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Effects of *N*-acyl homoserine lactone analogues on *Porphyromonas gingivalis* biofilm formation

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Background and Objective: The gram-negative anaerobic rod *Porphyromonas gingivalis* in oral biofilms is a primary etiological agent of periodontal disease. Biofilm formation of various gram-negative bacteria is regulated by a quorum-sensing circuit that relies on *N*-acyl homoserine lactones (HSLs). Some synthetic *N*-acyl HSL analogues act as quorum-sensing inhibitors and suppress biofilm formation in *Pseudomonas aeruginosa*. Development of chemical control agents against oral biofilms is necessary, because until now, biofilms have been removed only by mechanical debridement. The present study investigated the effect of *N*-acyl HSL analogues on *P. gingivalis* biofilm formation, with the aim of developing new drugs that inhibit oral biofilm formation.

Material and Methods: A flow-cell model was used for *P. gingivalis* biofilm formation. Seventeen synthetic *N*-acyl HSL analogues were quantitatively assessed by spectrophotometry. The effects of three antagonistic compounds against *P. gingivalis* biofilm formation were further examined by confocal laser scanning microscopy, and investigated for primary attachment using spectrophotometry and phase contrast microscopy.

Results: Ten out of 17 analogues affected *P. gingivalis* biofilm formation. Three out of 10 analogues significantly decreased biofilm-forming cells ($p < 0.05$), and these biofilm structures were less well formed three-dimensionally. There were no quantitative or qualitative differences in cell attachment between the control and the three analogue-treated groups.

Conclusion: Three synthetic *N*-acyl HSL analogues inhibited biofilm formation in *P. gingivalis*. We suggest that these analogues influence the development stage of *P. gingivalis* biofilm formation.

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Porphyromonas gingivalis is found in subgingival biofilms and at the bottom of periodontal pockets, which are the so-called plaque-free zone, and is difficult to remove by mechanical debridement (1–3). *P. gingivalis* can interact with various bacterial cells, saliva components and the extracellular matrix (4). Furthermore, *P. gingivalis* possesses a variety of virulence factors, such as secreted protease, lipopolysaccharide and a capsule, and is an important etiological agent of periodontal disease (4,5).

Quorum sensing is a system of bacterial cell-to-cell communication mediated by autoinducers that are secreted by bacteria. Quorum sensing is

involved in biofilm formation, virulence, bioluminescence, antibiotic production and competence (6,7). The autoinducer of gram-negative bacteria has been identified as *N*-acyl homoserine lactone (HSL; 8). Our group has reported previously that various synthetic *N*-acyl HSL analogues have acted as quorum-sensing inhibitors for *Pseudomonas aeruginosa* (9). Several compounds have been tested for agonistic and antagonistic activity using a quorum-sensing-controlled reporter gene assay, and new agonists and antagonists have been found (9). We have also found that subtle structural modifications to the agonists yield compounds with antagonistic activity, and these antagonists significantly reduce the production of virulence factors and biofilm formation (10). Quorum sensing regulates biofilm formation that is associated with a wide variety of chronic and refractory infections associated with gram-negative bacteria (11).

The details of the quorum-sensing mechanism in *P. gingivalis* are not clear. In fact, there is not much evidence to suggest that *P. gingivalis* employs *N*-acyl-HSL-mediated quorum sensing, although *P. gingivalis* is known to secrete functional autoinducer-2 signals (12). Recently, it has been reported that planktonic *P. gingivalis* cells respond to some *N*-acyl HSL molecules (13). However, it is unknown whether *N*-acyl HSLs and their analogues affect *P. gingivalis* biofilm formation.

Quorum-sensing-inhibitory compounds might be applicable in many fields, including medicine, agriculture and environmental engineering. *N*-Acyl HSL analogues have been investigated as control strategies for the inhibition of quorum sensing (14). It seems that the use of *N*-acyl HSL analogues is a potential novel strategy for controlling oral biofilms. The present study investigated the effects of *N*-acyl HSL analogues on *P. gingivalis* biofilm formation. This may be the first study to test the effect of *N*-acyl HSL analogues on periodontopathic bacteria. Moreover, we investigated the compounds that showed antagonistic activity against *P. gingivalis* biofilm formation for their effects on *P. gingivalis* cell attachment and three-dimensional structure.

