INFECTION AND IMMUNITY, Feb. 2006, p. 910–919 Vol. 74, No. 2
0019-9567/06/$08.00+0 doi:10.1128/IAI.74.2.910–919.2006

N-Acylhomoserine Lactones Antagonize Virulence Gene Expression and Quorum Sensing in *Staphylococcus aureus*

Saara Qazi,[1]† Barry Middleton,[1]†‡ Siti Hanna Muharram,[1] Alan Cockayne,[1] Philip Hill,[1,3] Paul O'Shea,[2] Siri Ram Chhabra,[1] Miguel Cámara,[1] and Paul Williams[1]*

Institute of Infection, Immunity and Inflammation, Centre for Biomolecular Sciences, University of Nottingham, Nottingham NG7 2RD,[1] School of Biology, University of Nottingham, Nottingham NG7 2RD,[2] and School of Biosciences, Sutton Bonington Campus, University of Nottingham, Loughborough, Leicestershire LE12 5RD,[3] United Kingdom

Received 9 June 2005/Returned for modification 14 July 2005/Accepted 24 October 2005

Many gram-negative bacteria employ *N*-acylhomoserine lactone (AHL)-mediated quorum sensing to control virulence. To determine whether gram-positive bacteria such as *Staphylococcus aureus* respond to AHLs, we used a growth-dependent *lux* reporter fusion. Exposure of *S. aureus* to different AHLs revealed that 3-oxo-substituted AHLs with C_{10} to C_{14} acyl chains inhibited light output and growth in a concentration-dependent manner, while short-chain AHLs had no effect. *N*-(3-Oxododecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL) inhibited the production of exotoxins and cell wall fibronectin-binding proteins but enhanced protein A expression. Since these processes are reciprocally regulated via the *S. aureus agr* quorum-sensing system, which in turn, is regulated via *sar*, we examined the effect of AHLs on *sarA* and *agr*. At sub-growth-inhibitory concentrations of 3-oxo-C_{12}-HSL, both *sarA* expression and *agr* expression were inhibited, indicating that the action of 3-oxo-C_{12}-HSL is mediated at least in part through antagonism of quorum sensing in *S. aureus*. Spent culture supernatants from *Pseudomonas aeruginosa*, which produces both 3-oxo-C_{12}-HSL and *N*-butanoyl-homoserine lactone (C_4-HSL), also inhibited *agr* expression, although C_4-HSL itself was inactive in this assay. Since quorum sensing in *S. aureus* depends on the activities of membrane-associated proteins, such as AgrB, AgrC, and AgrD, we investigated whether AHLs perturbed *S. aureus* membrane functionality by determining their influence on the membrane dipole potential. From the binding curves obtained, a dissociation constant of 7 μM was obtained for 3-oxo-C_{12}-HSL, indicating the presence of a specific saturable receptor, whereas no binding was observed for C_4-HSL. These data demonstrate that long-chain 3-oxo-substituted AHLs, such as 3-oxo-C_{12}-HSL, are capable of interacting with the *S. aureus* cytoplasmic membrane in a saturable, specific manner and at sub-growth-inhibitory concentrations, down-regulating exotoxin production and both *sarA* and *agr* expression.

The emergence of bacteria that are resistant to multiple antibiotic classes is a major public health threat (22). This threat has been compounded over the last few decades by the failure of drug discovery programs to develop new antibacterial agents with truly novel modes of action. As a consequence, there is renewed interest in antibacterial targets which disrupt the capacity of pathogenic bacteria to cause infection by attenuating virulence (1). Since pathogenicity is usually multifactorial, regulatory genes involved in the global control of virulence are potential targets. In this context, bacterial cell-to-cell communication (quorum-sensing) systems which control diverse physiological processes, including virulence, offer attractive antibacterial targets (49). Such systems rely on the interaction of a diffusible, low-molecular-weight signal molecule (sometimes referred to as an "autoinducer") with a sensor kinase or response regulator to activate or repress gene expression (44, 49). As such, they offer multiple target sites for intervention since the disruption of cell-to-cell communication can be accomplished either by blockade of signal synthesis, by signal molecule degradation, or by inhibition of signal reception (49).

A number of chemically distinct quorum-sensing signal molecule families have been identified, the most intensively investigated of which have been the *N*-acylhomoserine lactones (AHLs). These compounds are produced by gram-negative bacteria, including human pathogens belonging to the genera *Aeromonas*, *Brucella*, *Burkholderia*, *Pseudomonas*, *Serratia*, and *Yersinia* (44, 49). All AHLs that have been reported to date are characterized by a homoserine lactone ring not substituted in the β and γ positions which is N acylated with a fatty acyl group at α position 1. The acyl chains vary in length from 4 to 18 carbons, in saturation levels, and in oxidation states, and in most cases the chain has an even number of carbon atoms (10). For example, in *Pseudomonas aeruginosa*, the two main AHLs produced are the short-chain molecule *N*-butanoyl-L-homoserine lactone (C_4-HSL) and the long-chain molecule *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL), which are involved in coordinately regulating virulence and secondary metabolite production (10, 34, 44, 49, 50).

While no gram-positive AHL producers have been reported yet, a number of linear and posttranslationally modified pep-

* Corresponding author. Mailing address: Institute of Infection, Immunity and Inflammation, Centre for Biomolecular Sciences, University of Nottingham, Nottingham NG7 2RD, United Kingdom. Phone: 44-115-951-5047. Fax: 44-115-8467951. E-mail: paul.williams@nottingham.ac.uk.

† S.Q. and B.M. made equal contributions to this work.

‡ Present address: ITI Life Sciences, Innovation House, 17 Luna Place, Dundee Technology Park, Dundee DD2 1TP, United Kingdom.

tide-based quorum-sensing signal molecules associated with DNA uptake and processing (competence), conjugation, and virulence have been described (30). These include the peptide lactones and peptide thiolactones made by *Enterococcus faecalis* and the staphylococci, respectively, (30). For both pathogens, these posttranslationally modified peptide signal molecules are involved in virulence gene regulation.

In *Staphylococcus aureus*, the *agr* locus regulates the expression of diverse cell surface proteins (e.g., protein A, coagulase, fibronectin-binding proteins) and exoproteins (e.g., proteases, hemolysins, toxic shock syndrome toxin 1 [TSST-1], and enterotoxin B) in concert with cell population density (4, 29). As *S. aureus* reaches the stationary phase, *agr* represses genes coding for cell surface colonization proteins, such as protein A and the fibronectin-binding proteins, and activates expression of the genes for secreted exotoxins and tissue-degrading exoenzymes (4, 7, 29). In several different experimental animal models of *S. aureus* infection, *agr* mutants exhibit significantly reduced virulence, highlighting the key role of this regulatory locus in staphylococcal pathogenicity (7)

The staphylococcal *agr* locus consists of two divergent operons, which are controlled by the P2 and P3 promoters, respectively (26, 29). The P2 operon consists of four genes, *agrBDCA*, all of which are required for activation of transcription from the P2 and P3 promoters, while the P3 transcript, RNAIII, is itself the effector for the *agr* response (26, 29). AgrA and AgrC constitute a two-component system in which AgrC is the sensor kinase and AgrA is the response regulator. The system is activated through the interaction of an autoinducing peptide (AIP) with AgrC (16, 29). Structurally, the staphylococcal AIPs are peptide thiolactones consisting of seven to nine amino acid residues in which the central cysteine residue is covalently linked to the C-terminal amino acid carboxylate, forming a cyclic thioester (5, 20, 23). The expression of *agr* is, however, highly influenced by environmental conditions and other regulatory systems, including SarA, which, in common with *agr*, positively regulates the *agr* P2 and P3 promoters and hence numerous exotoxins and the fibronectin-binding proteins while repressing protein A production (4).

In most studies of bacterial quorum-sensing signal molecules the workers have largely concentrated on the function and action of these molecules in a given species or genus. Staphylococci, for example, can be subdivided into different groups on the basis of the ability of their AgrD-derived peptide thiolactones to cross-activate or -inhibit *agr* expression (16, 29). However, there have been few studies of the actions of quorum-sensing signal molecules produced by one organism on other unrelated bacterial genera. In such cases, the data obtained are often difficult to interpret because crude spent culture supernatants are used as sources of quorum-sensing signal molecules (13, 24). Furthermore, to our knowledge, the response of gram-positive bacteria to gram-negative AHL signal molecules has not been investigated previously. In this study we synthesized a range of AHL signal molecules that had different acyl chain lengths with or without a 3-oxo substituent and examined the response of *S. aureus* with respect to growth and virulence factor production. We found that long-chain AHLs, such as 3-oxo-C_{12}-HSL which is produced by *P. aeruginosa*, are capable of interacting with the *S. aureus* cytoplasmic membrane in a saturable, specific manner and at sub-growth-inhibitory concentrations, reducing exotoxin production and down-regulating both *sarA* and *agr* expression

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *S. aureus* strains RN6390 (16) and KH1187A (a TSST-1 producer) (23) were routinely grown at 37°C on L-agar, in L-broth, or in CYGP broth (28) in the presence or absence of AHLs or spent culture supernatants from *P. aeruginosa* PAO1. Plasmid pSB2030 (P_{xylA}::*gfp-luxABCDE*) confers chloramphenicol resistance and provides growth-dependent bioluminescence and fluorescence when it is used to transform *S. aureus* (36, 37), and it was used to monitor the effects of AHLs on *S. aureus* RN6390. To investigate the impact of AHLs on *agr* expression, the *agrP3*::*blaZ* reporter fusion *S. aureus* RN6390B(pRN6683) constructed by Ji et al. (16) was used. For experiments to determine the effect of *P. aeruginosa* culture supernatants on *agrP3* expression, strain PAO1 was grown to the stationary phase in CYGP broth with shaking at 37°C.

