expression of some virulence factors as occurs in staphylococci and enterococci.[45−49] However, unlike the situation in staphylococci, no interference mechanisms indicative of potential antagonists have yet been identified.[33,38] *De novo* antagonist design using the reverse alanine scanning approach as have done in this study may be useful for development of anti-pathogenic agents to attenuate virulence of these Gram-positive pathogens.

METHODS

Bacterial Strains and Growth Condition. *E. faecalis* OG1RF[50] was used as an isogenic gelatinase-positive strain for agonist/antagonist assay. *E. faecalis* OU510,[25] which carries *fsr* genes but lacks GBAP biosynthesis, was used to titrate gelatinase activity. All *E. faecalis* strains were cultured in 36.4 g L^{-1} Todd-Hewitt broth (THB) (Oxoid) at 37 °C with gentle agitation. *Lactococcus lactis* NZ9000 (pNZfsrC-his6)[39] was cultured in GM17MC medium at 30 °C with gentle shaking.

GBAP Agonist/Antagonist Assay. In order to briefly examine agonist/antagonist activity of synthetic peptides, *E. faecalis* OG1RF, which is GBAP- and gelatinase-positive, was used as an indicator strain. After OG1RF was cultured with synthetic peptides for 5 h, corresponding to the late-log phase, culture supernatant was collected and subjected to a gelatinase assay.[39] In this assay, Azocoll (<50 mesh, Calbiochem), an Azo dye-impregnated collagen, was used as a gelatinase substrate, and peptide fragments solubilized upon proteolysis were quantified by measuring A_{540} of the supernatant. The value was normalized as ratio to control cultured without synthetic peptide. The values greater than 1.0 and less than 1.0 represent agonist activity and antagonist activity, respectively. The IC_{50} was calculated from the sigmoidal dose−response model in Microsoft XLFit. In order to precisely examine antagonist activity, *E. faecalis* OU510,[25] which is a spontaneous mutant lacking in GBAP production but sensitive to exogenously added GBAP, was used as an indicator strain. After OU510 was cultured with various concentrations of sample and synthetic GBAP for 5 h, culture supernatant was collected and subjected to the Azocoll assay previously described.[39]

Peptides. All cyclic peptides used in this study, except for a series of thiolactone peptides produced by recombinant *Escherichia coli*, were synthesized according to a previously developed scheme,[24,39] in which N-terminally protected linear peptides were dehydrated between the hydroxyl group of Ser^3 and the carboxyl group at the C-terminus. A detailed description of synthesis procedure is provided in the Supporting Information.

A series of Ala^5-substitutes of [Cys^3, $Ala^{5,9,11}$]GBAP were prepared by the GyrA mini-intein system (New England Biolabs). The expression plasmid of each precursor protein, in which [Cys^3, Xaa^5, $Ala^{9,11}$]GBAP was fused to the N-terminus of GyrA mini-intein, was constructed with pTXB1 (New England Biolabs). Each precursor protein, [Cys^3, Xaa^5, $Ala^{9,11}$]GBAP-GyrA, was then expressed in *E. coli* Rosetta(DE3) (Novagen) and affinity-purified using chitin resin (New England Biolabs). [Cys^3, Xaa^5, $Ala^{9,11}$]GBAP was then cleaved from GyrA mini-intein by treating the fusion protein with 50 mM 2-mercaptoethanesulfonic acid in 100 mM sodium phosphate (pH 6.8), 50 mM NaCl, 1 mM EDTA for 1−7 days at 4 °C. The resultant [Cys^3, Xaa^5, $Ala^{9,11}$]GBAP-thioester was autonomously converted to the thiolactone form of [Cys^3, Xaa^5, $Ala^{9,11}$]GBAP.

Fluorescence-labeled GBAP (FITC-GBAP) for the receptor-binding assay was prepared by reacting unlabeled peptides with fluorescein-4-isothicyanate (FITC; Dojindo) as previously described.[39]

All synthetic peptides were finally purified by a reverse-phase high-performance liquid chromatography (RP-HPLC) and ascertained by mass spectrometry analysis as previously described.[39] The amount of each synthetic peptide was quantified by ultraviolet absorption at 280 nm ($\varepsilon_{280} = 5690a + 1280b$; a = the number of tryptophan residues, b = the number of tyrosine or tyrosine derivative).

