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Ajoene, a Sulfur-Rich Molecule from Garlic, Inhibits Genes Controlled by Quorum Sensing

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In relation to emerging multiresistant bacteria, development of antimicrobials and new treatment strategies of infections should be expected to become a high-priority research area. Quorum sensing (QS), a communication system used by pathogenic bacteria like *Pseudomonas aeruginosa* to synchronize the expression of specific genes involved in pathogenicity, is a possible drug target. Previous *in vitro* and *in vivo* studies revealed a significant inhibition of *P. aeruginosa* QS by crude garlic extract. By bioassay-guided fractionation of garlic extracts, we determined the primary QS inhibitor present in garlic to be ajoene, a sulfur-containing compound with potential as an antipathogenic drug. By comprehensive *in vitro* and *in vivo* studies, the effect of synthetic ajoene toward *P. aeruginosa* was elucidated. DNA microarray studies of ajoene-treated *P. aeruginosa* cultures revealed a concentration-dependent attenuation of a few but central QS-controlled virulence factors, including rhamnolipid. Furthermore, ajoene treatment of *in vitro* biofilms demonstrated a clear synergistic, antimicrobial effect with tobramycin on biofilm killing and a cease in lytic necrosis of polymorphonuclear leukocytes. Furthermore, in a mouse model of pulmonary infection, a significant clearing of infecting *P. aeruginosa* was detected in ajoene-treated mice compared to a nontreated control group. This study adds to the list of examples demonstrating the potential of QS-interfering compounds in the treatment of bacterial infections.

Infections that develop into chronic conditions are a fast-growing problem in the developed world. The underlying biology is thought to be the ability of bacteria to form biofilms (20), which consist of structured and aggregated (often surface-attached) communities of bacteria (18). Multiple studies have documented that such aggregated communities are more resistant to a variety of antibiotics and the action of the immune system compared to their planktonic counterparts (6, 19, 41, 68). Biofilm infections are often connected to patients with medical devices and implants as well as hospitalized patients. Lately, bacterial biofilms have also been associated with nonhealing, chronic wounds (9, 24, 38). There is thus an urgent need for development of new treatment strategies using a combination of drugs targeting a multitude of antimicrobial targets.

Several Gram-negative pathogens use *N*-acyl homoserine lactone (AHL)-mediated communication systems in a process termed quorum sensing (QS) to coordinate specific gene expression, thereby synchronizing expression of particular phenotypic features between the individual cells (28). QS is thought to play an important role during the initial event of infection for the common opportunistic Gram-negative human pathogen *Pseudomonas aeruginosa*, which is associated with nosocomial and wound infections, immunocompromise (48, 70), and the genetically inherited disease cystic fibrosis (CF) (22). By employing the QS system to control expression of its virulence factors (many of which are antigenic determinants), *P. aeruginosa* is able to operate in a stealthy manner until a certain cell density is reached, where the QS systems become activated. Upon activation of the QS systems, a coordinated release of tissue-damaging and immune defense-degrading virulence factors takes place (28, 36). It was recently documented by us that the QS-controlled virulence factor rhamnolipid (also known as heat-stable hemolysin) destroys polymorphonuclear leukocytes (PMNs) by lytic necrosis (36). Besides lysing neutrophils and macrophages, rhamnolipid has also been reported to impair chemotaxis of neutrophils (42, 62). As rhamnolipid is associated with bacteria living in biofilm, it is likely to function as a shield toward important cellular components of the host defense (1, 36, 71). Furthermore, QS promotes increased tolerance of *P. aeruginosa* biofilms to antibiotic treatments (6) and provides biofilms with structural rigidity through release of extracellular DNA (eDNA) (21, 56).

Two of the *P. aeruginosa* quorum sensors are based on the LuxRI homologues present in most Gram-negative bacteria with QS systems. The LuxI homologues function as an AHL synthetase producing the required signal molecules, and the LuxR homologues function as transcriptional activators which, upon binding of the cognate signal molecules, activate the transcription of the QS target genes (28). The *P. aeruginosa* QS system consists of *lasRI*

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and *rhlRI* hierarchically arranged with the *las*-encoded system, normally at the top level controlling the *rhl*-encoded system, and a third system that intervenes between the two denoted the pseudomonas quinolone signal (PQS). The three interacting QS systems LasRI, RhlRI, and PQS use the following signal molecules for activation: *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL), *N*-butanoyl homoserine lactone (C_4-HSL), and 2-heptyl-3-hydroxy-4-quinolone (PQS), respectively (50, 51, 54).