AHL and AIP-I synthesis. AHLs with N-linked acyl side chains consisting of 4, 6, 8, 10, 12, and 14 carbon atoms with 3-oxo substituents (3-oxo-alkanoyl series) or without 3-oxo substituents (alkanoyl series) were synthesized as described previously by Chhabra et al. (8, 9). Group I *S. aureus* autoinducing peptide (AIP-1) was synthesized as described previously by McDowell et al. (23).

Bioluminescence assays. An experiment to determine the influence of AHLs on *S. aureus* replication was carried out in 96-well microtiter plates in CYGP broth using *S. aureus* RN6390(pSB2030) essentially as described previously (35, 36). *S. aureus* RN6390(pSB2030) was grown overnight aerobically at 37°C in CYGP broth containing chloramphenicol (5 μg/ml). The resulting bacterial pellet was washed twice in fresh medium and then diluted 1/20 into fresh medium and grown for 3 h at 37°C aerobically. This preparation was further diluted 1/20 into fresh medium. A range of AHL concentrations was added to 5-ml aliquots of the diluted bacterial culture. Replicate samples (200 μl) were loaded into clear-bottom 96-well microtiter plates, which were incubated at 37°C in an Anthos Lucy 1 luminometer (Anthos Labtech, Salzburg, Austria), and the optical density at 492 nm (OD_{492}) and luminescence were measured every 30 min for 24 h. The data were plotted as relative light units divided by OD_{492}, which accounted for the influence of increased growth on the total bioluminescence. To simplify presentation of the data showing the effects of different AHLs on *S. aureus*(pSB2030), the maximal relative light unit/OD_{492} values recorded were plotted as a bar chart since the shapes of the curves for each AHL tested were similar.

Analysis of exotoxins and cell wall colonization factors. The influence of AHLs on staphylococcal virulence factor production was determined as follows. The production of α-hemolysin in cell-free staphylococcal culture supernatants was assayed by using the microtiter plate format with a 0.5% (vol/vol) suspension of rabbit erythrocytes and by N-terminal sequencing of proteins that were separated on 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels and electroblotted onto polyvinylidene difluoride membranes. δ-Hemolysin production was analyzed by reversed-phase high-performance liquid chromatography (HPLC) as described by Otto and Gotz (33). TSST-1 was detected by Western blotting of cell-free culture supernatants of stationary-phase cells using a rabbit polyclonal antibody. Protein A and fibronectin-binding proteins were detected in staphylococcal cell wall fractions that were prepared by digestion of whole cells (standardized by resuspension to an OD_{600} of 1.0) with lysostaphin (100 μg/ml) as described by Morrissey et al. (27). After SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting, protein A was detected using a horseradish peroxidase–rabbit anti-rat immunoglobulin G conjugate (Sigma), and the fibronectin-binding proteins were detected after incubation with human fibronectin (30 μg/ml), followed by incubation with a mouse antibody to human fibronectin (Sigma). Western blots were developed using an ECL chemiluminescence system (Amersham Biosciences, United Kingdom)

Northern blot analysis. *S. aureus* RN6390(pSB2035) was grown overnight in CYGP broth containing chloramphenicol (5 μg/ml). The resulting bacterial pellet was washed twice in fresh medium and then diluted 1/20 into fresh medium and grown aerobically for 3 h at 37°C. Then the culture was further diluted 1/20 into fresh medium in the presence or absence of either 3-oxo-C_{12}-HSL or C_4-HSL. AHLs were added at the time of inoculation, at 4, 6, and 9 h 20-ml culture samples were removed, and the bacterial cells were harvested. For RNAIII, 3-oxo-C_{12}-HSL (5 μM) was also added after induction, and samples were taken at hourly intervals from 3.5 h to 8.5 h. The OD_{600} of bacterial suspensions were normalized to 0.45 in 400 μl prior to RNA extraction. Staphylococcal RNA was extracted using an RNeasy total RNA kit (QIAGEN) with 800 μg/ml lysostaphin added in the initial cell lysis step. RNA concentrations were determined using a Genequant spectrophotometer (Pharmacia Biotech).

Samples were adjusted to provide 1.5 μg (*sarA*) or 3 μg (RNAIII) RNA per lane and together with RNA markers (Promega) were electrophoresed on 1.5% agarose–formaldehyde gels and then transferred to a Hybond N^+ membrane as described in the *Promega Protocols and Applications Guide* (3rd ed.). The Northern blots were incubated overnight at 50°C with digoxigenin-labeled DNA probes (Boehringer Mannheim) obtained by random priming of PCR products from *S. aureus* RN6390 for *sarA* and RNAIII using primers based on the corresponding previously published sequences. After hybridization, the filters were washed sequentially in 2× SSC–0.1% (wt/vol) SDS at room temperature for 5 min (two washes) and in 0.1× SSC–0.1% (wt/vol) SDS at 68°C for 15 min (two washes) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The bound probe was visualized using CDP-star (Boehringer Mannheim) according to the manufacturer's protocol.

***S. aureus agrP3*::*blaZ* reporter assays.** *S. aureus agrP3*::*blaZ* reporter assays were carried out using *S. aureus* RN6390B(pRN6683) as described by Ji et al. (16) and McDowell et al. (23) and using the chromogenic cephalosporin nitrocefin as a substrate. *S. aureus* strain RN6390B(pRN6683) was grown overnight at 37°C with shaking in CYGP broth (28). The culture was diluted 1/100 into fresh CYGP broth and grown at 37°C to the logarithmic phase (OD_{600}, 0.4). To 45 μl of this staphylococcal cell suspension in a microtiter plate, 5 μl of CYGP broth (as a control) or a range of concentrations of 3-oxo-C_{12}-HSL or C_4-HSL were added. For some experiments, 5 μl of filter-sterilized spent stationary-phase supernatant (undiluted or diluted 1/10 or 1/100 with CYGP broth) from *P. aeruginosa* was added to the staphylococcal cell suspension, or, as a positive control, 5 μl of the synthetic staphylococcal autoinducing group I peptide (20, 23) was added at concentrations of 0.27 μM, 0.027 μM, and 0.0027 μM (23). The microtiter plates were incubated for 55 min at 37°C in a Labsystems II microplate reader, 50 μl of CYGP broth containing 5 mM sodium azide was added to stop the reaction, and β-lactamase activity was determined using the chromogenic cephalosporin nitrocefin as the substrate as described previously (16, 23). For these experiments an increase in $\varepsilon_{492} - \varepsilon_{690}$ of 0.001 min^{-1} was defined as 1 U of β-lactamase activity. Values for the 50% inhibitory concentration (IC_{50}) were extracted from the sigmoidal dose-response curves using the Prism2 program (Graphpad, San Diego, Calif.). All assays were carried out in triplicate at least three times.

Dipole potential analysis. *S. aureus* membranes were prepared from cells grown in CYGP broth to an OD_{540} of ~0.8 and were lysed with lysostaphin (80 μg/ml). After sonication, the membrane pellet was washed three times using 5 mM Tris HCl (pH 7.5) containing 280 nM sucrose. Monodisperse 100-nm-diameter unilammellar phospholipid vesicles were prepared using phosphatidylcholine as described by Wall et al. (48) Membranes were labeled with the dipole potential fluorescent sensor 1-(3-sulfonatopropyl)-4-[β-2-(di-*n*-octylamino)-6-naphthylvinyl] pyridinium betaine (di-8-ANEPPS) (Molecular Probes, Leiden, The Netherlands) (11, 12) in ethanol at a final concentration of 1 μM. Fluorescence spectra and dual-wavelength recordings were obtained using an Aminco Bowman (New York, N.Y.) series 2 luminescence spectrophotometer. Excitation spectra were collected with an emission wavelength of 580 nm. The variation in the ratio of fluorescence at 460 nm to fluorescence at 520 nm [R(460/520)] as a function of 3-oxo-C_{12}-HSL concentration was measured in the dual-wavelength excitation mode. The data obtained were fitted to a simple hyperbolic function using Easyplot (Cherwell Scientific, United States), which describes a single-binding-site model according to: $R(460/520) = \text{capacity} \cdot [\text{3-oxo-C}_{12}\text{-HSL}]/K_d + [\text{3-oxo-C}_{12}\text{-HSL}]$, where K_d is the dissociation constant and the capacity is the maximum value of R(460/520).