Material and methods

Synthesis of *N*-acyl HSL analogues

N-Acyl HSL analogues were synthesized to replace the HSL moiety with a variety of amines and alcohols, as described previously (9). The 17 *N*-acyl HSL analogues synthesized were classified into three groups according to the number of carbons in their acyl side-chains, namely, five C_4-HSL (analogue nos 1–5), four C_6-HSL (analogue nos 6–9) and eight C_{12}-HSL analogues (analogue nos 10–17; Fig. 1).

Bacterial strains and culture conditions

The source of *P. gingivalis* strain 381 has been described previously (3). Mass cultures were grown anaerobically in Gifu anaerobic medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with hemin (Sigma-Aldrich, St Louis, MO, USA; 5 mg/L) and menadione (Wako Pure Chemical Industries, Ltd, Osaka, Japan, 1 mg/L) at 37°C.

Quantitative analysis of *P. gingivalis* biofilm-forming cells

Details of the biofilm culture model using a modified Robbins device and hydroxyapatite disks (6 mm diameter and 1 mm thick) have been described previously (15). Three separate modified Robbins devices and 30 hydroxyapatite disks (10 disks per modified Robbins device) were prepared for each analogue. In the two experimental groups, the analogues were dissolved in

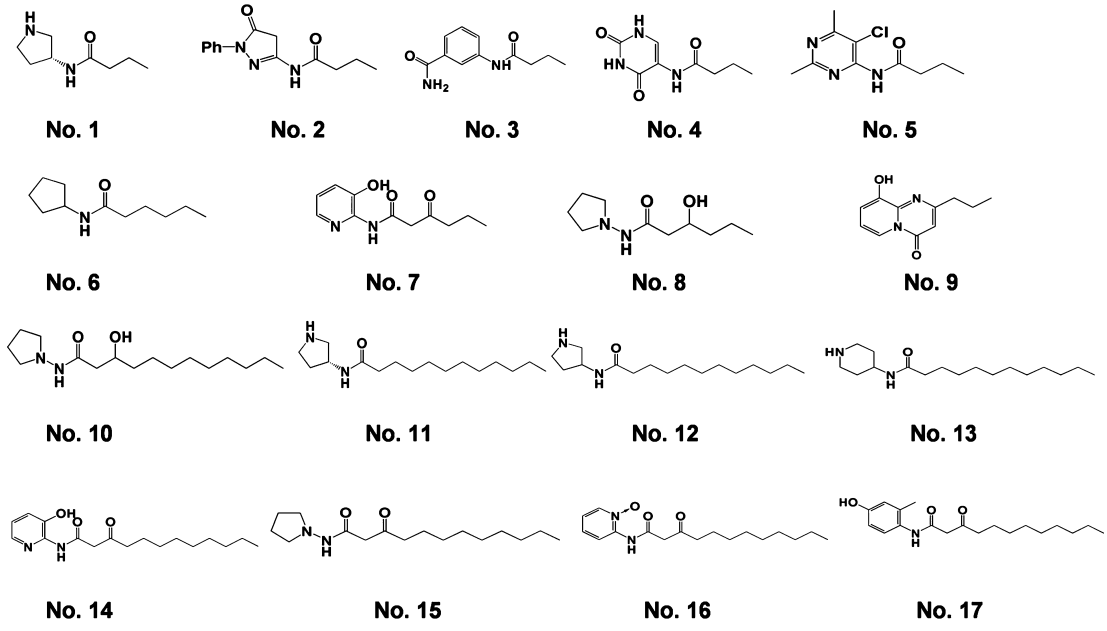

Fig. 1. Chemical structure of *N*-acyl HSL analogues tested. The analogue sample numbers correspond to those used in Figs 2–4.

