

## Inhibition of Quorum Sensing in *Serratia marcescens* AS-1 by Synthetic Analogs of *N*-Acylhomoserine Lactone<sup>∇</sup>

Tomohiro Morohoshi,<sup>1\*</sup> Toshitaka Shiono,<sup>1</sup> Kiyomi Takidouchi,<sup>1</sup> Masashi Kato,<sup>1</sup> Norihiro Kato,<sup>1</sup> Junichi Kato,<sup>2</sup> and Tsukasa Ikeda<sup>1</sup>

Department of Applied Chemistry, Utsunomiya University, Utsunomiya, Tochigi 321-8585, Japan,<sup>1</sup> and Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, Higashi-Hiroshima, Hiroshima 739-8530, Japan<sup>2</sup>

Received 14 March 2007/Accepted 27 July 2007

**Quorum sensing is a regulatory system for controlling gene expression in response to increasing cell density. *N*-Acylhomoserine lactone (AHL) is produced by gram-negative bacteria, which use it as a quorum-sensing signal molecule. *Serratia marcescens* is a gram-negative opportunistic pathogen which is responsible for an increasing number of serious nosocomial infections. *S. marcescens* AS-1 produces *N*-hexanoyl homoserine lactone (C<sub>6</sub>-HSL) and *N*-(3-oxohexanoyl) homoserine lactone and regulates prodigiosin production, swarming motility, and biofilm formation by AHL-mediated quorum sensing. We synthesized a series of *N*-acyl cyclopentylamides with acyl chain lengths ranging from 4 to 12 and estimated their inhibitory effects on prodigiosin production in AS-1. One of these molecules, *N*-nonanoyl-cyclopentylamide (C<sub>9</sub>-CPA), had a strong inhibitory effect on prodigiosin production. C<sub>9</sub>-CPA also inhibited the swarming motility and biofilm formation of AS-1. A competition assay revealed that C<sub>9</sub>-CPA was able to inhibit quorum sensing at four times the concentration of exogenous C<sub>6</sub>-HSL and was more effective than the previously reported halogenated furanone. Our results demonstrated that C<sub>9</sub>-CPA was an effective quorum-sensing inhibitor for *S. marcescens* AS-1.**

*Serratia marcescens* is a gram-negative opportunistic human pathogen which is responsible for an increasing number of serious nosocomial infections (8). *S. marcescens* produces a range of secreted products, including proteases, nucleases, lipases, chitinases, the biosurfactant serrawettin, and hemolysin (8). *S. marcescens* also produces prodigiosin (2-methyl-3-pentyl-6-methoxyprodigiosin), which is a red pigment and has antibacterial, antifungal, antiprotozoan, and immunosuppressant activities (7, 27). Prodigiosin production is regulated by a range of environmental signals, such as temperature, phosphate limitation, and medium components (7, 27). Nosocomial infections due to some clinical isolates of *S. marcescens* are also problematic because this pathogen is commonly multidrug resistant (27). Thus, it is necessary to seek a novel treatment technique for *Serratia* infections without overuse of antibiotics.

Quorum sensing is a regulatory system for controlling gene expression in response to increasing cell density (2, 6). *N*-Acylhomoserine lactone (AHL) (Fig. 1A) is produced by gram-negative bacteria, which use it as a quorum-sensing signal molecule (2). The LuxI protein family synthesizes AHL, and the LuxR protein family binds AHL and regulates expression of many genes responsible for bioluminescence, production of pigments or antibiotics, etc. (2, 6). In particular, many gram-negative pathogens control the expression of virulence factors, secretion of extracellular protease, pectinase, and rhamnolipid, and biofilm formation via the quorum-sensing system (2).