RESULTS

Long-chain 3-oxo-substituted AHLs inhibit the growth of *S. aureus*. When organisms were grown in CYGP broth in 96-well microtiter plates in the presence of 3-oxo-C_{12}-HSL (Fig. 1A), an AHL produced by *P. aeruginosa*, the growth of *S. aureus* was inhibited in a concentration-dependent manner; this was particularly marked at concentrations of 30 μM or above, and growth was completely inhibited by 75 μM (Fig. 1B).

Previously, we developed a bioluminescent *luxABCDE*-based reporter plasmid (pSB2030) which confers growth-dependent bioluminescence when it is transformed into *S. aureus* and offers a rapid and sensitive means of assessing the efficacy of antibacterial agents (36, 37). LuxAB-dependent bioluminescence requires energy, in the form of reduced flavin mononucleotide obtained from bacterial metabolism, a long-chain aldehyde synthesized and recycled by LuxCDE, and molecular oxygen (37). As the production of bioluminescence from recombinant bacteria containing the *lux* genes depends on biochemically active bacterial cells, it can be assumed that any compound that impairs the biochemistry and thus compromises cellular viability leads to a rapid reduction in luminescence. The effects of antimicrobial compounds on *lux*-containing recombinant bacteria can therefore be readily assessed (36, 37, 39, 41, 46). Figure 1C shows that the growth-dependent bioluminescence of *S. aureus* RN6390(pSB2030) was completely inhibited by 10 μM 3-oxo-C_{12}-HSL.

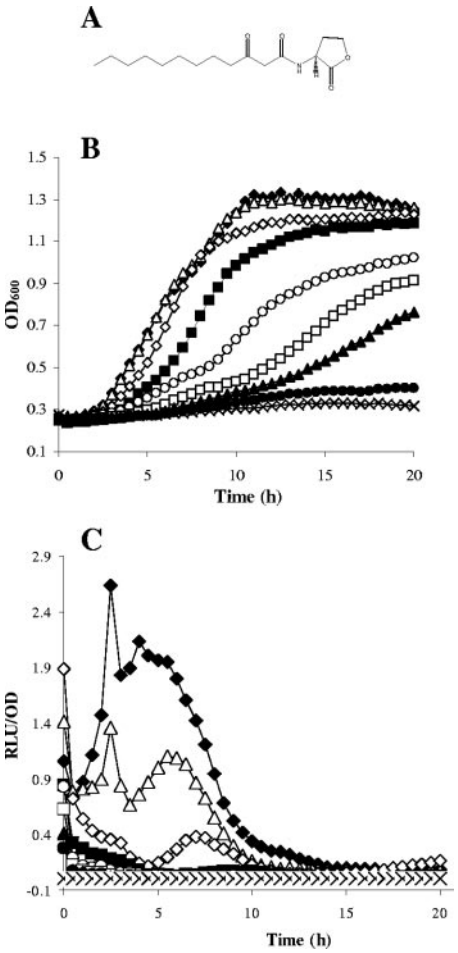

FIG. 1. Inhibition of *S. aureus* RN6390(pSB2030) growth (B) and bioluminescence (C) in the presence of no 3-oxo-C_{12}-HSL (A) (◆) and in the presence of 3-oxo-C_{12}-HSL at concentrations of 1 μM (△), 5 μM (◇), 10 μM (■) 30 μM (○o), 50 μM (□), 60 μM (▲) 75 μM (●), and 100 μM (×). RLU, relative light units.

Since the *S. aureus* RN6390(pSB2030) bioreporter responded sensitively to 3-oxo-C_{12}-HSL, we examined the effects of a range of AHLs having different acyl chain lengths with and without a 3-oxo substituent at concentrations of 5 and 50 μM (Fig. 2). Apart from *N*-decanoyl-L-homoserine lactone (C_{10}-HSL), unsubstituted AHLs, such as C_4-HSL (which is also produced by *P. aeruginosa*) (48), did not reduce the light output (Fig. 2A). For the 3-oxo series, however, AHLs with C_8, C_{10}, C_{12}, and C_{14} chains all significantly reduced bioluminescence

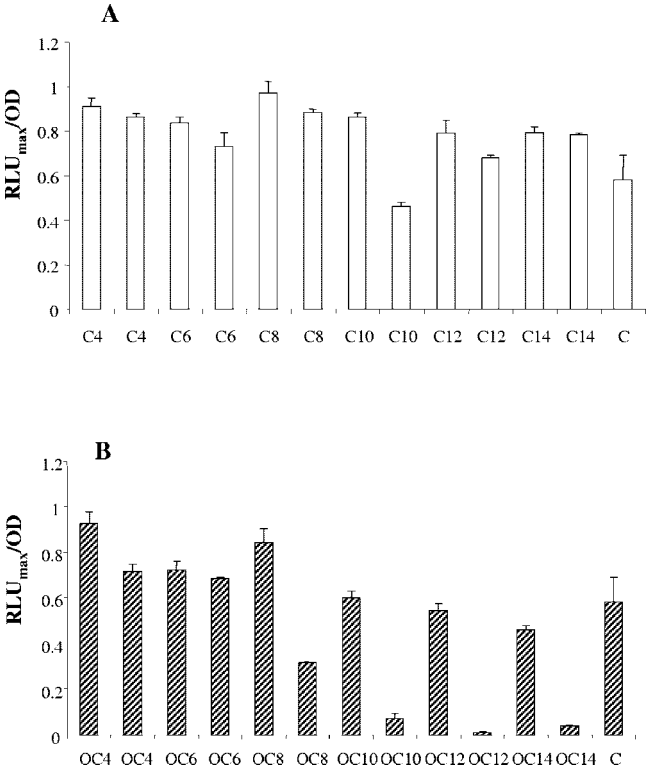

FIG. 2. Inhibition of *S. aureus* RN6390(pSB2030) bioluminescence by AHLs having different acyl chain lengths and C-3 substituents. (A) Unsubstituted AHLs; (B) 3-oxo-substituted AHLs. Compounds with C_4, C_6, C_8, C_{10}, C_{12}, and C_{14} acyl chains were evaluated. Bar C indicates the results for the control (diluent only). For each AHL, the first bar indicates the results obtained with 5 μM and the second bar indicates the results obtained with 50 μM. RLU, relative light units.

cence at a concentration of 50 μM, and 3-oxo-C_{12}-HSL and 3-oxo-C_{14}-HSL were the most active molecules (Fig. 2B). Interestingly, *S. aureus* RN6390(pSB2030) responded to AHLs such as C_4-HSL and 3-O-C_4-HSL with slightly enhanced rather than reduced bioluminescence compared with the solvent control (Fig. 2)

3-oxo-C_{12}-HSL inhibits exotoxin and fibronectin-binding protein production but enhances protein A synthesis. When added to *S. aureus* cultures, 3-oxo-C_{12}-HSL abolished the hemolytic activity of spent stationary-phase culture supernatants in a concentration-dependent manner, suggesting that this long-chain AHL was capable of inhibiting exotoxin synthesis (data not shown). The results of an SDS-PAGE analysis of the extracellular proteins of *S. aureus* RN6390 grown in the absence or presence of 3-oxo-C_{12}-HSL are shown in Fig. 3A. The presence of two major proteins with molecular masses of approximately 36 kDa and 45 kDa was abolished in the cells grown in the presence of 3-oxo-C_{12}-HSL (10 μM). N-terminal sequencing of the 34-kDa band identified the 34-kDa protein as α-hemolysin. We were unable to determine the identity of the 45-kDa band by N-terminal sequencing. 3-oxo-C_{12}-HSL also abolished production of δ-hemolysin by RN6390, as determined by HPLC analysis (Fig. 3B). Furthermore, Western blot analysis of TSST-1 production by *S. aureus* strain KH1187A also revealed that levels of this toxin are substantially reduced by 3-oxo-C_{12}-HSL (Fig. 3C).

To determine whether 3-oxo-C_{12}-HSL also inhibited cell wall colonization factors, immunoblot analysis was used to assay for immunoglobulin-binding protein A and Western ligand blot analysis was used to assay for fibronectin-binding proteins. Figure 4A shows that after 6 and 9 h of growth, cell wall protein A synthesis was enhanced in *S. aureus* RN6930 by 3-oxo-C_{12}-HSL at sub-growth-inhibitory concentrations (5 and 15 μM) compared with control, untreated cultures. Conversely, the levels of cell wall fibronectin-binding proteins were reduced during growth in the presence of 3-oxo-C_{12}-HSL (Fig. 4B). Interestingly, 3-oxo-C_{12}-HSL had no effect on two *agr*-independent, iron-regulated, cell wall-associated *S. aureus* proteins, FrpA and FrpB (22; data not shown).