1 mL of dimethyl sulfoxide, and the final concentration of each analogue was adjusted to 10 and 100 μM with culture medium. The third group, used as a control, did not have any added analogue. The hydroxyapatite disks were placed face down in the modified Robbins device after being incubated with human saliva. Culture medium (450 mL) that contained *P. gingivalis* cells (10^8 colony-forming units (CFU)/mL) and each analogue was stirred for 14 d using a peristaltic pump (SJ-1220; Atto Co., Tokyo, Japan) at a flow rate of 3.3 mL/min, as described previously (15). The *P. gingivalis* culture medium was changed every 2 d.

Quantitative analysis of the biofilm-forming cells ($n = 9$ per group) was carried out by spectrophotometry. A residual one disk per group was observed to confirm biofilm structures by scanning electron microscopy. After obtaining biofilm samples from the modified Robbins device, the specimens were rinsed with distilled water to remove non-adherent cells and ultrasonicated at 4°C for 30 min in 300 μL distilled water, to remove the *P. gingivalis* biofilms from the hydroxyapatite disks, as described in our previous study (15). Biofilm-forming cells were quantified by measuring optical density (OD) at 550 nm. In *P. gingivalis*, a relationship was previously found between OD and the number of biofilm-forming cells (in CFU/mL; $r = 0.8994$; 15).

Three-dimensional reconstruction of biofilms

P. gingivalis biofilms were formed as described above using the modified Robbins device on celluloid disks (Sumilon Celltite C-1; Sumitomo Bakelite Co. Ltd, Tokyo, Japan), in order that the laser light could penetrate. The three analogues were prepared at a concentration which showed antagonism against *P. gingivalis* biofilm formation. After 14 d, the biofilm samples on the disks were obtained from the modified Robbins device and stained with the Live/Dead® BacLight™ Bacterial Viability kit (L7007; Molecular Probes, Eugene, OR, USA) to evaluate the number of viable or dead biofilm-forming cells. A BacLight™ assay solution was adjusted according to the manufacturer's instructions, and biofilm samples were stained in the dark at room temperature for 15 min. After rinsing with water, samples were observed by confocal laser scanning microscopy (LSM 510; Carl Zeiss, München-Hallbergmoos, Germany). The scanning images were analyzed three-dimensionally by imaging software (Imaris®; Bitplane AG, Zurich, Switzerland).

Quantitative and qualitative analysis of *P. gingivalis* cell attachment

This assay was used to measure the degree of primary attachment and accumulation on a polystyrene surface, and was performed as described previously (16). A total of 100 μL of 10^8 CFU/mL *P. gingivalis* cells and each analogue (nos 9, 12 and 17) were inoculated into 96-well polystyrene microtiter plates (Becton-Dickinson, Sparks, MD, USA). The final concentration of the three *N*-acyl HSL analogues was adjusted to 10 and 100 μM. No analogues were added to the control group. The plates were incubated at 37°C under anaerobic conditions for 30 min. After 1% crystal violet was added to each well, the plates were incubated at room temperature for 30 min and washed with sterile water. The stain was dissolved in 100% ethanol, and the OD was measured at 595 nm using a spectrophotometer. Adherent cells were observed by phase contrast microscopy (ECLIPSE E600; Nikon, Tokyo, Japan).

The effects of the three analogues on the ability to adhere to three extracellular matrices were measured according to our previous method (17). Ninety-six-well plates with immobilized type I collagen, fibronectin or laminin were used. The adherent cells were stained with 1% crystal violet. After washing with distilled water, the plates were dried, and OD was measured at 570 nm.

Statistical analysis

The results of each series of experiments are shown as the means ± SEM.