It has been shown that *Serratia* strains employ quorum sensing for the regulation of genes encoding extracellular virulence factors (31). *Serratia* sp. strain ATCC 39006 produces *N*-butanoyl homoserine lactone (C<sub>4</sub>-HSL) and *N*-hexanoyl homoserine lactone (C<sub>6</sub>-HSL) and regulates production of prodigiosin and carbapenem with AHLs (30). *S. liquefaciens* (*S. marcescens*) MG1 produces C<sub>4</sub>-HSL and C<sub>6</sub>-HSL and regulates swimming motility and biofilm formation (14, 23). *S. marcescens* SS-1 produces four AHLs, *N*-(3-oxohexanoyl) homoserine lactone (3-oxo-C<sub>6</sub>-HSL), C<sub>6</sub>-HSL, *N*-heptanoyl homoserine lactone, and *N*-octanoyl homoserine lactone, and regulates sliding motility, synthesis of biosurfactant, prodigiosin, and nuclease (10). It was reported previously that signal generation mutants of *Serratia* strains were deficient in production of exoenzymes and prodigiosin and in biofilm formation (10, 14, 30). Thus, interfering with AHL-mediated quorum sensing could be an effective means of preventing infectious diseases caused by *Serratia* strains.

It was also reported previously that natural or nonnatural inhibitors were effective for preventing the expression of the genes controlled by the quorum-sensing system. Synthetic compounds modeled on the natural AHLs were evaluated for both their inducing activity and their ability to competitively inhibit the action of AHL in *Vibrio fischeri*, *Burkholderia cenocepacia*, and *Pseudomonas aeruginosa* (1, 3, 13, 22, 24, 28, 29). Halogenated furanones, produced by the red alga *Delisea pulchra*, inhibit quorum sensing in a number of bacteria (4, 5, 9, 20, 21). In *Serratia*, it was reported that halogenated furanone affected swarming motility in *S. liquefaciens* MG1 (4, 21). However, inhibitory effects on other features in *Serratia* strains, including prodigiosin production and biofilm formation, were not identified.

In a previous study, we found that *N*-acyl cyclopentylamide

\* Corresponding author. Mailing address: Department of Applied Chemistry, Utsunomiya University, 7-1-2 Yoto, Utsunomiya 321-8585, Japan. Phone: 81-28-689-6176. Fax: 81-28-689-6176. E-mail: morohoshi@cc.utsunomiya-u.ac.jp.

<sup>∇</sup> Published ahead of print on 3 August 2007.

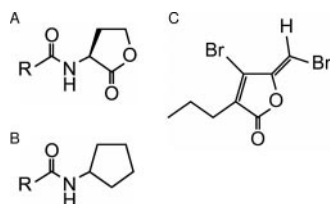


FIG. 1. Structures of AHL and quorum-sensing inhibitors used in this study. (A) AHL. (B) Cn-CPA. (C) 4-Bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone.

(Cn-CPA) (Fig. 1B) inhibited expression of the *lasB-lacZ* transcriptional fusion gene and biofilm formation without affecting the growth of *P. aeruginosa* PAO1 (12). In this paper, we demonstrate that Cn-CPA is an effective quorum-sensing inhibitor which interferes with expression of *S. marcescens* virulence factors regulated by the quorum-sensing system. Additionally, we also compared the inhibitory effects of Cn-CPA and halogenated furanone on quorum sensing.

#### MATERIALS AND METHODS

**Bacterial strains, plasmids, compounds, and growth conditions.** Selected bacterial strains and plasmids used in this study are listed in Table 1. All bacterial strains were cultured in Luria-Bertani (LB) medium (25). *S. marcescens* AS-1 and AS-1S were grown at 25°C for stable production of prodigiosin. *Escherichia coli* and *Chromobacterium violaceum* were grown at 30°C. AHL standards and Cn-CPA were synthesized by a previously described method (11, 12). 4-Bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone was also synthesized by a previously described method (16). Antibiotics were added as required at final concentrations of 100 µg/ml for ampicillin and 50 µg/ml for kanamycin.