3-oxo-C_{12}-HSL but not C_4-HSL inhibits *sarA* and *agr* expression. Since 3-oxo-C_{12}-HSL modulated the production of a number of *sar*- and *sarA*- dependent virulence determinants,

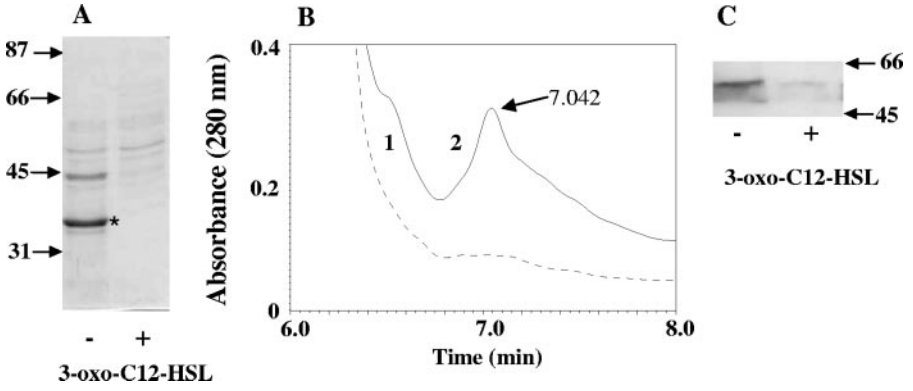

FIG. 3. 3-oxo-C_{12}-HSL inhibits exotoxin production in *S. aureus*. (A) SDS-PAGE of RN6390 culture supernatants grown in the absence (lane −) or in the presence (lane +) of 3-oxo-C_{12}-HSL (10 μM). The position of α-hemolysin was confirmed by N-terminal sequencing of the band indicated by an asterisk. (B) HPLC chromatogram showing the effect of 3-oxo-C_{12}-HSL on δ-toxin production in *S. aureus* RN6390. Upper HPLC trace, control (δ-toxin retention time, 7.04 min); lower HPLC trace, 3-oxo-C_{12}-HSL (10 μM) added to the growth medium prior to inoculation. (C) Western blot showing the effect of 3-oxo-C12-HSL on TSST-1 production in *S. aureus* strain KH1187A grown to the stationary phase in the absence (lane −) or in the presence (lane +) of 3-oxo-C_{12}-HSL (20 μM).

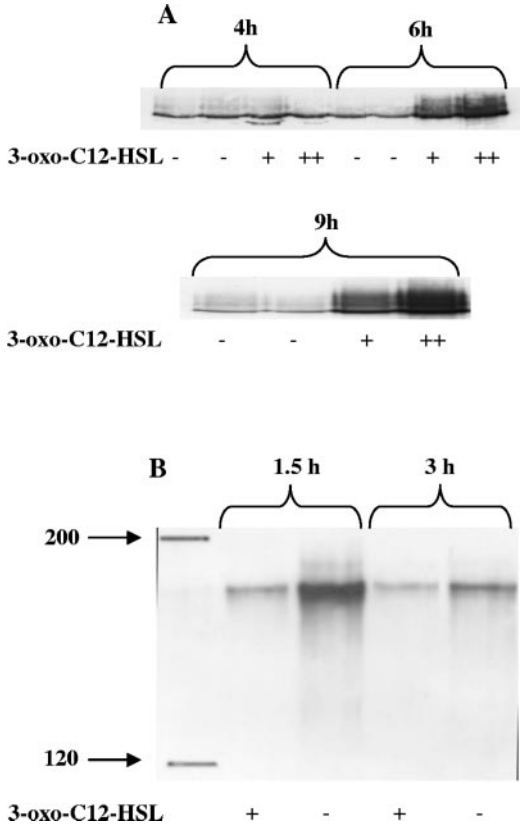

FIG. 4. (A) Western blot of cell wall proteins of *S. aureus* grown for 4, 6, and 9 h in the absence of 3-oxo-C_{12}-HSL (lanes −) or in the presence of 3-oxo-C_{12}-HSL at concentrations of 5 μM (lanes +) and 15 μM (lanes ++) and probed with horseradish peroxidase-conjugated rabbit-anti-rat immunoglobulin G for detection of protein A. (B) Western ligand blot of cell wall proteins of *S. aureus* grown for 1.5 h or 3 h in the presence (lanes +) or in the absence (lanes −) of 3-oxo-C_{12}-HSL and probed with human fibronectin. The lane on the left shows the positions of molecular mass marker proteins (in kDa).

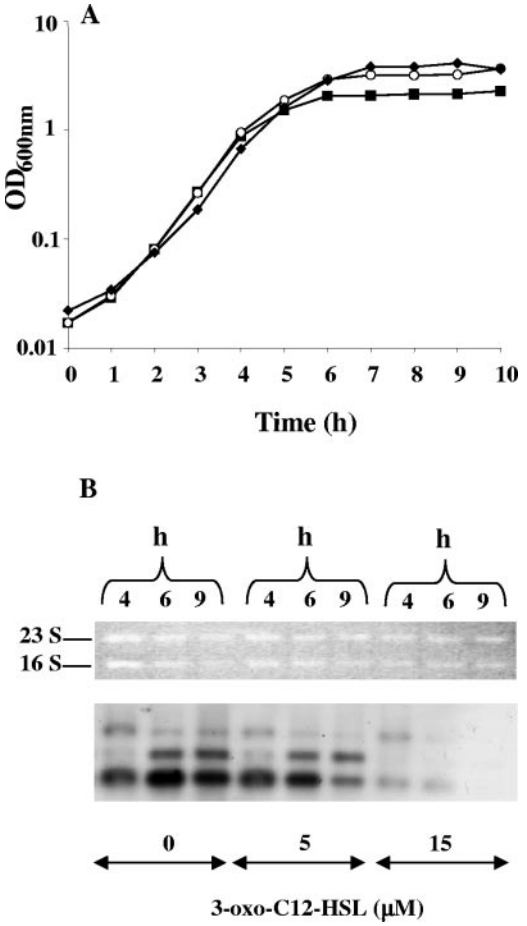

FIG. 5. 3-oxo-C_{12}-HSL inhibits *sarA* expression at sub-growth-inhibitory concentrations. (A) Growth of *S. aureus* RN6390 in shake flask cultures in the absence of 3-oxo-C_{12}-HSL (■) or in the presence of 3-oxo-C_{12}-HSL at a concentration of 5 μM (◆) or 15 μM (○). (B) Northern blot analysis of *sarA* transcripts prepared from *S. aureus* RN6390 grown for 4, 6, or 9 h in the absence of 3-oxo-C_{12}-HSL (lanes 0) or in the presence of 3-oxo-C_{12}-HSL at a concentration of 5 μM (lanes 5) or 15 μM (lanes 15). The top panel shows the loading gel with the positions of the 23S and 16S rRNAs indicated; the bottom panel shows the Northern blot.

including the hemolysins, TSST-1, protein A, and the fibronectin-binding proteins, the data suggested that the mode of action of this gram-negative AHL signal molecule may be a consequence of the loss of *sarA* or *agr* functionality. *S. aureus* was grown in shake flasks in the absence or presence of 5 or 15 μM 3-oxo-C_{12}-HSL, concentrations which under these growth conditions do not inhibit growth (Fig. 5A). The *sarA* locus is expressed in a growth phase-dependent manner as three overlapping transcripts (approximately 0.56 kb, 0.8 kb, and 1.2 kb) (2). Figure 5B shows the expression of the three *sarA* transcripts 4, 6, or 9 h after inoculation in the absence or presence of 5 μM or 15 μM 3-oxo-C_{12}-HSL. At the higher concentration, 3-oxo-C_{12}-HSL substantially reduced (after 4 and 6 h) the expression of the 0.56- and 1.2-kb transcripts, whereas at 9 h none of three *sarA* transcripts was apparent (Fig. 5B).

To evaluate the influence of 3-oxo-C_{12}-HSL on *agr* expression in *S. aureus*, Northern blot analyses of the RNAIII transcript and an *agrP3*::*blaZ* reporter (pRN6683) gene fusion assay were performed. When 3-oxo-C_{12}-HSL was added at a concentration of 5 μM at the time of inoculation and RNA samples were obtained after 4, 6, or 9 h of growth, the RNAIII levels were clearly reduced (Fig. 6A). Similar results were obtained when 3-oxo-C_{12}-HSL (5 μM) was added after induction of *agr* (Fig. 6B). To obtain quantitative data, *S. aureus* RN6390B(pRN6683) was grown to the logarithmic phase to induce *agr* expression prior to exposure to a range of 3-oxo-C_{12}-HSL concentrations. Figure 6C clearly demonstrates that 3-oxo-C_{12}-HSL inhibited *agr* expression in a concentration-dependent manner, and from the data an IC_{50} of 2.0 ± 0.83 μM was calculated (as determined by four separate assays performed in triplicate).