It has been shown that some terrestrial and marine organisms have evolved a system of specific molecules with AHL-antagonistic activity capable of interfering with the bacterial QS system in a possible prevention of colonization (8, 55, 58, 59, 64). Hentzer et al. (33) and Wu et al. (76) demonstrated that the QS system could be used as an effective antimicrobial drug target by altering the tolerance of biofilms to antibiotic treatment and promote a faster clearance of a *P. aeruginosa* lung infection in mice by using the chemically modified QS inhibitor (QSI) furanone C-30. Crude extracts of garlic (*Allium sativum* L.) have been shown to inhibit the expression of a large number of QS-controlled genes (58), and Bjarnsholt et al. (8) demonstrated the ability of garlic extracts, similar to C-30 treatments, to promote a rapid clearing of a pulmonary *P. aeruginosa* infection in mice. Garlic is widely accepted as a herb that through dietary intake can improve human health (61). Epidemiological studies have shown that a daily intake of garlic lowers the risk of certain cancers (25, 63), and several studies have documented an antithrombotic and lipid-lowering cardiovascular effect of some of the constituents in garlic (27).

By means of a bioassay-directed purification procedure, we identified the sulfur-containing compound ajoene (4,5,9-trithiadodeca-1,6,11-triene 9-oxide) to be a QSI present in garlic extract. When garlic is crushed, ajoene and several other organosulfides are produced as degradation products of allicin (diallyl thiosulfinate) (11). Ajoene has been reported to display conventional antimicrobial activities against a number of Gram-positive bacteria and the Gram-negative bacteria *Escherichia coli*, *Klebsiella pneumoniae*, and *Xanthomonas maltophilia* but not *P. aeruginosa* (45). To further exploit the QSI activity *in vitro* and *in vivo*, we employed chemically synthesized ajoene (M. Givskov, 8 December 2010, European patent application no. 10194154.0 and US provisional application no. 61/420,922). The *in vitro* experiments showed significant inhibition of a subclass of QS-regulated *P. aeruginosa* genes and a significant synergistic action with tobramycin with respect to the reduction of viability of biofilm cells. Furthermore, a mouse model of pulmonary infection was employed to demonstrate the antimicrobial effect of ajoene on *P. aeruginosa* infections.

MATERIALS AND METHODS

Bacterial strains. Sequenced *P. aeruginosa* PAO1 wild type was obtained from the Pseudomonas Genetic Stock Center (www.pseudomonas.med.ecu.edu; PAO0001). For detection of QSI activity, the following reporter strains were used: a QSI selector 1 strain (QSIS1; *E. coli*) and monitor strains for *lasB-gfp*, *rhlA-gfp* (*P. aeruginosa*), and *luxI-gfp* (*E. coli*), described in references 58, 32, 77, and 4, respectively. Production of AHLs was detected by using reporter strains for *lasB-gfp* (*E. coli*) (32) and *ahyI-gfp* (*E. coli*) (30). Animal experiments were performed with the wild-type *P. aeruginosa* strain PAO1, obtained from Barbara Iglewski (University of Rochester Medical Center, Rochester, NY). The strain is QS proficient, except for the reduced production of C_4-HSL previously noted for this *P. aeruginosa* variant (39). The clinical isolate CF438 was obtained from a child with CF and kindly provided by Helle K. Johansen and Oana Ciofu. The mucoid and nonmucoid isogenic strains are described elsewhere (44, 72).

Growth media and conditions for *in vitro* and *in vivo* experiments. ABT minimal medium (B medium [17] plus 2.5 mg thiamine liter^{-1} and 10% A10 [17]) supplemented with 0.5% (wt/vol) glucose and 0.5% (wt/vol) Casamino Acids was used for growing the monitor strains (overnight cultures) for the QSI indicator screens. All strains were incubated at 30°C with shaking (180 rpm) and supplemented with antibiotics where appropriate. For animal experiments, bacteria from freezer stocks were plated onto blue agar plates (State Serum Institute, Denmark) and incubated at 37°C overnight. Blue agar plates are selective for Gram-negative bacilli (34). One colony was used to inoculate overnight cultures grown in Luria-Bertani (LB) medium at 37°C with shaking.

Extraction and purification of QS-containing fractions from garlic. Fractions containing QS activity were identified by a QSIS1 bioassay-guided fractionation described previously (58). Garlic cloves were skinned, homogenized in toluene, and stirred overnight with an equal volume of water. The garlic pulp was filtered, and the solvents were separated, dried under vacuum, and tested for QSI activity. Activity was observed only from the toluene extract. The extract was fractionated on a C_{18} column (125 g, 200 by 50 mm) on a Biotage Isolera flash purification system (Isolera) with a flow rate of 30 ml/min and with sample added as dry load. Samples were collected (without detection) as 100-ml fractions; the first sample was collected at 10% methanol (MeOH) in H_2O, the following 10 samples were eluted with a 10 to 100% MeOH gradient (1000 ml), and all subsequent samples were eluted with 100% MeOH (typically, 500 ml). Activity was detected in the 50 to 60% MeOH fractions, which were combined and further purified by semipreparative high-performance liquid chromatography (HPLC) on a Chromolith RP-18e column (100 by 4.6 mm) with a flow rate of 2 ml per min, eluting with 30% acetonitrile (MeCN) and increasing to 37% MeCN over 20 min. A single fraction eluting at 9.5 min was determined to have QSI activity. Positive electrospray (ESI^+) high-resolution mass spectrometry (HRMS) gave a mass of 235.0282 Da, corresponding to a formula of $C_9H_{15}OS_3$. Comparison of ^{1}H nuclear magnetic resonance (NMR) data identified this fraction as a 60:40 mixture of *E*- and *Z*-ajoenes (11).