**Cloning and disruption of the AHL synthase gene of AS-1.** Chromosomal DNA of AS-1 was extracted to construct a genomic library by the standard protocol (25). DNA was digested partially with *Sau3AI*, and the fragments were inserted into the *Bam*HI site of cloning vector pSTV28. A genomic library of AS-1 was transformed into *E. coli* XL1-Blue, and the ability to produce AHL was checked by cross-streaking with a CV026 biosensor. One of the AHL-producing plasmids, pASS01, was digested with various restriction enzymes for construction of a restriction map. Sequencing was performed by using BigDye Terminator version 3.1 and an ABI Prism 3100 genetic analyzer (Applied Biosystems). To disrupt the AHL synthase gene designated *spnI*, pASS01 was digested partially with *PstI* and inserted into the 1.3-kb kanamycin cassette (*kan*) from *PstI*-

digested pUC4K for construction of pASS01K. The *spnI::kan* region of pASS02K was amplified by PCR using primers 5'-GATCCGAGGCTCAGCAAACA-3' and 5'-TATTGTCTCCAAACTGGGCG-3' and inserted into the *EcoRV* site of pGP704Sac38 for construction of pGP704SK. Disruption of the chromosomal *spnI* gene in AS-1 was performed by bacterial conjugation (19). Conjugation between *E. coli* S17-1  $\lambda$ pir with pGP704SK and AS-1 was performed. The chromosomal disruption of *spnI* was checked by PCR using the same primers, and the mutant was designated AS-1S.

**Extraction and bioassays of AHLs.** Bacteria were grown for 15 h, inoculated into 100 ml fresh LB medium (1% inoculum), and incubated for 20 h. Cells were removed by centrifugation, and the supernatant was mixed with an equal volume of acidified ethyl acetate. The ethyl acetate layer was transferred to a new flask, evaporated to dryness, and dissolved in 1 ml of dimethyl sulfoxide. For AHL detection, 0.25 ml of an overnight culture of the CV026 biosensor was mixed with 25 ml of 1.5% LB agar and poured into a petri dish. Eight-millimeter-diameter paper disks (Advantec Inc.) were placed on an agar plate, and 10 µl of culture extract was applied to the disks. The plates were incubated overnight at 28°C, and the area containing a purple pigment was measured to determine the amount of AHLs. AHL samples were also subjected to analytical and preparative thin-layer chromatography (TLC) by using a previously described method (19).

**Prodigiosin production assay.** *S. marcescens* strains were grown for 15 h, inoculated into fresh LB medium (1% inoculum) with or without inhibitors, and incubated for 20 h. Intracellular prodigiosin was extracted from the cells in an acidified ethanol solution (4% 1 M HCl in ethanol) (27). Prodigiosin production was determined by determining the ratio of the absorbance of the extracted prodigiosin solution at 534 nm to the turbidity of the culture suspension as optical density at 600 nm. The effects of inhibitors were evaluated using the relative prodigiosin production ( $A_{534}$ /optical density at 600 nm), for which the control value was 100%.

**Motility assays.** The medium used for motility assays was LB medium. Swimming assay plates contained 0.3% (wt/vol) agar, and swarming assay plates contained 0.5% (wt/vol) agar (17). Assay plates were inoculated with bacteria from an overnight culture in LB agar (1.5% [wt/vol]) at 25°C with a sterile toothpick. The plates were incubated at 25°C for 20 h.

**Biofilm formation assay.** Biofilm formation was determined by a previously described method, with a slight modification (14). The biofilm formation medium used was Difco minimal broth Davis with 0.2% glucose and 0.5% Casamino Acids (8). A full-grown culture of AS-1S was diluted 100-fold in biofilm formation medium, and 200 µl of the dilution was added to each well of a polypropylene microtiter plate (Corning Inc.). After incubation at 25°C for 20 h, 25 µl of a 1% crystal violet solution was added to each well. The plates were incubated at room temperature for 15 min and rinsed with distilled water. The crystal violet was dissolved in 200 µl of 95% ethanol, and biofilm formation was analyzed at 570 nm by using a Spectra Max 250 spectrophotometer (Molecular Devices).