Since 3-oxo-C_{12}-HSL is produced by *P. aeruginosa*, we also sought to determine whether spent stationary-phase culture supernatants prepared from this gram-negative pathogen could inhibit *agr* expression at dilutions which do not inhibit staphylococcal growth. Figure 7A shows that at a dilution of 1/10, *P. aeruginosa* spent culture supernatants reduced *agr* expression by approximately 60% compared with the control (CYGP broth) and that this inhibition was lost if there was

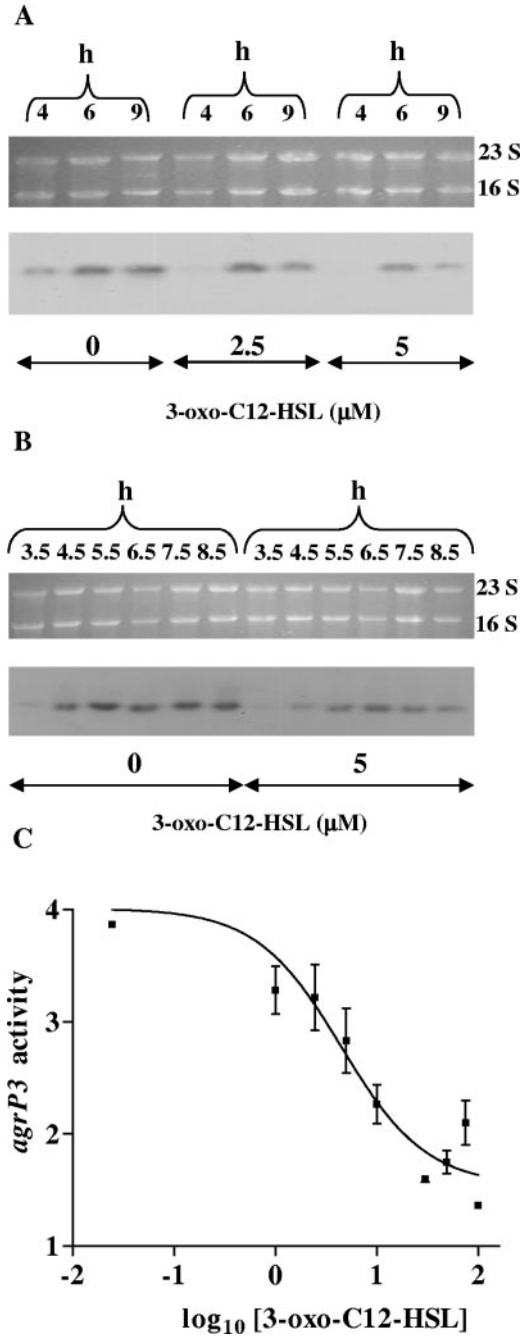

FIG. 6. 3-oxo-C_{12}-HSL inhibits *agr* expression. (A) Northern blot analysis of RNAIII transcripts prepared from *S. aureus* RN6390 grown for 4, 6, or 9 h in the absence of 3-oxo-C_{12}-HSL (lanes 0) or in the presence of 3-oxo-C_{12}-HSL at a concentration of 2.5 μM (lanes 2.5) or 5 μM (lanes 5). The top panel shows the loading gel with the positions of the 23S and 16 S rRNAs indicated, and the bottom panel shows the Northern blot. (B) Northern blot analysis of RNAIII transcripts prepared from *S. aureus* RN6390 grown for 3.5 h to induce *agr* prior to addition of no 3-oxo-C_{12}-HSL (lanes 0) or 3-oxo-C_{12}-HSL at a concentration of 5 μM (lanes 5) and sampled every hour for a further 5 h. The top panel shows the loading gel with the positions of the 23S and 16S rRNAs indicated, and the bottom panel shows the Northern blot. (C) Dose-response curve showing the inhibition of an *S. aureus agrP3*::*blaZ* reporter by 3-oxo-C_{12}-HSL. The error bars indicate standard deviations ($n = 3$).

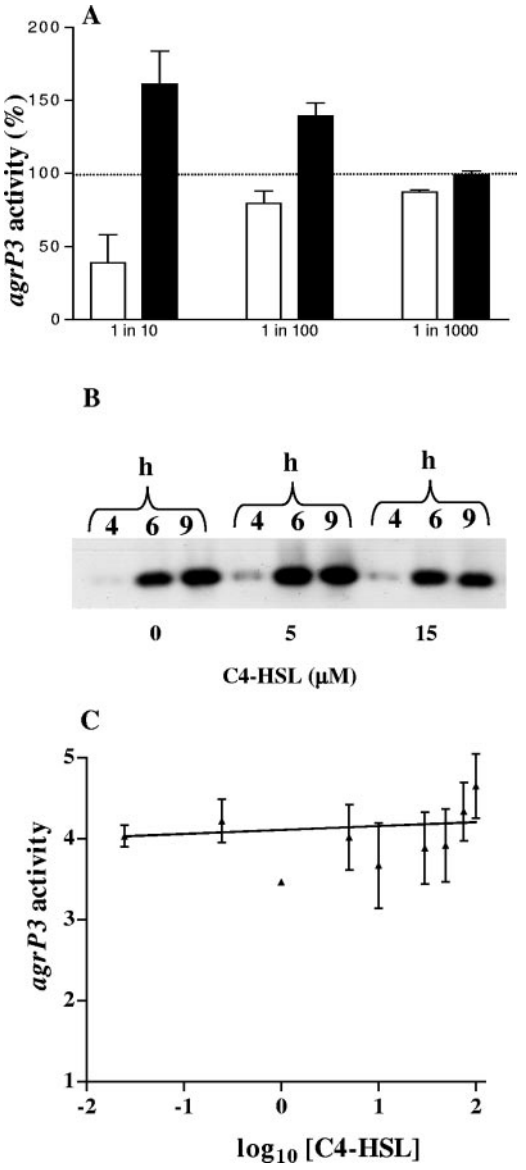

FIG. 7. Cell-free *P. aeruginosa* stationary-phase culture supernatants but not C_4-HSL inhibit *agr* expression in *S. aureus*. (A) Influence of *P. aeruginosa* culture supernatants (open bars) diluted 1/10, 1/100, or 1/1,000 and staphylococcal AIP-1 at concentrations of 0.27 μM, 0.027 μM, and 0.0027 μM (solid bars) on the expression of *agrP3*::*blaZ*. The data are expressed as percent changes compared with the reporter gene fusion exposed to CYGP broth alone. (B) Northern blot analysis of the RNAIII transcript prepared from *S. aureus* RN6390 grown for 4, 6, or 9 h in the absence of C_4-HSL (lanes 0) or in the presence of C_4-HSL at a concentration of 5 μM (lanes 5) or 15 μM (lanes 15). (C) Dose-response curve showing that C_4-HSL does not inhibit the *S. aureus agrP3*::*blaZ* reporter. The error bars indicate standard deviations ($n = 3$).

further dilution of the pseudomonas supernatant. *P. aeruginosa* also produces a second AHL quorum-sensing signal molecule (C_4-HSL), which, although it had no effect on staphylococcal growth (Fig. 2A), potentially influences *agr* expression. Figure 7B shows that C_4-HSL at concentrations of 5 and 15 μM had no effect on *agr* as determined by Northern blot analysis of RNAIII levels. Similar results were obtained using the

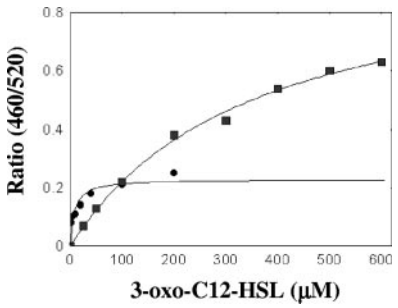

FIG. 8. 3-oxo-C_{12}-HSL disturbs the membrane dipole potential. Changes in the dipole potential were determined spectrofluorometrically using the dipole potential fluorescent sensor di-8-ANEPPS to measure the variation in the fluorescence ratio, *R*(460/520), as a function of 3-oxo-C_{12}-HSL concentration using phospholipid liposomes (■) and *S. aureus* membranes ●.

agrP3::*blaZ* reporter with C_4-HSL concentrations up to 100 μM; i.e., this short-chain *P. aeruginosa* AHL did not inhibit *agr* expression (Fig. 7C).