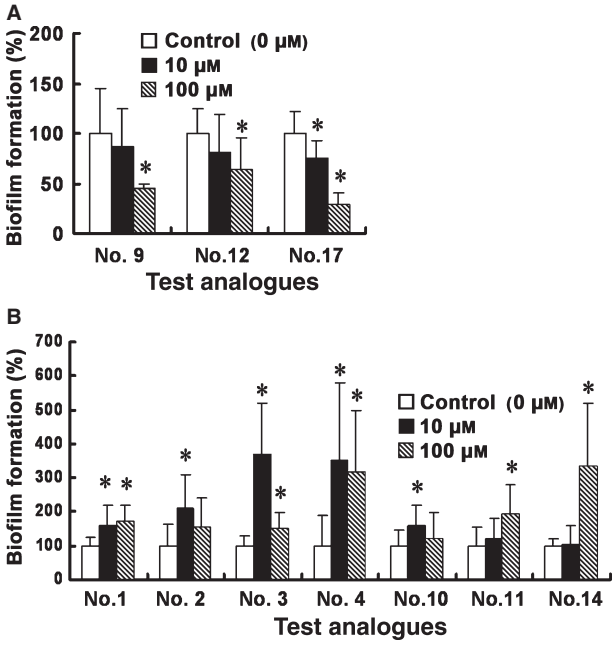

Fig. 2. Effects of analogues on *P. gingivalis* biofilm formation. In A and B, each number indicates the number of the analogue, corresponding to Fig. 1. (A) For three analogues at 100 μM and for analogue 17 at 10 μM, the OD values decreased and were significantly lower than control values. (B) Analogues 1–4, 10, 11 and 14 increased the OD values. Data are presented as the means ± SEM ($n = 9$). * Significant difference compared with the control biofilms ($p < 0.05$).

The significant differences between each experimental group and the control group for the biofilm formation and adherent cells were analyzed using Student's unpaired *t* test.

Results

Effects of *N*-acyl HSL analogues on *P. gingivalis* biofilm formation

Ten out of 17 *N*-acyl HSL analogues affected *P. gingivalis* biofilm formation (Fig. 2), while the other seven had no such effect (analogue nos 5–8, 13, 15 and 16). For biofilms treated with analogues 9 and 12 (100 μM) and 17 (10 and 100 μM), the OD values were significantly decreased compared with those of the control group ($p < 0.05$) (Fig. 2A). Analogue no. 9 was a C_6-HSL analogue, and nos 12 and 17 were C_{12}-HSL analogues. In biofilms treated with seven out of 17 *N*-acyl HSL analogues, the OD values were significantly increased compared with those of the control group ($p < 0.05$; Fig. 2B), and four out of five (analogue nos 1–4) of the C_4-HSL analogues acted as agonists.

Observation of *P. gingivalis* biofilm using confocal laser scanning microscopy

In the control group, we observed a mature biofilm of 50 μm in height that consisted of viable *P. gingivalis* cells stained green, but dead cells were not seen (Fig. 3A). In Fig. 3B–E, we observed a few red-stained dead cells in the scanning images. The volume of *P. gingivalis* biofilms on the plate surfaces in the presence of analogues 9, 12 and 17 was smaller than that of the control group (Fig. 3B–E). In the presence of 100 μM analogue 17, there was very little *P. gingivalis* biofilm formation (Fig. 3E).