**Nucleotide sequence accession numbers.** The nucleotide sequences of the *spnI/spnR* locus and the 16S rRNA gene from strain AS-1 have been deposited

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Description	Source or reference
<i>Serratia marcescens</i> strains		
AS-1	Laboratory-maintained strain, natural isolate	This study
AS-1S	AS-1 <i>spnI::kan</i> , AHL-negative strain	This study
<i>Escherichia coli</i> strains		
XL1-Blue	<i>recA1 endA1 gyrA46 thi hsdR17 supE44 relA1 lac/F'</i> [ <i>proAB</i> <sup>+</sup> <i>lacI</i> <sup>q</sup> <i>lacZ</i> ΔM15::Tn10 (Tet <sup>r</sup> )]	Stratagene
S17-1 $\lambda$ pir	<i>thi pro hsdR hsdM</i> <sup>+</sup> <i>recA</i> RP4 2Tc::Mu-Km::Tn7	26
<i>Chromobacterium violaceum</i>		
CV026	Mini-Tn5 mutant derived from <i>C. violaceum</i> ATCC 31532, Hg <sup>r</sup> <i>cviI::Tn5 xylE Kan</i> <sup>r</sup> plus spontaneous Sm <sup>r</sup> , AHL biosensor	18
Plasmids		
pSTV28	Cloning vector, Cm <sup>r</sup>	Takara Bio
pUC4K	Cloning vector, Kan <sup>r</sup> Ap <sup>r</sup>	Pharmacia
pASS01	3.0-kb <i>Sau3AI</i> fragment from AS-1 genomic DNA in pSTV28	This study
pASS01K	pASS01 containing <i>kan</i> in <i>PstI</i> site	This study
pGP704Sac38	pBR322 derivative with R6K <i>ori</i> , <i>mob</i> RP4, polylinker from M13 tg131 containing <i>sacB</i> ; Ap <sup>r</sup>	19
pGP704SK	pGP704Sac38 containing <i>spnI::kan</i> from pASS01K	This study

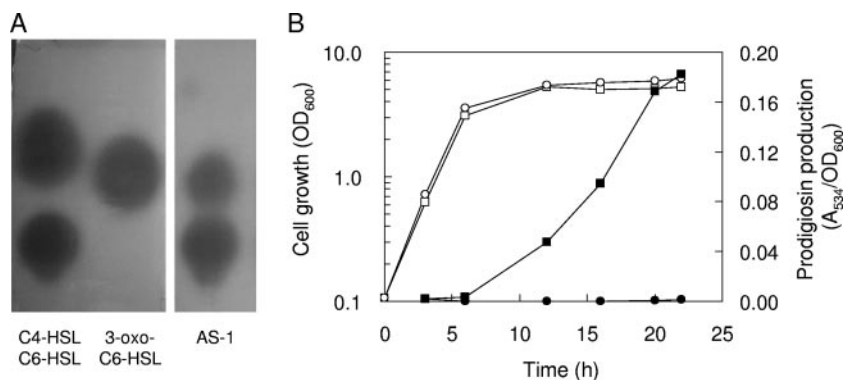


FIG. 2. Identification and characterization of AHLs produced by *S. marcescens* AS-1 and AS-1S. (A) TLC analysis of AHLs produced by strain CV026 and its mutants. AHLs were visualized as pigments produced by strain CV026 and were identified after comparison with the standards C<sub>4</sub>-HSL, C<sub>6</sub>-HSL, and 3-oxo-C<sub>6</sub>-HSL. (B) Production of prodigiosin throughout growth in LB medium in AS-1 (squares) and AS-1S (circles). Samples were taken at various times and analyzed to determine growth (open symbols) and prodigiosin production (filled symbols). OD<sub>600</sub>, optical density at 600 nm.

in the DDBJ/EMBL/GenBank databases under accession no. AB234869 and AB270613, respectively.

## RESULTS

**Effect of the *spnI* mutation on AHL and prodigiosin production.** *S. marcescens* AS-1 was isolated from a soil sample as an AHL producer. The nucleotide sequence of the 16S rRNA fragment from AS-1 showed 99.9% identity with that of *S. marcescens* strain AU736 (accession no. AY043386). First, we attempted to clone the AHL synthase gene from AS-1. To clone the AHL synthase gene, we prepared a genomic library of AS-1. One of the approximately 3,000 plasmids, designated pASS01, showed obvious AHL-producing activity. The nucleotide sequence of a 3.0-kb fragment of pASS01 revealed the presence of two open reading frames, which were the AHL synthase gene (*spnI*) and the gene homologous to the gene encoding LuxR (*spnR*). The amino acid sequences of the putative gene products of *spnI* and *spnR* showed 75.4 and 94.3% identity to SpnI and SpnR from *S. marcescens* SS-1, respectively (10). The 159 bp upstream of the translation initiation site of *spnR* contained a dyad, ACCTGACCGAAAGGTCAGGT, which contains conserved bases of the consensus lux box sequence (5'-RNSTGYAXGATNXTRCASRT-3') (6). This sequence exhibited 100% sequence identity to the lux box-like sequence present upstream of *spnR* from SS-1 (10). In addition, the *spnI* and *spnR* open reading frames have a 23-bp overlapping region at their 3' ends. This overlap can be observed in the SS-1 *spnI/spnR* locus (data not shown).