Interaction of 3-oxo-C_{12}-HSL and C_4-HSL with staphylococcal membranes and synthetic phospholipid vesicles. Since 3-oxo-C_{12}-HSL is capable of disrupting the function of Agr, which includes three membrane-associated components (namely, AgrB, AgrC, and AgrD) (29, 53), we employed a novel spectrofluorometric technique to investigate the interaction of AHLs with both staphylococcal and synthetic unilamellar phospholipid membrane vesicles. This method assesses changes in one of the major membrane electrostatic potentials, the dipole potential (31). The dipole potential originates via the molecular dipoles associated with carbonyl, oxygen-bonded phosphate moieties of phospholipids and is orientated toward water molecules at the membrane interface (31). Changes in the magnitude of the dipole potential as a consequence of membrane-ligand interactions, therefore, have important consequences for membrane structure and function. By incorporating the dipole potential fluorescent sensor di-8-ANEPPS into the membranes, a dual-wavelength ratiometric method (10, 11, 31) could be used to measure changes in the membrane dipole potential following exposure to the AHLs C_4-HSL and 3-oxo-C_{12}-HSL. Figure 8 shows the resulting curves for binding of 3-oxo-C_{12}-HSL to staphylococcal and synthetic membranes. From these data, dissociation constants of 7 μM and 352 μM, respectively, were calculated, indicating that 3-oxo-C_{12}-HSL has a much higher affinity for staphylococcal membranes than for synthetic phospholipid membranes. In both cases the data fit a hyperbolic function which described a noncooperative, single-site binding model. C_4-HSL had no effect on the membrane dipole potential, which is consistent with its lack of inhibitory activity.

DISCUSSION

Although AHLs are considered primarily in the context of quorum-sensing-dependent control of gene expression in the gram-negative producer organism (44, 49), these molecules clearly possess biological properties beyond their role in bacterial cell-to-cell communication. In gram-negative bacteria, long-chain AHLs ($>C_{10}$) may antagonize the functions of shorter-chain AHLs ($<C_8$). For example, the C_4-HSL-dependent expression of exoproteases in *Aeromonas hydrophila* can be inhibited by AHLs with C_{10} and C_{12} acyl side chains (43). Similarly, production of the C_6-HSL-dependent purple pigment violacein in *Chromobacterium violaceum* is inhibited by AHLs with a C_8 or longer side chain (21). Recently, Hogan et al. (15) reported that 3-oxo-C_{12}-HSL produced by *P. aeruginosa* suppressed filamentation in the pathogenic yeast *Candida albicans* at a concentration of 200 μM. Furthermore, 3-oxo-C_{12}-HSL exhibits both proinflammatory and immune modulatory activities (38, 42, 45) For example, 3-oxo-C_{12}-HSL modulates leukocyte function (38, 45) and induces interleukin-8 in respiratory epithelial cells (42). It also has a profound effect on the heart rate, inducing bradycardia in live conscious rats (14). Thus, 3-oxo-C_{12}-HSL may play a role not only in regulating *P. aeruginosa* virulence gene expression but also in the manipulation of eukaryotic cells and host tissues to maximize the provision of nutrients via the bloodstream while down-regulating host defense mechanisms.

Here we show that 3-oxo-C_{12}-HSL antagonizes growth and virulence factor production in the gram-positive human pathogen *S. aureus*. 3-oxo-C_{12}-HSL may therefore confer on *P. aeruginosa* a competitive advantage in host tissues that are also infected by *S. aureus*, such as tissues with wound infections, and in the respiratory tracts of individuals with cystic fibrosis (CF). With respect to the latter, AHLs, including 3-oxo-C_{12}-HSL, have been detected directly in sputum samples from CF patients infected with *P. aeruginosa* (25). In CF lungs, *S. aureus* is often replaced by *P. aeruginosa*, although the reason(s) for this is not clear; it has been suggested that this replacement is a consequence of the prolonged use of antistaphylococcal antibiotics (19). However, our in vitro data showing that physiologically relevant concentrations of 3-oxo-C_{12}-HSL can modulate the behavior of *S. aureus* provide a possible alternative explanation. In planktonic cultures, *P. aeruginosa* has been estimated to produce 3-oxo-C_{12}-HSL at concentrations up to 5 μM (33), while in the biofilm mode of growth concentrations as high as 600 μM have been reported (6). Thus, *P. aeruginosa* is likely to produce sufficient 3-oxo-C_{12}-HSL to modulate the adaptive behavior of *S. aureus*. Indeed, using stationary-phase *P. aeruginosa* culture supernatants at sub-growth-inhibitory dilutions, we observed a 60% inhibition of *agr* expression.

AHL structure-activity analysis indicated that long-chain AHLs with a 3-oxo substituent are more active against *S. aureus* than short-chain or unsubstituted short- or long-chain compounds are. This is also true with respect to the immune modulatory and cardiovascular activities of 3-oxo-C_{12}-HSL. For example, C_{12}-HSL was 13-fold less active than 3-oxo-C_{12}-HSL, whereas 3-oxo-C_{10}-HSL was approximately 5-fold less active in a mouse splenocyte proliferation assay (9). C_4-HSL, which, like 3-oxo-C_{12}-HSL, is produced by *P. aeruginosa*, is inactive in immunological assays against *C. albicans* and against *S. aureus* with respect both to growth and *agr* expression.

Long-chain fatty acids, such as capric acid (C_{10}) and lauric acid (C_{12}), but not short-chain fatty acids have been reported to effectively inhibit *S. aureus* growth at a concentration of 10 mM (3), which is more than 100-fold higher than the concentration of 3-oxo-C_{12}-HSL (75 μM) required to completely inhibit staphylococcal growth. Other workers have reported

MICs for lauric and capric acids of 400 and 800 μg/ml, respectively, against a range of methicillin-resistant and methicillin-sensitive *S. aureus* strains, although the corresponding fatty amines were significantly more active (MICs of 100 and 6.25 μg/ml for caprylamine and laurylamine, respectively) (18). While the mechanism of action of these compounds has not been established, it has been suggested that it involves destabilization of the cytoplasmic membrane (18). Interestingly, the surfactant glycerol monolaurate, which contains a C_{12} fatty acid, inhibits *S. aureus* virulence factor production, as does lauric acid itself (35, 40). In contrast to 3-oxo-C_{12}-HSL, glycerol monolaurate inhibits protein A synthesis and does not antagonize *agr* expression (35).

AHLs, such as 3-oxo-C_{12}-HSL, are readily inactivated as quorum-sensing signal molecules by exposure to alkaline pHs or by enzyme-induced opening of the homoserine lactone ring to generate the corresponding 3-oxo fatty amine derivative (e.g., 3-oxo-dodecanoylhomoserine in the case of 3-oxo-C_{12}-HSL) (52). During preparation of this paper, Kaufmann et al. (17) reported that alkali-mediated hydrolysis of 3-oxo-AHLs could also generate a tetrameric acid derivative [3-(1-hydroxydecylidene)-5-(2-hydroxyethyl)pyrrolidine-2,4-dione] which, like 3-oxo-C_{12}-HSL, has antibacterial activity against gram-positive bacteria, including the staphylococci. Thus, both 3-oxo-C_{12}-HSL and its breakdown products have antistaphylococcal activity, indicating that the homoserine lactone ring is not required for the growth-inhibitory effects of this *P. aeruginosa* quorum-sensing signal molecule.

The amphipathic nature of 3-oxo-C_{12}-HSL suggested that it may exert its activity against *S. aureus* by perturbing membrane function. To investigate this possibility, we used the dipole potential fluorescent sensor di-8-ANEPPS (10, 11) to examine the interactions of 3-oxo-C_{12}-HSL and C_4-HSL with synthetic phosphatidylcholine and staphylococcal membranes. The binding curves obtained revealed that 3-oxo-C_{12}-HSL bound to *S. aureus* membranes with high affinity. This contrasts with C_4-HSL, which did not interact with the membranes. Furthermore the almost 50-fold increase in the affinity of 3-oxo-C_{12}-HSL for staphylococcal membranes compared to synthetic phospholipid membranes implies that there is a specific staphylococcal membrane receptor, the nature of which remains to be established.

Exogenous 3-oxo-C_{12}-HSL exerted a marked effect on staphylococcal exotoxin and cell wall protein A in a manner which is consistent with a mechanism of action involving direct or indirect antagonism of the *agr* quorum-sensing system. Using the *agr*P3::*blaZ* reporter assay, an IC_{50} of 2.0 ± 0.83 μM was calculated from the dose-response curve, which is consistent with a mechanism involving perturbation of *agr*-dependent quorum sensing. Since 3-oxo-C_{12}-HSL efficiently inserts into staphylococcal membranes with high affinity, this may prevent the processing of AgrD by AgrB within the membrane by effectively inhibiting AIP synthesis or by interfering with the capacity of AgrC to sense the AIP since AgrB, AgrC, and AgrD are all inserted into the membrane (53) However, the phenotype observed for fibronectin binding (a reduction in fibronectin-binding protein levels was noted following exposure to 3-oxo-C_{12}-HSL) is more characteristic of a defect in *sarA* expression since SarA, in contrast to *agr*, positively regulates fibronectin-binding protein expression in an *agr*-independent manner (51). In support of this, we noted that transcription of all three *sarA* transcripts was virtually abolished in *S. aureus* cultured in the presence of 15 μM 3-oxo-C_{12}-HSL. Since SarA regulates *agr* (7), this in turn would also account for the reduction in *agr* expression observed in the presence of this long-chain AHL. However, if the action of 3-oxo-C_{12}-HSL is to inhibit *sarA* expression via perturbation of membrane function, this raises the question of the mechanism involved. It is therefore tempting to speculate that 3-oxo-C_{12}-HSL may antagonize the function of other membrane-associated regulators, such as the sensor components of two-component sensor kinase response regulator systems, of which there are 16 in *S. aureus* (7). Potential candidates in this context include *arlRS*, *saeRS*, and *srrAB*, which are known to control expression of cell wall colonization factors and exotoxins directly and/or indirectly via *sar* and *agr* (4, 7). Thus, it is likely that 3-oxo-C_{12}-HSL, in a concentration-dependent manner, perturbs the function of a number of membrane sensors which collectively result in modulation of virulence factor expression in *S. aureus*. The precise mechanism(s) of action of 3-oxo-C_{12}-HSL on *S. aureus*, however, remains to be experimentally determined since it is possible that the effects observed on growth, *agr* and *sarA* expression, and virulence factor production are all independently affected and not directly related to membrane perturbation.