Chemically synthesized ajoene. Ajoene was synthesized from commercially available distilled allyl disulfide as a 1:4 mixture of E and Z isomers as described by Givskov (European patent application no. 10194154.0 and US provisional application no. 61/420,922). Synthetic ajoene was purified by silica gel chromatography and characterized by ^{1}H NMR, ^{13}C NMR, and HRMS. The purity was greater than 98%. Synthetic ajoene was used in all experiments conducted in the study described in this paper.

Determination of inhibitor strength. The following bioassays were used to determine inhibitor activity of ajoene. ABT medium (150 μl) supplemented with 0.5% (wt/vol) glucose and 0.5% (wt/vol) Casamino Acids was added to all wells in a 96-well microtiter dish (Black Isoplate; Perkin Elmer). To the first column, ajoene was added to a final concentration of 200 μg/ml and a 2-fold serial dilution was made. No ajoene was added to the last column, which was used as a reference. At last, 150-μl overnight cultures of the QSI monitors (*lasB-gfp* [32], *rhlA-gfp* [77], or *luxI-gfp* [4]) were added to all the wells to a final optical density at 450 nm (OD_{450}) of 0.1. Additionally, the signal molecule *N*-(3-oxohexanoyl)-L-homoserine lactone was added to the *luxI-gfp* reporter screen in a final concentration of 100 nM. The growth of the bacterial cells (OD_{450}) and green fluorescent protein (GFP) expression (excitation wavelength, 485 nm; emission wavelength, 535 nm) were measured on a multilabel plate reader (Wallac 1420 VICTOR^2; Perkin Elmer) every 15 min over 14 h. The temperature was held constant at 34°C.

Production of QS signal molecules. Production of C_4-HSL and 3-oxo-C_{12}-HSL was detected in the supernatant from an overnight culture of a clinical isolate (CF438) by using the AHL-specific reporter strains and the method described by Hentzer et al. (32).

RNA preparation for DNA microarray analysis. *P. aeruginosa* PAO1 exponentially growing (OD_{600}, 0.5) at 37°C and 180 rpm in AB medium (B medium plus 10% A10) supplemented with 0.5% Casamino Acids was diluted to an OD_{600} of 0.05. When an OD_{600} of 0.5 was reached, the culture was divided into 5 cultures of 50 ml and the following four concentrations of ajoene were added: 10 μg/ml, 20 μg/ml, 40 μg/ml, and 80 μg/ml. No ajoene was added to one culture. At an OD_{600} of 2.0, samples were retrieved and 2 volumes of RNAlater (Ambion) was added. Isolation of RNA was performed using an RNeasy minipurification kit (Qiagen) with on-column DNase treatment. The following synthesis of cDNA and hybridization were performed by the microarray core unit at Rigshospitalet, Denmark. The gene expressions were analyzed by the use of the software ArrayStar (version 3.0; DNAStar). DNA microarray analysis of global gene expression was performed according to guidelines provided by Affymetrix and repeated three times with RNA from three individual growth experiments.

RT-PCR. The purified RNA used for DNA microarray analysis was also used for real-time PCR (RT-PCR). cDNA was made from 1 μg of RNA using high-capacity RNA-to-cDNA master mix (Applied Biosystems). For quantitative real-time PCR, amplification was performed with Power SYBR green master mix in a Step One Plus thermal cycler (Applied Biosystems). The primers were designed using Primer Express software (version 3.0; Applied Biosystems). Forty cycles were run with denaturation at 95°C for 15 s, annealing at 55°C for 30 s, and extension at 60°C for 45 s. The gene *rpoD* was used as a control for standardization. Primer sequences were as follows: *rhlA* forward, 5′-GGCGATCGGCCATCT-3′; *rhlA* reverse, 5′-AGCGAAGCCATGTGCTGAT-3′; *lasB* forward, 5′-CG ACAACGCGTCGCAGTA-3′; *lasB* reverse, 5′-AGGTAGAACGCACGGT TGTACA-3′; *rpoD* forward, 5′-ACAAGATCCGCAAGGTACTGAAG-3′; and *rpoD* reverse, 5′-CGCCCAGGTGCGAATC-3′.