Using a gene replacement method, we constructed the *spnI* mutant designated AS-1S (*spnI::kan*). To check whether deletion of the *spnI* gene affected the biosynthesis of AHLs, the amount of AHLs produced in the culture supernatant was determined. AHLs were extracted with acidified ethyl acetate and fractionated by TLC. A TLC-overlaid CV026 biosensor revealed two AHL spots in the culture supernatant of AS-1, but no spot was observed in the culture supernatant of AS-1S. As determined by comparison with synthetic AHLs, these spots corresponded to 3-oxo-C<sub>6</sub>-HSL and C<sub>6</sub>-HSL (Fig. 2A). SpnI from SS-1 produced two AHLs, 3-oxo-C<sub>6</sub>-HSL and C<sub>6</sub>-HSL, as major products (10). It was assumed

that these two SpnI proteins had similar functions in AHL biosynthesis.

Prodigiosin production by AS-1 and AS-1S was determined throughout growth (Fig. 2B). Although cultures of these two strains had similar growth rates, prodigiosin production was increased at early stationary phase in strain AS-1 but not observed at any time in strain AS-1S. Various AHLs which have different acyl chain lengths were added to the cultures of AS-1S, and prodigiosin production was determined. Addition of C<sub>6</sub>-HSL at a concentration of 5 μM stimulated prodigiosin production in AS-1S, but addition of C<sub>4</sub>-HSL or *N*-octanoyl homoserine lactone did not (data not shown). Prodigiosin production by AS-1S was completely restored to wild-type levels by supplying 20 μM C<sub>6</sub>-HSL (data not shown). It was hypothesized that prodigiosin production was controlled by AHL-mediated quorum sensing and that this molecule could be used as a marker for screening quorum-sensing inhibitors.

**Effects of Cn-CPA on prodigiosin production.** In a previous study, we demonstrated that Cn-CPA had an inhibitory effect on induction of the quorum-sensing-controlled genes in *P. aeruginosa* PAO1 (12). Therefore, we checked whether Cn-CPA had the ability to interfere with prodigiosin production controlled by quorum sensing in AS-1. A series of Cn-CPAs with acyl chain lengths ranging from 4 to 12 were synthesized and dissolved in dimethyl sulfoxide. Each Cn-CPA was added to an AS-1 culture at a final concentration of 200 μM. After determination of prodigiosin production, two Cn-CPAs, *N*-octanoyl-cyclopentylamide and *N*-nonanoyl-cyclopentylamide (C<sub>9</sub>-CPA), showed high levels of inhibitory effects on prodigiosin production (Fig. 3). The most effective inhibitor was C<sub>9</sub>-CPA, which inhibited prodigiosin production 87% at a final concentration of 200 μM. Prodigiosin production assays were performed using a range of concentrations of C<sub>9</sub>-CPA. The results revealed that C<sub>9</sub>-CPA inhibited prodigiosin production in a dose-dependent manner (Fig. 4). The concentration of C<sub>9</sub>-CPA required for half-maximal inhibition was approximately 24 μM. The growth of AS-1 was not affected by addition of C<sub>9</sub>-CPA at any concentration up to 200 μM (data not shown). These results indicate that C<sub>9</sub>-CPA specifically inhibits the quorum-sensing system.



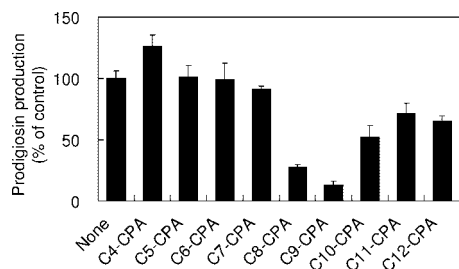


FIG. 3. Effects of Cn-CPA on prodigiosin production in *S. marcescens* AS-1. Intracellular prodigiosin was extracted after 20 h of cultivation. The results were reproduced in three experiments, and the error bars indicate standard deviations.