Thus, the data described above suggest that long-chain AHLs, such as 3-oxo-C_{12}-HSL, may not only activate gene expression in the producer organism but also inhibit gene expression in other microorganisms occupying or competing for the same ecological niche. Consequently, 3-oxo-C_{12}-HSL is a potential structural backbone for the design of chemical libraries of novel antistaphylococcal agents which block virulence gene expression in this important group of gram-positive human pathogens. The anti-infective potential of AIP antagonists which block *agr*-mediated quorum sensing has been demonstrated in a murine abscess model (20). However, AIP antagonists which inhibit AgrC have been reported to promote biofilm formation, and thus, while they may prove to be useful for treating certain acute infections, may promote chronic infections, a highly undesirable outcome in, for example, patients with indwelling medical devices (32). This is, however, less likely to occur with agents based on the 3-oxo-C_{12}-HSL structural backbone since this compound inhibits not only *agr* but also *sarA*, which is essential for biofilm development in *S. aureus* (47) This is because *sarA* mutants exhibit impaired biofilm formation under both static and flow conditions and show decreased polysaccharide intercellular adhesion via an *agr*-independent mechanism (47). Further work is required to determine the influence, if any, of 3-oxo-C_{12}-HSL on biofilm development in the staphylococci.

ACKNOWLEDGMENTS

We thank Chris Harty and Mavis Daykin for AHL synthesis and for the HPLC analysis of δ-hemolysin, respectively, James Cottam and Josep Cladera for assistance with the *agrP3*::*blaZ* assays and dipole fluorescence measurements, respectively, and R. Novick for providing the *agrP3*::*blaZ* reporter.

This work was funded by grants and a studentship (to B.M.) from the Medical Research Council, UK, and the Biotechnology and Biological Sciences Research Council, UK, respectively.