Measurements of total rhamnolipid production. Samples for measurements of total rhamnolipid concentration were retrieved from the cultures grown for DNA microarray analysis and RT-PCR at OD_{600}s of 1.5 and 2.0 and kept at −80°C until further examinations. HPLC with ESI^+ HRMS detection (47) was used to quantify rhamnolipids as their $[M + NH_4]^+$ (peak area) on the basis of external standard quantification of a NMR-validated rhamnolipid B standard. A series of diluted standard was analyzed before and after the samples, in order to minimize potential differences in ionization levels of rhamnolipid between the samples. Other rhamnolipids were assumed to give the same ionization efficiency as rhamnolipid B. Total rhamnolipid concentration was derived from six major rhamnolipids with the following masses $[M + NH_4]^+$: 668.4 (rhamnolipid B [C_{10}-C_{10}-rha-rha]), 694.4 (C_{10}-C_{12}D-rha-rha), 696.4 (C_{10}-C_{12}-rha-rha), 522.4 (C_{10}-C_{10}-rha), 548.4 (C_{10}-C_{12}D-rha), and 550.4 (C_{10}-C_{12}-rha).

Stability of ajoene. Overnight cultures of *P. aeruginosa* strain PAO1 and the Δ*lasR*-Δ*rhlR* and Δ*lasI*-Δ*rhlI* mutants were diluted to an OD_{600} of 0.2 and incubated with 100 μg/ml ajoene at 37°C and 4°C for 18 h. The samples were sterile filtrated (pore size, 0.22 μm), and to measure the QSI activity of ajoene, the supernatants were tested in the *P. aeruginosa* QSI screen using the *lasB-gfp* monitor strain (see "Determination of inhibitor strength" above).

Effect of ajoene on *P. aeruginosa* QS signal molecule content. Production of AHL was quantified by HPLC with tandem mass spectrometry (MS/MS) as described in reference 60. *P. aeruginosa* was grown as described for DNA array analysis and RT-PCR, with the same concentrations of ajoene added. At OD_{600}s of 1.5, 1.8, and 2.0, samples were retrieved and sterile filtrated (pore size, 0.22 μm). Acidified ethyl acetate was added to the supernatant in a 1:1 ratio and left at room temperature overnight. The top phase was withdrawn, concentrated under nitrogen gas, and resuspended in 500 μl ethanol, with 1 μl analyzed by HPLC-MS/MS (60). In this case, external standard quantification was done. The method detects C_4-HSL, open lactone-C_4-HSL, 3-oxo-C_{12}-HSL, and open lactone-3-oxo-C_{12}-HSL. Detection limits were in the 10 to 30 nM range.

Effect of serum albumin on QSI activity of ajoene. The *P. aeruginosa* QSI screen (with the *lasB-gfp* monitor strain) was used to test the effect of serum albumin on ajoene activity. Bovine serum albumin was dissolved in ABT medium to a concentration of 100 mg/ml. Three hundred microliters was added to the first row of a 96-well microtiter dish (Black Isoplate; Perkin Elmer). To the rest of the rows, 150 μl medium without serum albumin was added. A 2-fold serial dilution of serum albumin was made, and ajoene was added to the following final concentrations: 12.5 μg/ml and 25 μg/ml. Finally, 150 μl of the *lasB-gfp* monitor strain was added to all the wells (for a more detailed description, see "Determination of inhibitor strength" above).

***In vitro* biofilms.** Biofilms were grown at 37°C in continuous-culture, once-through, three-channel flow cells with individual channel dimensions of 1 by 4 by 40 mm perfused with sterile AB trace minimal medium containing 0.3 mM glucose as described by Christensen et al. (15) and Pamp and Tolker-Nielsen (49). Overnight cultures were diluted to an OD_{600} of 0.1 in 0.9% NaCl, and 250 μl per channel was used for inoculation. All microscopic observations and image acquisitions were performed using a confocal laser scanning microscope (Leica TCS SP5; Leica Microsystems, Germany). Images were obtained with a ×40 dry objective and ×100 oil objective. To visualize dead bacterial cells and lysed PMNs, propidium iodide (PI; P-4170; Sigma, Steinheim, Germany) was used, whereas expression of GFP was used as a measure for live bacterial cells. Image scanning was carried out at 488-nm (green) and 543-nm (red) laser lines from an Ar/Kr laser. An Imaris software package (Bitplane AG) was used to generate pictures of the biofilm. Tobramycin was diluted in 0.9% NaCl. The medium containing ajoene was kept on ice during the experiment.

Preparation of PMNs. Isolation of PMNs was performed as described by Bjarnsholt et al. (6), with modifications. Human blood was collected from healthy volunteers in BD Vacutainer tubes containing 0.129 M sodium citrate. PMNs were resuspended in RPMI 1640 with $NaHCO_3$ to obtain a concentration of 1.5×10^7 PMNs/ml.