Fluorescence-Based Receptor-Binding Assay. For the fluorescence-based receptor-binding assay, a recombinant stain of *Lactococcus lactis* NZ9000(pNZfsrC-his6) was used as previously described.[39] After the culture of NZ9000(pNZfsrC-his6) reached an OD_{600} of 0.5, nisin A (Sigma-Aldrich, Inc.) dissolved in water and adjusted to pH 3.0 with HCl was added to achieve a final concentration of 10 ng mL^{-1} and incubated for overnight for the expression of FsrC. After the induction, the cells were harvested and washed with 50 mM Tris-HCl (pH 7.8), and the cell concentration was adjusted to an OD_{600} of 10. One hundred microliters of cell suspension was added to a 1.5-mL tube containing an equal volume of 2 μM of FITC-GBAP with or without 20 μM of ZBzl-YAA5911, and the mixture was kept standing for 1 h at 4 °C. The mixture was then centrifuged at 6,000*g* for 3 min, and the cell pellet was washed thoroughly two times with ice-cold 50 mM Tris-HCl (pH 7.8). The washed cells were then resuspended in 100 μL of 50 mM Tris-HCl (pH 7.8) containing 0.25% (v/v) Triton X-100 and 2.5 μM Alexa Fluor 430 carboxylic acid succinimidyl ester as an internal standard was measured at an absorbance (emission wavelength) of 520 nm, with excitation wavelengths ranging from 300 to 500 nm in a fluorophotometer (F-7000; Hitachi). The fluorescence intensity of FITC at excitation wavelength of 495 nm was normalized to that of the internal standard at 440 nm. The autofluorescence of bacterial cells was measured using the cells prepared in the same way without adding FITC-GBAP. The binding of FITC-GBAP to FsrC expressed on the *L. lactis* cells was calculated by subtracting the 440 nm/495 nm value of FITC-GBAP-nontreated cells from that of the FITC-GBAP-treated cells.

Evaluation of ZBzl-YAA5911 in an Aphakic Rabbit Endophthalmitis Model. Male Japanese White rabbits weighing 2.0−2.4 kg (Kitayama Labes Co., Ltd.) were maintained in accordance with the Institutional Animal Care and Use Committee guidelines and the Association for Research in Vision and Ophthalmology Statement for the Use of Laboratory Animals in Ophthalmic and Vision Research. Rabbits were anesthetized by an intramuscular injection with an equal mixture of 5% ketamine (Ketalar intramuscular 500 mg; Sankyo Co., Ltd.) and 2% xylazine (Selactar; Bayer Ltd.) at 1 mL kg^{-1} for all procedures. Rabbits were euthanized with an overdose of pentobarbital sodium.

Rabbits underwent experimental endophthalmitis by inoculation with *E. faecalis* as previously described.[14] Briefly, lensectomy was

performed on the rabbit's left eye by phacoemulsification (AMO Prestige; AMO JAPAN K. K.). Then, using a 27 gauge blunt needle on a 1-mL disposable syringe, the eyes were inoculated from a lens capsular bag containing 0.05 mL of the *E. faecalis* OG1S strain (5.49 $\log_{10}$ CFU mL^{-1}). Rabbits were treated with intracameral injection of 0.05 mL of ZBzl-YAA5911 (10^{-4} M) in 2% DMSO/saline or vehicle (2% DMSO/saline) at 6, 12, and 18 h after bacterial inoculation using a 30 gauge needle on a 1-mL disposable syringe. To estimate anti-infectious efficacy of ZBzl-YAA5911, retinal function was verified using electroretinography (ERG) and count bacteria recovered from the anterior chamber or the vitreous at 36 h after bacterial inoculation. Bacterial counts in ocular tissues were determined by plating serial dilutions on brain heart infusion agar, as previously described.[14] The degree of retinal function was evaluated by recording the scotopic b-wave amplitude for each eye using flash ERG (LE-3000; Tomey Corp.).[14] Retinal function was determined as b-wave amplitude in the experimental eye, expressed as a percentage of that in the contralateral control eye. Statistical analysis was performed using JMP9.0.2 (SAS institute). Dunnett test was used to compare drug toxicity *in vitro* between the ZBzl-YAA5911 and control groups. Student's *t* test was used to compare viable bacteria in ocular tissue and changes in retinal responsiveness between the ZBzl-YAA5911 and control groups. Values of $p < 0.05$ were considered statistically significant.

■ ASSOCIATED CONTENT

Supporting Information

This information is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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