To investigate the competition between C<sub>6</sub>-HSL and C<sub>9</sub>-CPA during prodigiosin production, we used an AHL-negative strain, AS-1S. For the competition assay, C<sub>6</sub>-HSL was used at a final concentration of 5 μM, which was required for 50% maximal induction of prodigiosin production under our conditions (data not shown). The competition assay was performed using a range of concentrations of C<sub>9</sub>-CPA and 5 μM C<sub>6</sub>-HSL. In the presence of exogenous C<sub>6</sub>-HSL, C<sub>9</sub>-CPA inhibited prodigiosin production in a dose-dependent manner. When C<sub>9</sub>-CPA was added at a concentration of 20 μM, which was four times the concentration of C<sub>6</sub>-HSL, prodigiosin production almost disappeared (Fig. 5). In *S. marcescens* SS-1, long-chain AHLs (*N*-decanoyl homoserine lactone and *N*-dodecanoyl homoserine lactone) are capable of antagonizing sliding motility controlled by quorum sensing (10). However, AHLs with acyl chain lengths of 4 to 10 did not competitively inhibit prodigiosin production induced by 5 μM C<sub>6</sub>-HSL in AS-1S (data not shown). It was reported previously that halogenated furanone inhibited quorum sensing in *S. liquefaciens* MG1. Thus, we also compared the inhibitory effects on prodigiosin production of C<sub>9</sub>-CPA and halogenated furanone. However, although halogenated furanone had an inhibitory effect on prodigiosin production, some prodigiosin was produced at a concentration of 20 μM (Fig. 5). These results indicated that C<sub>9</sub>-CPA had a

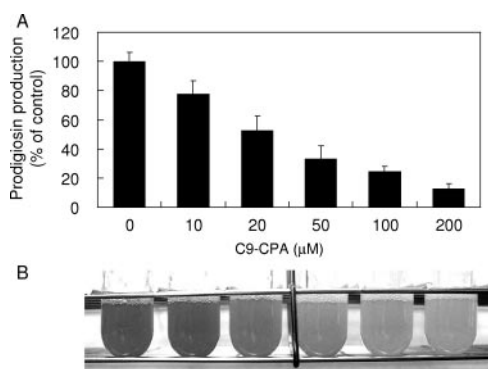


FIG. 4. (A) Dose response showing inhibitory effects of C<sub>9</sub>-CPA on prodigiosin production in *S. marcescens* AS-1. Intracellular prodigiosin was extracted after 20 h of cultivation. The results were reproduced in three experiments, and the error bars indicate standard deviations. (B) Red-pigmented cultures with various concentrations of C<sub>9</sub>-CPA.

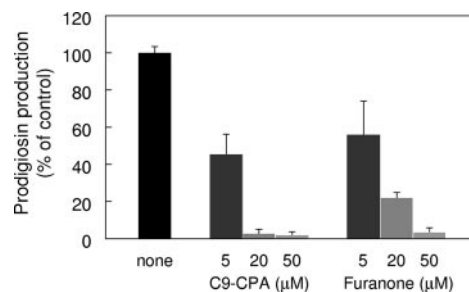


FIG. 5. Competition assay with C<sub>6</sub>-HSL and quorum-sensing inhibitors of prodigiosin production in *S. marcescens* AS-1. AS-1 was cultivated with 5 μM C<sub>6</sub>-HSL and various concentrations of C<sub>9</sub>-CPA or halogenated furanone. Intracellular prodigiosin was extracted after 20 h of cultivation. The results were reproduced in three experiments, and the error bars indicate standard deviations.

greater inhibitory effect on quorum sensing than halogenated furanone.