REFERENCES

1. **Alksne, L. E., and S. J. Projan.** 2000. Bacterial virulence as a target for antimicrobial chemotherapy. Curr. Opin. Microbiol. **11:**625–626.
2. **Bayer, M. G., J. H. Heinrichs, and A. L. Cheung.** 1996. The molecular architecture of the *sar* locus in *Staphylococcus aureus*. J. Bacteriol. **178:**4563–4570.
3. **Bergsson, G., J. Arnfinnsson, O. Steingrimsson, and H. Thormar.** 2001. Killing of Gram-positive cocci by fatty acids and monoglycerides. APMIS **109:**670–678.
4. **Bronner, S., H. Monteil, and G. Prevost.** 2004. Regulation of virulence determinants in *Staphylococcus aureus*: complexity and applications. FEMS Microbiol. Rev. **28:**183–200.
5. **Chan, W. C., B. Coyle, and P. Williams.** 2004. Virulence regulation and quorum sensing in staphylococcal infections: competitive AgrC antagonists as quorum sensing inhibitors. J. Med. Chem. **47:**4633–4641.
6. **Charlton, T. S., R. de Nys, A. Netting, M. Kumar, M. Givskov, and S. Kjellerberg.** 2000. A novel and sensitive method for the quantification of *N*-3-oxoacyl homoserine lactones using gas chromatography-mass spectrometry: application to a model bacterial biofilm. Environ. Microbiol. **2:**530–541.
7. **Cheung, A. L., A. S. Bayer, G. Zhang, H. Gresham, and Y. Q. Xiong.** 2004. Regulation of virulence determinants *in vitro* and *in vivo* in *Staphylococcus aureus*. FEMS Immunol. Med. Microbiol. **40:**1–9.
8. **Chhabra, S. R., P. Stead, N. J. Bainton, G. P. C. Salmond, G. S. A. B. Stewart, P. Williams, and B. W. Bycroft.** 1993. Autoregulation of carbapenem biosynthesis in *Erwinia carotovora* by analogues of *N*-(3-oxohexanoyl)-L-homoserine lactone. J. Antibiot. **46:**441–454.
9. **Chhabra, S. R., C. Harty, D. S. W. Hooi, M. Daykin, P. Williams, D. I. Pritchard, and B. W. Bycroft.** 2003. Synthetic analogues of bacterial quorum sensing molecules as immune modulators. J. Med. Chem. **46:**97–104.
10. **Chhabra, S. R., B. Philip, L. Eberl, M. Givskov, P. Williams, and Cámara, M.** 2005. Extracellular communication in bacteria. Top. Curr. Chem. **240:**279–315.
11. **Cladera, J., and P. O'Shea.** 1998. Intramembrane molecular dipoles affect the membrane insertion and folding of a model amphiphilic peptide. Biophys. J. **74:**2434–2442.
12. **Cladera, J., L. Martin, J. M. Ruysschaert, and P. O'Shea.** 1999. Characterization of the sequence of interactions of the fusion domain of the simian immunodeficiency virus with membranes. J. Biol. Chem. **274:**29951–29959.
13. **Duan, K., C. Dammel, J. Stein, H. Rabin, and M. G. Surette.** 2003. Modulation of *Pseudomonas aeruginosa* gene expression by host microflora through interspecies communication. Mol. Microbiol. **50:**1477–1491.
14. **Gardiner, S., S. R. Chhabra, C. Harty, D. I. Pritchard, B. W. Bycroft, P. Williams, and T. Bennett.** 2001. Haemodynamic properties of bacterial quorum sensing signal molecules Br. J. Pharmacol. **133:**1047–1054.
15. **Hogan, D. A., A. Vik, and R. Kolter.** 2004. A *Pseudomonas aeruginosa* quorum sensing molecule influences *Candida albicans* morphology. Mol. Microbiol. **54:**1212–1223.
16. **Ji, G., R. Beavis, and R. P. Novick.** 1997. Bacterial interference caused by autoinducing peptide variants. Science **276:**2027–2030.
17. **Kaufmann, G. F., R. Sartorio, S.-Y Lee, C. J. Rogers, M. M. Meijler, J. A. Moss, B. Clapham, A. P. Brogan, T. J. Dickerson, and K. D. Janda.** 2005. Revisiting quorum sensing: discovery of additional chemical and biological functions for 3-oxo-*N*-acylhomoserine lactones. Proc. Natl. Acad. Sci. USA **102:**309–314.
18. **Kitihara, T., N. Koyama, J. Matsuda, Y. Aoyama, Y. Hirakata, S. Kamihira, S. Kohno, M. Nakashima, and H. Sasaki.** 2004. Antimicrobial activity of saturated fatty acids and fatty amines against methicillin resistant *Staphylococcus aureus*. Biol. Pharm. Bull. **27:**1321–1326.
19. **Lyczak, J. B., C. L. Cannon, and G. B. Pier.** 2002. Lung infections associated with cystic fibrosis. Clin. Microbiol. Rev. **15:**194–222.
20. **Mayville, P., G. Ji, R. Beavis, H. Yang, M. Goger, R. P. Novick, and T. W. Muir.** 1999. Structure-activity analysis of synthetic autoinducing thiolactone peptides from *Staphylococcus aureus* responsible for virulence. Proc. Natl. Acad. Sci. USA **96:**1218–1223.
21. **McClean, K. H., M. K. Winson, L. Fish, A. Taylor, S. R. Chhabra, M. Cámara, M. Daykin, S. Swift, J. Lamb, B. W. Bycroft, G. S. A. B. Stewart, and P. Williams.** 1997. Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. Microbiology **143:**3703–3711.
22. **McCormick, J. B.** 1998. Epidemiology of emerging/re-emerging antimicrobial-resistant bacterial pathogens. Curr. Opin. Microbiol. **1:**125–129.
23. **McDowell, P., Z. Affas, C. Reynolds, M. T. G. Holden, S. J. Wood, S. Saint, A. Cockayne, P. J. Hill, C. E. R. Dodd, B. W. Bycroft, W. C. Chan, and P. Williams.** 2001. Structure, activity and evolution of the group I thiolactone peptide quorum sensing system of *Staphylococcus aureus*. Mol. Microbiol. **41:**503–512.
24. **McKenney, D., K. E. Brown, and D. G. Allison.** 1995. Influence of *Pseudomonas aeruginosa* exoproducts on virulence factor production in *Burkholderia cepacia*: evidence of interspecies communication. J. Bacteriol. **177:**6989–6992.
25. **Middleton, B., H. C. Rodgers, M. Cámara, A. J. Knox, P. Williams, and A. Hardman.** 2002. Direct detection of *N*-acylhomoserine lactones in cystic fibrosis sputum. FEMS Microbiol. Lett. **207:**1–7.
26. **Morfeldt, E., K. Tegmark, and S. Arvidson.** 1996. Transcriptional control of the *agr*-dependent virulence gene regulator, RNAIII, in *Staphylococcus aureus*. Mol. Microbiol. **21:**1227–1237.
27. **Morrissey, J. A., A. Cockayne, J. Hammacott, K. Bishop, A. Denman-Johnson, P. J. Hill, and P. Williams.** 2002. Conservation, surface exposure, and in vivo expression of the Frp family of iron-regulated cell wall proteins in *Staphylococcus aureus*. Infect. Immun. **70:**2399–2407.
28. **Novick, R. P.** 1991. Genetic systems in staphylococci. Methods Enzymol. **204:**587–636.
29. **Novick, R. P.** 2003. Autoinduction and signal transduction in the regulation of staphylococcal virulence Mol. Microbiol. **48:**1429–1449.
30. **Novick, R. P., and T. W. Muir.** 1999. Virulence gene regulation by peptides in staphylococci and other Gram positive bacteria. Curr. Opin. Microbiol. **2:**40–45.
31. **O'Shea, P.** 2005. Physical landscapes in biological membranes: physicochemical terrains for spatio-temporal control of biomolecular interactions and behaviour. Phil. Trans. R. Soc. Lond. A **363:**575–588.
32. **Otto, M.** 2004. Quorum-sensing control in staphylococci—a target for antimicrobial drug therapy? FEMS Microbiol. Lett. **241:**135–141.
33. **Otto, M., and F. Gotz.** 2002. Analysis of quorum sensing activity in staphylococci by RP-HPLC of staphylococcal δ-toxin. BioTechniques **28:**1088–1096.
34. **Pearson. J. P., K. M. Gray, L. Passador, K. D. Tucker, A. Eberhard, B. H. Iglewski, and E. P. Greenberg.** 1994. Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. Proc. Natl. Acad. Sci. USA **91:**197–201.
35. **Projan, S. J., S. Brown-Skrobot, P. M. Schlievert, F. Vandenesch, and R. P. Novick.** 1994. Glycerol monolaurate inhibits production of β-lactamase, toxic shock syndrome toxin-1 and other staphylococcal exoproteins by interfering with signal transduction. J. Bacteriol. **176:**4204–4209.
36. **Qazi, S. N. A., E. Counil, J. Morrisey, C. E. D. Rees, A. Cockayne, K. Winzer, W. C. Chan, P. Williams, and P. J. Hill.** 2001. *agr* expression precedes escape from the host endosome of internalized *Staphylococcus aureus*. Infect. Immun. **69:**7074–7082.
37. **Qazi, S. N. A., S. E. Harrison, T. Self, P. Williams, and P. J. Hill.** 2004. Real-time monitoring of intracellular *Staphylococcus aureus* replication. J. Bacteriol. **186:**1065–1077.
38. **Ritchie, A. J., A. O. W. Yam, K. M. Tanabe, S. A. Rice, and M. A. Cooley.** 2003. Modification of in vivo and in vitro T- and B-cell-mediated immune responses by the *Pseudomonas aeruginosa* quorum-sensing molecule *N*-(3-oxododecanoyl)-L-homoserine lactone. Infect. Immun. **71:**4421–4431.
39. **Rocchetta, H. L., C. J. Boylan, J. W. Foley, P. W. Iversen, D. L. Letourneau, C. L. McMillian, P. R. Contag, D. E. Jenkins, and T. R. Parr.** 2001. Validation of a non-invasive, real time imaging technology using bioluminescent *Escherichia coli* in the neutropenic mouse thigh model of infection. Antimicrob. Agents Chemother. **45:**129–137.
40. **Ruzin, A., and R. P. Novick.** 2000. Equivalence of lauric acid and glycerol monolaurate as inhibitors of signal transduction in *Staphylococcus aureus*. J. Bacteriol. **182:**2668–2671.
41. **Salisbury, V., A. Pfoestl, H. Wiesinger-Mayr, R. Lewis, K. E. Bowker, and A. P. MacGowan.** 1999. Use of a clinical *Escherichia coli* isolate expressing *lux* genes to study the antimicrobial pharmacodynamics of moxifloxacin. J. Antimicrob. Chemother. **43:**829–832.
42. **Smith, R. S., E. R. Fedyk, T. A. Springer, N. Mukaida, B. H. Iglewski, and R. P. Phipps.** 2001. IL-8 production in human lung fibroblasts and epithelial cells activated by the pseudomonas autoinducer *N*-3-oxododecanoyl homoserine lactone is transcriptionally regulated by NF-kappa B and activator protein-2. J. Immunol. **167:**366–374.
43. **Swift, S., M. J. Lynch, L. Fish, D. F. Kirke, J. M. Tomas, G. S. A. B. Stewart, and P. Williams.** 1999. Quorum sensing-dependent regulation and blockade of exoprotease production in *Aeromonas hydrophila*. Infect. Immun. **67:**5192–5199.
44. **Swift, S., J. A. Downie, N. Whithead, A. M. L. Barnard, G. P. C. Salmond, and P. Williams.** 2001. Quorum sensing as a population density dependent determinant of bacterial physiology Adv. Microb. Physiol. **45:**199–270.
45. **Telford, G., D. Wheeler, P. Williams, P. T. Tomkins, P. Appleby, H. Sewell, G. S. A. B. Stewart, B. W. Bycroft, and D. I. Pritchard.** 1998. The *Pseudomonas aeruginosa* quorum-sensing signal molecule *N*-(3-oxododecanoyl)-L-homoserine lactone has immunomodulatory activity. Infect. Immun. **66:**36–42.
46. **Tenhami, M., K. Hakkila, and M. Karp.** 2001. Measurement of effects of antibiotics in bioluminescent *Staphylococcus aureus* RN4220 Antimicrob. Agents Chemother. **45:**3456–3461.
47. **Valle, J., A. Toldeo-Arana, C. Berasain, J. M. Ghigo, B. Amorena, J. R. Penades, and I. Lasa.** 2003. SarA and not SigB is essential for biofilm development by *Staphylococcus aureus*. Mol. Microbiol. **48:**1075–1087.
48. **Wall, J., C. Golding, M. van Veen, and P. O'Shea.** 1995. The use of fluoresceinphosphatidylethanolamine (FPE) as a real-time probe for peptide-membrane interactions. Mol. Membr. Biol. **12:**183–192.

49. **Williams, P.** 2002. Quorum sensing: an emerging target for antibacterial chemotherapy? Expert Opin. Ther. Targets **6:**257–274.
50. **Winson, M. K., M. Cámara, A. Latifi, M. Foglino S.R. Chhabra, M. Daykin, M. Bally, M., V. Chapon, G. P. C. Salmond, B. W. Bycroft, A. Lazdunski, G. S. A. B. Stewart, and P. Williams.** 1995. Multiple quorum sensing modulons interactively regulate virulence and secondary metabolism in *Pseudomonas aeruginosa*: identification of the signal molecules *N*-butanoyl-L-homoserine lactone and *N*-hexanoyl-L-homoserine lactone. Proc. Natl. Acad. Sci. USA **92:**9421–9431.
51. **Wolz, C., P. Pohlmann-Dietze, A. Steinhuber, Y. T. Chien, A. Manna, W. van Wamel, and A. Cheung.** 2000. *agr*-independent regulation of fibronectin-binding protein(s) by the regulatory locus *sar* in *Staphylococcus aureus*. Mol. Microbiol. **36:**230–243.
52. **Yates, E. A., B. Philipp, C. Buckley, S. Atkinson, S. R. Chhabra, R. E. Sockett, M. Goldner, Y. Dessaux, M. Cámara, H. Smith, and P. Williams.** 2002. *N*-acyl homoserine lactone undergo lactonysis in a pH-, temperature-, and acyl chain length-dependent manner during growth of *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa*. Infect. Immun. **70:**5635–5646.
53. **Zhang, L., J. Lin, and G. Ji.** 2004. Membrane anchoring of the AgrD N-terminal amphipathic region is required for its processing to produce a quorum-sensing pheromone in *Staphylococcus aureus*. J. Biol. Chem. **279:** 19448–19456.

Editor: J. B. Bliska