PMN exposure of biofilms. The exposure experiment was performed as described by Bjarnsholt et al. (6). We evaluated the biofilm and PMN interactions every 30 min for 2 h. Necrotic PMNs were demonstrated as PMNs with increased red fluorescence from the supplemented DNA stain PI.

Animals. Female BALB/c mice were purchased from Taconic M&B A/S (Ry, Denmark) at 9 to 11 weeks of age and were maintained on standard mouse chow and water *ad libitum* for a minimum period of 1 week before the challenge.

The animal studies were carried out in accordance with the European convention and directive for the protection of vertebrate animals used for experimental and other scientific purposes and the Danish law on animal experimentation. All experiments were authorized and approved by the National Animal Ethics Committee, Denmark (the Animal Experiments Inspectorate, dyreforsoegstilsynet.dk) and given permit number 2008/561-1466. All surgery was performed using fentanyl (Hypnorm)-midazolam, and pentobarbital was used to euthanize the mice at the termination of the experiments. All efforts were made to minimize suffering.

Pulmonary infection model. The pulmonary infection model in mice was prepared and performed as described previously (10). The ajoene solution used for the treatment group was prepared as follows: ajoene was dissolved in 96% ethanol to a concentration of 100 mg/ml and diluted 40 times in a 20% vehicle solution [(2-hydroxypropyl)-β-cyclodextrin (catalog no. C0926; Sigma) dissolved in 0.9% NaCl] to a concentration of 2.5 mg/ml, which reduced the concentration of ethanol to 2.4%. Each mouse was treated with 25 mg ajoene kg^{-1} body weight (BW) subcutaneously (s.c.) once a day as prophylactic treatment for 2 days, right after infection, and subsequently at 2 days postinfection. The placebo group received 96% ethanol diluted in the vehicle corresponding to the amount of ethanol that the ajoene-treated group received.

Statistical analysis. The number of mice in each group was calculated to provide a power of 0.8 or higher for continuous data. For analyzing quantitative data, the Mann-Whitney U test was used for calculating *P*

values in the statistical program GraphPad Prism (version 5.0; GraphPad Software, Inc., San Diego, CA). *P* values of ≤0.05 were considered significant.

Apoptosis assay. Apoptosis tests in the lung epithelial cell line A549 (purchased from the German Collection of Microorganisms and Cell Lines [DMSZ], Braunschweig, Germany) was performed by flow cytometry using a fluorescein isothiocyanate (FITC)-conjugated antibody to cleaved poly(ADP-ribose) polymerase (PARP; Cell Signaling Technology, Denver, CO). Samples of 5×10^5 cells were incubated in the presence of various concentrations of ajoene in six-well plates for 4 h. Subsequently, cells were detached with trypsin-EDTA, washed twice, fixed in 2% formaldehyde for 15 min, permeabilized by 0.1% saponin (Roth GmbH, Karlsruhe, Germany) in PBS for 60 min, and incubated with anti-cleaved PARP according to the manufacturer's protocol for 30 min. For positive control, cells were cultured in the presence of apoptosis inducer tetrandrine (Sigma-Aldrich, Steinheim, Germany) for 4 h (12).

Proliferation assay. Interference of ajoene with A549 cell proliferation was measured employing MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt] proliferation assays (Promega, Mannheim, Germany) following the manufacturer's protocol.

Toxicity assay. Cytotoxicity testing was performed using a lactate dehydrogenase (LDH) release-based assay kit purchased from Roche Applied Science (Mannheim, Germany). A549 lung epithelial cells were exposed to concentration series of test substances for 24 h before LDH release was determined according to the manufacturer's instructions.

RESULTS

Ajoene is the major bioactive QSI compound in garlic extract. Previously, demonstrations of inhibition of QS in *P. aeruginosa* with crude and partially purified garlic extracts (8) encouraged us to further identify and assess the efficacy of the pure QS inhibitor. In our hands, Spanish garlic appeared to contain a higher level of bioactivity, with respect to QS inhibition, than garlic obtained from other countries, including China and Argentina (data not shown). The compounds present in garlic bulb extracts were stable to time, protease activity, and various evaporation techniques at room temperature, and it was only at high temperatures that degradation was observed. An iterative process of fractionation and assaying was applied to crude garlic extracts to determine the presence of any potential QSI compounds (bioassay-guided fractionation).