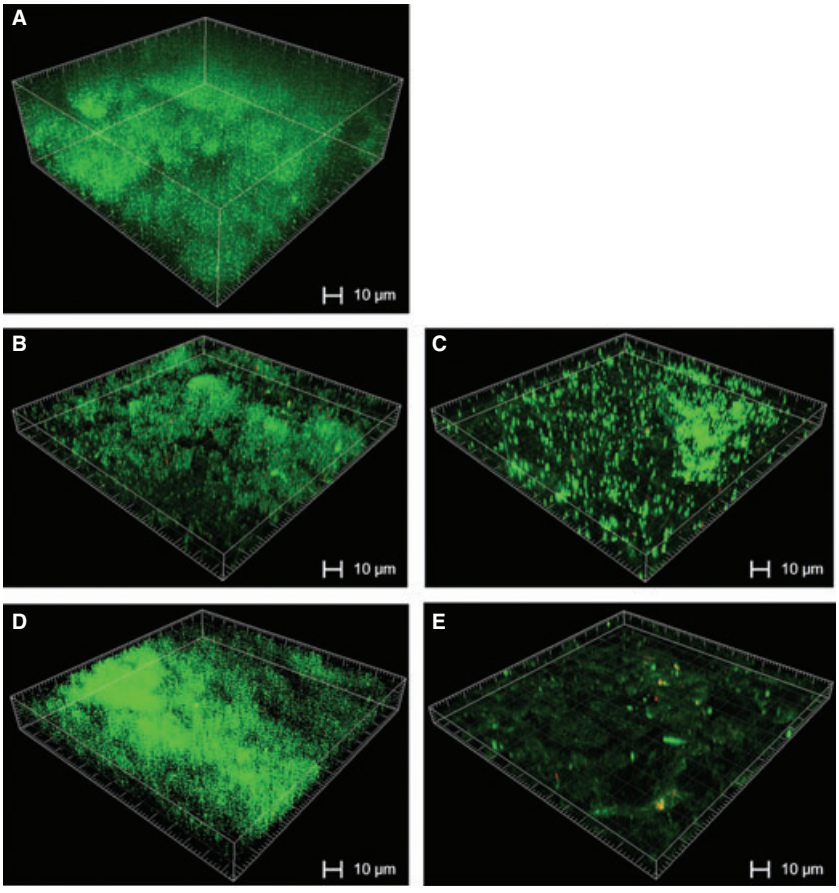

Fig. 3. Confocal laser scanning microscopy images of 14 d *P. gingivalis* biofilms grown with culture medium (A), 100 μM analogue no. 9 (B), 100 μM analogue no. 12 (C), and 10 and 100 μM analogue no. 17 (D and E, respectively). Viable cells in the biofilm were stained green, but there were very few red-stained dead cells. In the presence of analogues 9, 12 and 17, the area of biofilm formation was narrower than that in the control conditions, and the thickness was 10–25 μm (B–E).

Effects of *N*-acyl HSL analogues on *P. gingivalis* cell attachment

To evaluate the inhibitory effects of the *N*-acyl HSL analogues on cell attachment during *P. gingivalis* biofilm formation, the three analogues (nos 9, 12 and 17) that showed antagonistic action against biofilm formation were examined further. There was no significant difference in the OD values between the control group and the three analogue-treated groups by comparison of *P. gingivalis* cell attachment (Fig. 4A). Under phase contrast microscopy, there was a small qualitative difference in cell attachment or microcolony formation between the control and the three analogue-treated groups (Fig. 4B). There was no significant difference between the control and the three analogue-treated groups in the number of *P. gingivalis* cells that adhered to type I collagen, fibronectin and laminin (Fig. 4C).

Discussion

We found that 10 out of 17 analogues examined had a biological effect on *P. gingivalis* biofilm formation, although no *N*-acyl HSLs were detected in the supernatant of *P. gingivalis* culture medium (data not shown). *P. gingivalis* is known only to secrete functional autoinducer-2 signals (18), but the details of the quorum-sensing mechanism in *P. gingivalis* are not clear. Recently, it has been reported that planktonic *P. gingivalis* cells respond to some *N*-acyl HSL molecules (13). In contrast, *Escherichia coli* cannot synthesize any HSLs, but possesses an *N*-acyl HSL transcriptional activator, SdiA. *E. coli* responds to *N*-acyl-HSL-associated signals mediated by SdiA, which show a distinct effect on its biofilm formation (19).