**Effects of C<sub>9</sub>-CPA on motility and biofilm formation.** In a previous report, it was shown that in *Serratia* strains various phenotypes other than prodigiosin production are regulated. For instance, biofilm formation and sloughing in *S. liquefaciens* MG1 are controlled by quorum sensing (14, 23). Swarming motility in *S. liquefaciens* MG1 and sliding motility in *S. marcescens* SS-1 are regulated by AHL-mediated quorum sensing (4, 10). To examine inhibition of these features, workers have investigated whether halogenated furanone affects the swarming motility of *S. liquefaciens* MG1 (4, 21). Therefore, we checked whether Cn-CPA had the ability to interfere with surface motility on agar plates and biofilm formation in AS-1. Assays were performed on LB medium with 0.3% agar for swimming motility assays or with 0.5% agar for swarming motility assays. AS-1 and AS-1S showed obvious swimming activity with or without AHL or Cn-CPA (data not shown). In contrast, AS-1 showed surface translocation on a swarming assay plate, but AS-1S did not. This defect in swarming motility in AS-1S was restored by adding 5 μM C<sub>6</sub>-HSL, which was required for 50% maximal induction of prodigiosin production (Fig. 6). These data indicated that quorum sensing in AS-1 controlled swarming motility but did not control swimming motility. A competition assay with C<sub>6</sub>-HSL was performed at a C<sub>9</sub>-CPA concentration of 50 μM, and surface translocation of AS-1S disappeared completely (Fig. 6). We also tested biofilm formation on a polypropylene plastic surface. AS-1 and AS-1S produced certain amounts of biomass that adhered to the polypropylene plastic after 20 h. The biofilm formation by AS-1S was reduced to approximately 45% of the parental level. When 5 μM C<sub>6</sub>-HSL was added to each well, biofilm formation was increased to a level that was approximately 62% of the parental biofilm level (Fig. 7). This behavior implied that biofilm formation by AS-1 was influenced by AHL-mediated quorum sensing. A competition assay with C<sub>6</sub>-HSL was performed using various concentrations of C<sub>9</sub>-CPA, and the adherent biomass decreased in a dose-dependent manner (Fig. 7). In particular, the amount of adherent biomass decreased until it was at the control level when C<sub>9</sub>-CPA was added at a concentration of 50 μM.

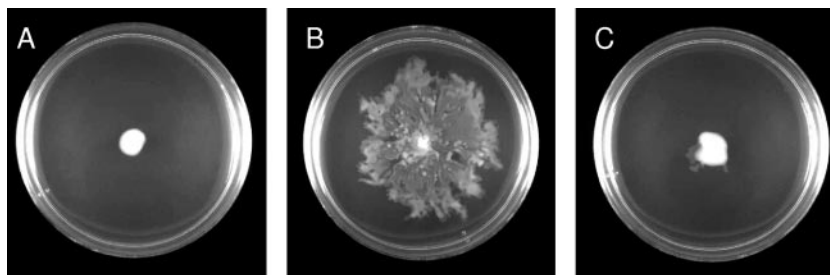


FIG. 6. Analysis of swarming motility of AS-1S on swarming assay plates with 0.5% agar. Plates were incubated at 25°C and monitored after 20 h. (A) AS-1S without any compounds. (B) AS-1S with 5  $\mu\text{M}$  C<sub>6</sub>-HSL. (C) AS-1S with 5  $\mu\text{M}$  C<sub>6</sub>-HSL and 50  $\mu\text{M}$  C<sub>9</sub>-CPA.

## DISCUSSION

In this work, we demonstrated that C<sub>9</sub>-CPA was an effective quorum-sensing inhibitor for *S. marcescens* AS-1. Although several quorum-sensing inhibitors have been evaluated for some bacteria, there have been few reports on quorum-sensing inhibitors for *Serratia* strains. It was reported previously that halogenated furanone affected swarming motility in *S. liquefaciens* MG1. Our work provides the first report of nonnatural quorum-sensing inhibitors for *S. marcescens*. C<sub>9</sub>-CPA was able to inhibit not only swarming motility but also prodigiosin production and biofilm formation in AS-1. In addition, the inhibitory effect of C<sub>9</sub>-CPA on quorum sensing in AS-1 was greater than the effect reported for halogenated furanone. We have previously demonstrated that C<sub>9</sub>-CPA had an inhibitory effect on prodigiosin production in another *Serratia* strain, *S. rubidaea* N-1, which is a producer of prodigiosin and a highly salt-tolerant strain (32). It is possible that Cn-CPA inhibits quorum sensing in a broad range of *Serratia* strains.