Several different water extracts were investigated, with several different columns for fractionation and purification initially tested. These columns included matrices consisting of C_{18}, Sephadex G10 and LH20, and hydrophilic interaction chromatography (HILIC) matrix; however, in the end, ajoene was isolated on C_{18} material, as described in the Materials and Methods section. During examination of the initial water extract, the activity was lost. Investigations showed that the active compound was most likely degraded or adsorbed to sodium sulfate (used as a drying agent). Consequently, the focus was switched to the toluene phase. The toluene extracts continuously showed a high level of QSI activity. The treatment of these extracts was similar to that of the water extracts; however, all drying agents were avoided in case of absorption of the active compounds. In this case, the primary column matrices were silica based (SI, DIOL) or C_{18}. Bioassay-guided fractionation based on QSI screens (with QSIS1 and the *lasB-gfp* and *rhlA-gfp* reporter strains) of these extracts isolated a single primary compound responsible for the *in vitro* activity. This compound was isolated, examined by MS and NMR (data not shown), and identified as ajoene (Fig. 1). In addition, several ajoene derivatives (Fig. 1) were shown to be present. Ajoene is a lipid-soluble allyl sulfide formed from allicin, which is converted from alliin by an enzymatic process when garlic is crushed (11). A range of different organosulfur compounds is formed in this process, with ajoene being among the most abundant (37). To further investigate the QSI bioactivity, we used chemically synthesized ajoene prepared by a recently published method (Givskov, European patent application no. 10194154.0 and US provisional application no. 61/420,922). Both naturally occurring and chemically synthesized ajoene exist as two isomers, the *Z* and *E* isomers, in different ratios dependent on the preparation method (Fig. 1).

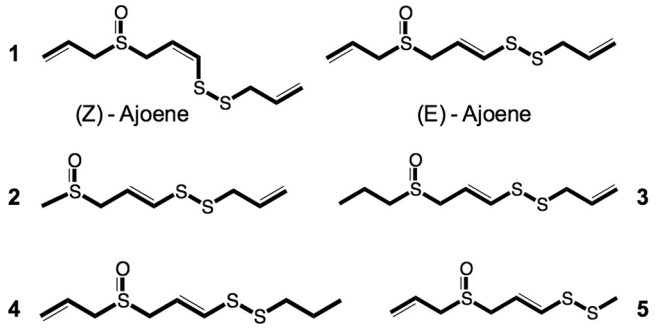

FIG 1 Ajoene and derivatives. Compound 1, ajoene, present in two isomers, *E* and *Z*; compounds 2 to 5, ajoene derivatives.

To determine the QSI activity of ajoene, dose-response curves were created using two QSI reporter systems which contain fusions of the QS-controlled *lasB* promoter and *rhlA* promoter to *gfp* (ASV), encoding an unstable GFP variant in a *P. aeruginosa* background (32, 77). We also used a QS reporter system harbored in an *E. coli* background where the *luxR* gene and the promoter region of the *luxI* are fused to *gfp* (ASV) (4). In all three reporter systems, induction of the QS system can be measured as increasing fluorescence. The presence of an antagonist decreases GFP expression, and thus, fluorescence is proportional to the concentration or effectiveness of the QSI present. Growth of the reporter strains was monitored to make sure that the concentrations of added ajoene were not affecting primary metabolic functions and thereby altering growth rate. Fifty percent inhibitory concentrations (IC_{50}s) were calculated from the curves expressing the specific fluorescence (GFP expression/cell density) (Fig. 2), giving the following values: *lasB-gfp* reporter, 15 μM; *rhlA-gfp* reporter, 50 μM; and *luxI-gfp* reporter, 100 μM. The calculations were performed by plotting the maximal slopes from the curves obtained with the different reporter strains as a function of the concentrations of ajoene. The slope represents the synthesis rate (change in the number of relative fluorescence units/OD_{450}/change in time [ΔRFU/OD_{450}/Δtime]).

Target gene specificity. DNA microarray analysis was used to identify the target gene specificity of ajoene. As a reference, we have used the QS regulon previously identified by Hentzer et al. (33). In the past, this data set has been used to validate the target specificity of putative QSI compounds. The study by Hentzer et al. (33) defines QS-regulated genes to be those genes for which the expression is altered more than 5-fold in a Δ*lasI*-Δ*rhlI* mutant in response to the addition of exogenous C_4-HSL and 3-oxo-C_{12}-HSL. Genes with a less than 5-fold alteration in expression between treated and nontreated cultures were not included in this