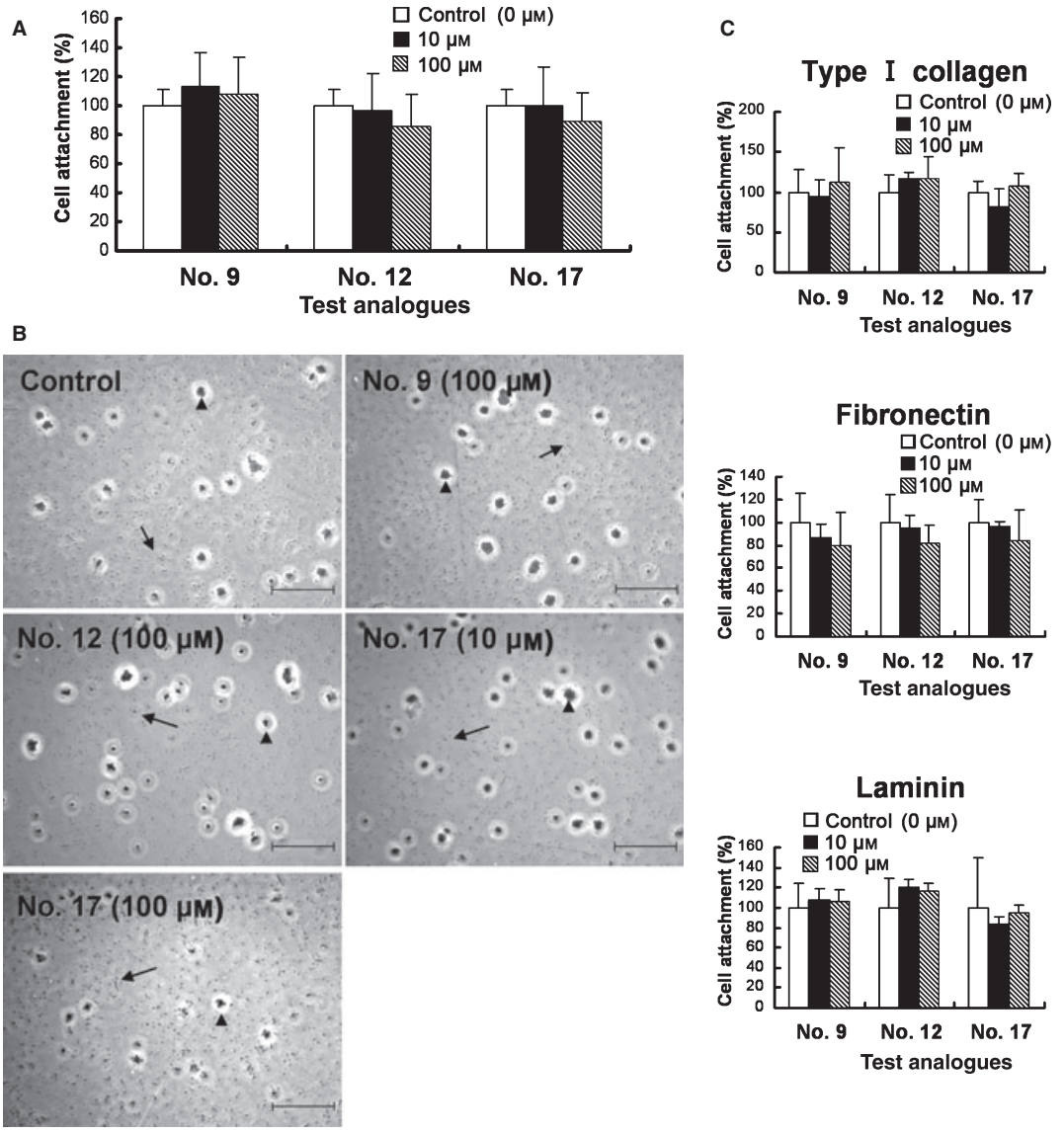

Fig. 4. Effects of the three antagonistic analogues (nos 9, 12 and 17) on *P. gingivalis* cell attachment. (A) *P. gingivalis* cell attachment to the polystyrene microtiter plates. In the presence of the analogues at 10 and 100 μM, there was no significant difference from the each control group ($n = 8$). (B) Phase contrast images of *P. gingivalis* cell attachment to the polystyrene plates. Adherent *P. gingivalis* cells (arrows) were scattered, and small numbers of microcolonies (arrowheads) were formed on the surface in all groups. (C) *P. gingivalis* cell attachment to extracellular matrices. Analogues 9, 12 and 17 had no effect on the attachment to type I collagen, fibronectin and laminin, and no significant difference was seen. Data are presented as the means ± SEM ($n = 8$).

Although *Bacteroides fragilis* has no *luxI* orthologues, it contains putative *luxR* orthologues, which can respond to exogenous *N*-acyl HSLs and modulate biofilm formation (20). In *E. coli* K-12, the SdiA receptor binds four different HSL signals, and the signals control biofilm formation in a dose-dependent manner (19). In this study, three of the 17 compounds showed antagonistic activity against *P. gingivalis* biofilm formation in a dose-dependent manner (Fig. 2A). *P. gingivalis* might possess a specific receptor that functions as an *N*-acyl HSL transcriptional activator and may receive the HSL signals in a manner similar to that of other bacteria.