The length of the acyl chain of the Cn-CPA most effective for inhibition of quorum sensing in AS-1 was nine. Although the length of the acyl chain of AHLs produced by AS-1 was six (C<sub>6</sub>-HSL and 3-oxo-C<sub>6</sub>-HSL), *N*-hexanoyl-cyclopentylamide did not affect quorum sensing in AS-1. In *P. aeruginosa* PAO1, which produces two AHLs, C<sub>4</sub>-HSL and *N*-(3-oxododecanoyl) homoserine lactone, the most effective quorum-sensing inhibitor was *N*-decanoyl-cyclopentylamide (12). *N*-decanoyl-cyclo-

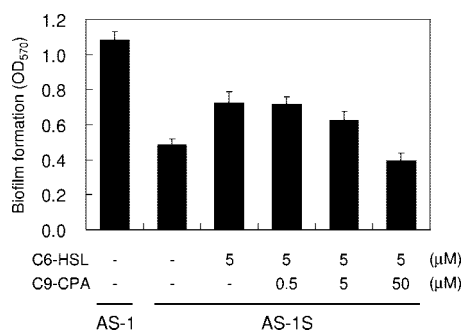


FIG. 7. Quantification of bacteria in biofilms formed on polypropylene plastic by strains AS-1 and AS-1S. Biofilms were allowed to form in a 96-well polypropylene microtiter dish, stained with crystal violet, and estimated by analyzing the absorbance at 570 nm (OD<sub>570</sub>). The amounts of C<sub>6</sub>-HSL and C<sub>9</sub>-CPA used in the reactions are indicated at the bottom. Seven wells of each sample were used for measuring biofilm formation, and the error bars indicate standard deviations.

pentylamide had a great inhibitory effect on the *las* system, which was controlled by *N*-(3-oxododecanoyl) homoserine lactone. The length of the acyl chain of the most effective Cn-CPA for AS-1 and PAO1 differed considerably from the lengths of the acyl chains of their own AHLs. The interaction between R-protein and the Cn-CPA inhibitor has not been elucidated clearly, so it is thought that investigation of the interaction between purified SpnR and C<sub>9</sub>-CPA is needed. On the other hand, it was reported that halogenated furanone accelerated LuxR turnover (15). The genes coding for prodigiosin, biosurfactant, and nuclease biosynthesis in *S. marcescens* SS-1 are negatively regulated by SpnIR (10). In *S. marcescens* SS-1, AHLs produced via SpnI interact with SpnR, resulting in de-repression of these genes (10). The amino acid sequence of SpnR from AS-1 showed high homology with that of SpnR from SS-1 (94% identity). If SpnR from AS-1 works as a negative regulator in quorum sensing as well as SpnR from SS-1, acceleration of SpnR turnover could lead to activation of the genes regulated by quorum sensing. Thus, it was assumed that C<sub>9</sub>-CPA interferes with the quorum-sensing system by acting as an effective competitor of AHL binding to SpnR.

Many kinds of quorum-sensing inhibitors modeled on the natural AHLs have been designed, synthesized, and evaluated for inhibition of quorum sensing. However, chemosynthesis of these compounds was complicated and required a number of synthetic steps. Cn-CPA can be synthesized easily by using appropriate acyl chlorides and cyclopentylamine through one coupling reaction and several extraction processes (12). Although the relationship between quorum sensing and opportunistic infection by *Serratia* is not clear, C<sub>9</sub>-CPA was able to inhibit biofilm formation and motility, which were considered some of the major virulence factors. In the future, it may be possible to use C<sub>9</sub>-CPA as an antipathogen drug for *Serratia* infections, as a substitute for current antibiotic drugs.

## ACKNOWLEDGMENTS

We are grateful to Satoshi Ito of Utsunomiya University and Takemori Ishida of Hiroshima University for technical advice.

This work was supported in part by Grant-in-Aid for Young Scientists (B) 18760588 from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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