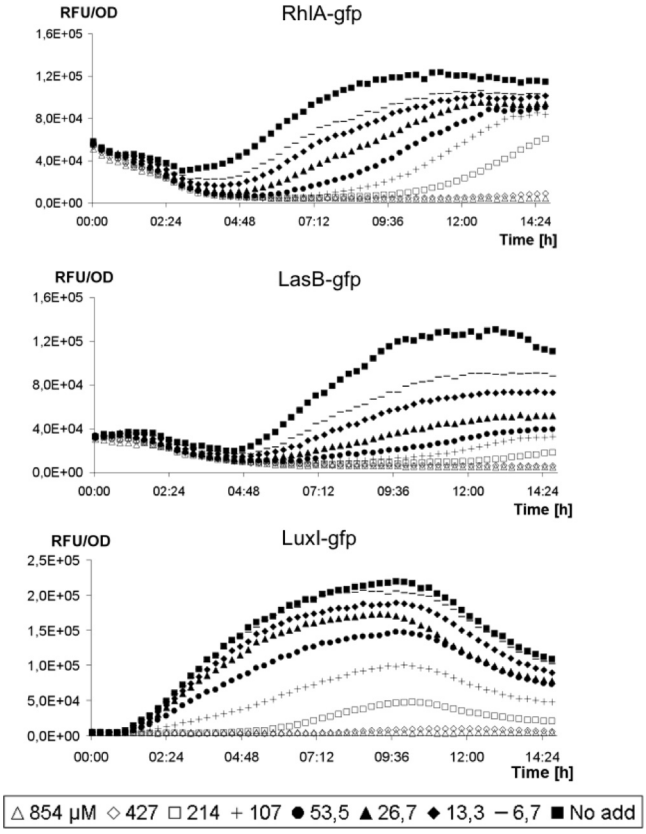

FIG 2 Expression of QS-controlled specific fluorescence (GFP expression/cell density). The QS bioassays used were with *P. aeruginosa* harboring either the *rhlA-gfp* or the *lasB-gfp* fusion and *E. coli* harboring the *luxI-gfp* fusion incubated with synthesized ajoene.

study. Exponentially growing *P. aeruginosa* cultures were treated with the following four concentrations of ajoene: 10 μg/ml (42.7 μM), 20 μg/ml (85.4 μM), 40 μg/ml (170.8 μM), and 80 μg/ml (341.6 μM). None of these concentrations affected growth (see Fig. S1 in the supplemental material). The samples were retrieved at an OD_{600} of 2.0, as previous investigations have shown that the highest activity among the QS genes is at this particular cell density.

Only a small number of genes were significantly ($P < 0.05$) downregulated more than 5-fold by the four different concentrations of ajoene: 0 at 10 μg/ml, 0 at 20 μg/ml, 2 at 40 μg/ml, and 11 at 80 μg/ml. According to the QS regulon defined by Hentzer et al. (33), 10 of the 11 genes and, according to Rasmussen et al. (59), all the genes downregulated more than 5-fold by ajoene are defined as QS regulated. There is a clear relationship between the increasing concentration of ajoene used for treatment and the degree of regulation of the target genes. The transcriptomic analysis indicated that the optimum concentration for repression of the target genes is close to 80 μg/ml ajoene. Attempts to repress more than those 11 genes (out of a total of 5,570 *P. aeruginosa* genes) were not possible without supplementing cultures with concentrations that would also affect growth. This means that ajoene administered at this optimum concentration exhibits a high degree of target specificity toward a small subgroup of the QS regulon. Five genes were significantly ($P < 0.05$) upregulated more than 5-fold in response to treatment with 80 μg/ml ajoene, and with the three lower ajoene concentrations, there were only a few genes for which expression was significantly altered. Three of the genes encode components of a type VI secretion system (*tagQ1*, PA0070; *tssB1*, PA0083; *hcp1*, PA0085), and the other two genes are *exaC* (PA1984) and PA0182, which is a probable short-chain dehydrogenase. Among the genes significantly downregulated by ajoene were the following QS-regulated important virulence factors: LasA protease (*lasA*, PA1871); chitinase (*chiC*, PA2300); the cytotoxic galactophilic lectin (*lecA*, PA2570); the rhamnosyltransferase AB operon (*rhlA*, PA3478; *rhlB*, PA3479); the PvdS-regulated endoprotease (*prpL*, PA4175) that degrades casein, elastin, lactoferrin, transferrin, and decorin (75); and the associated chitin-binding protein *cbpD* (PA0852), which mediates attachment to chitin-containing substrates and presumably assists in biofilm formation (26) (Table 1). None of the treatments seemed to affect transcription of the genes encoding central regulatory genes of the QS circuit. This is similar to other previously published QSI compounds, including furanone C-30 (33), patulin, and penicillic acid (59), and indicates that interaction of the inhibitor with its target may occur at the posttranscriptional level.