Among the three *N*-acyl HSL analogues that reduced *P. gingivalis* biofilm formation, there were no structure–activity relationships with regard to the length of the acyl side-chains and the types of substitutions. There was also no structure–activity relationship among the seven agonistic analogues. No structure–activity relationships among the inhibitory HSLs or their analogues against the quorum-sensing system in *E. coli* or *P. aeruginosa* biofilm formation have been reported (9,10,19); therefore, more research is needed to understand the impact of various chemical structures on different bacteria. In contrast, exogenous *N*-acyl HSLs are capable of

regulating the production of virulence factors by interacting with cytoplasmic membranes (21). *N*-acyl HSLs produced by *P. aeruginosa* have been shown to interact with eukaryotic cells (22). This depends on the fact that such bacterial signaling compounds as *N*-acyl HSLs are functionally and structurally similar to eukaryotic lipid-based hormones. Now, there is growing evidence that *N*-acyl HSLs can elicit biological effects in eukaryotic cells (23). Another possibility is that *P. gingivalis* biofilm-forming cells receive *N*-acyl HSL analogues by non-specific reactions in the same manner as eukaryotic cells do. The 17 HSL analogues in the present study were used at concentrations of 10 and 100 μM. The latter concentration was used to confirm the influence of the analogues, although it was necessary to examine its cytotoxicity. Also, the analogues can be used *in vivo* at 10 μM.

It has been reported that tobramycin has bactericidal effects on *P. aeruginosa* biofilm-forming cells, using a staining technique for viable and dead cells (24). In this study, we considered that the three antagonistic compounds would not have bactericidal activity against *P. gingivalis* biofilm-forming cells, because a low number of red-stained cells was seen by confocal laser scanning microscopy (Fig. 3B–E), and most analogues did not influence planktonic *P. gingivalis* cell growth under perfusion for 14 d at 10 and 100 μM (data not shown). These three analogues might exert their antibiofilm activity by inhibition of growth of biofilm-forming cells, which promotes detachment from part of the biofilm.

Biofilm communities are achieved through a programmed series of processes, which include cell attachment, microcolony formation and differentiation of the mature biofilm, and then biofilm-forming bacteria detach from the structured biofilm matrix (25). In this study, we selected three extracellular matrices (collagen, laminin and fibronectin), which exist in periodontal tissue, to examine the effects of analogues on initial attachment. It is possible that these surface matrices might be unaffected by the three antagonistic analogues (nos 9, 12 and 17) at the concentrations examined in this study. The three *N*-acyl HSL analogues showed antagonistic action against *P. gingivalis* biofilm formation, but did not affect the initial attachment of the *P. gingivalis* cells (Fig. 4A,C). A quorum-sensing-defective mutant of *P. aeruginosa* forms flat and undifferentiated biofilms, and it has been suggested that quorum-sensing systems are involved in biofilm differentiation (26). Under the control of a quorum-sensing inhibitor, *P. aeruginosa* forms a flat and undifferentiated biofilm that is similar to that of the quorum-sensing-defective mutant (24). It seemed that the biofilms in the three groups treated with analogues 9, 12 and 17 were three-dimensionally less mature (Fig. 3B–E). Moreover, when the three antagonistic analogues were applied respectively for 1 wk after formation of mature *P. gingivalis* biofilms, using the same flow cell model, there was no significant reduction in the number of *P. gingivalis* biofilm-forming cells in each analogue-treated group (data not shown). Since initial attachment and mature biofilms were unaffected by the three antagonistic analogues, it is likely that they exert their effects on microcolony formation or biofilm development in *P. gingivalis*.

We found that the three *N*-acyl HSL analogues significantly reduced the number of biofilm-forming cells during *P. gingivalis* biofilm formation. Some of the *N*-acyl HSL analogues decreased *P. gingivalis* biofilm formation; therefore, the analogues may be useful against other oral bacterial species that cannot produce any *N*-acyl HSLs. However, we must still address some questions concerning the effects of *N*-acyl HSL analogues on *P. gingivalis* biofilm formation. Thus quorum-sensing signaling systems are highly attractive targets for the development of novel prophylactic and therapeutic agents against oral biofilm infections.

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