To verify the microarray data, RT-PCR was performed with the

TABLE 1 Alterations in gene expression by ajoene[a]

Gene no.	Gene	Description	Fold change in gene expression with ajoene concn (μg/ml) of[b]:			
			10	20	40	80
PA0852	*cbpD*	Chitin-binding protein	−2.8	−2.5	−3.9*	−6.9*
PA1871	*lasA*	LasA protease precurser	−2.6	−2.2	−3**	−8.7**
PA2069		Probable carbamoyl transferase	−2.3	−2.4	−4	−5.3*
PA2146		Conserved hypothetical protein	−1.3	−1.8*	−2.6*	−7.3**
PA2300	*chiC*	Chitinase	−2.5	−2.1*	−5.1**	−24.6**
PA2570	*pa1L*	LecA	−1.8	−2	−3.3	−6.3*
PA3478	*rhlB*	Rhamnosyltransferase chain B	−2.6	−2	−3.3**	−8.7**
PA3479	*rhlA*	Rhamnosyltransferase chain A	−2.2	−1.5	−2.6	−8.8**
PA4141		Hypothetical protein	−1	1.1	−1.3	−5.4**
PA4142		Probable secretion protein	−2	−2.2	−2.7	−5.1*
PA4175	*prpL*	Pvds-regulated endoprotease	−3.7	−3.3	−5.3*	−6.8*

[a] Genes included are >5 times downregulated by 80 μg/ml ajoene treatment.
[b] Fold change in gene expression compared to an untreated control. Data represent the averages of three individual experiments. *, $P < 0.05$, Student's *t* test; **, $P < 0.01$, Student's *t* test.

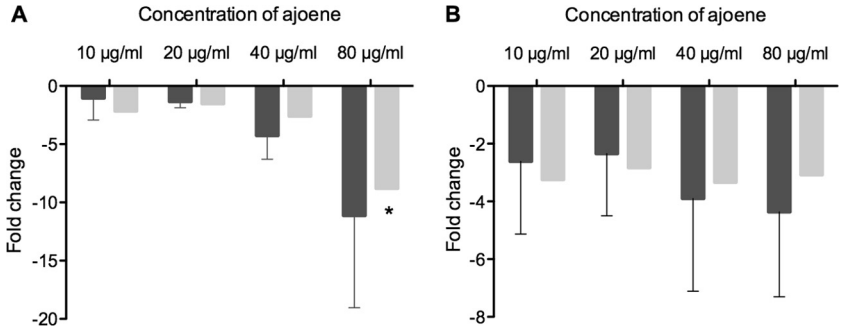

FIG 3 Fold change in gene expression of *rhlA* and *lasB* measured by RT-PCR (dark gray bars) and DNA microarray (light gray bars). Data represent the average of three individual experiments. *, $P < 0.05$, Student's *t* test. Error bars are means ± SDs.

two stringently QS-regulated genes *lasB* and *rhlA* (Fig. 3). When comparing the two experimental methods, repression of the two genes followed the same trend, with a slightly stronger reduction observed with the RT-PCR-based method. The RT-PCR data showed that a concentration of 80 μg/ml ajoene lowered expression of *rhlA* almost 12-fold and that of *lasB* almost 5-fold. According to Rasmussen et al. (59), the genes listed in Table 1 (except for *prpL*, which is exclusively regulated by the Las QS system) are subject to regulation by both the Las and Rhl QS systems. This suggests that ajoene may primarily target the Rhl system.

Attenuation of rhamnolipid production by ajoene. To exemplify the actual efficacy of ajoene in downregulating one of the important virulence factors, the concentration of rhamnolipid present in the cultures grown for DNA array analysis and RT-PCR was directly quantified by liquid chromatography (LC)-MS. The production of rhamnolipids encoded by the *rhlA*, *rhlB*, and *rhlC* genes (PA3479, PA3478, and PA1131, respectively) is initiated in early stationary phase and coordinately regulated by the Rhl and the PQS systems (57, 73). Samples where therefore retrieved at OD_{600}s of 1.5 and 2.0 to monitor rhamnolipid production before and after the synthesis was fully induced. The data showed clearly that there was an increase in rhamnolipid production from an OD_{600} of 1.5 to one of 2.0. The concentrations of rhamnolipid in the samples correlated inversely with increasing concentrations of ajoene. When treated with 20 μg/ml ajoene at an OD_{600} of 2.0, the rhamnolipid content was reduced to approximately 1/3 of that for the untreated culture, and there was almost no detectable rhamnolipid present in the sample when the cells were treated with 80 μg/ml ajoene (Fig. 4).

Effect of ajoene on *P. aeruginosa* QS signal molecule production. According to the transcriptomic analysis, the genes inhibited by ajoene treatment could indicate that a posttranscriptional effect on gene products is responsible for generation of C_4-HSL. To test this, the concentrations of C_4-HSL and 3-oxo-C_{12}-HSL in untreated and ajoene-treated cultures were determined by HPLC-MS/MS at three different cell densities (OD_{600}s, 1.5, 1.8, and 2.0). The concentration of C_4-HSL was found to inversely correlate with increasing concentration of ajoene, and at a concentration of 80 μg/ml of ajoene, the level was reduced almost 3-fold compared to the untreated control. With respect to the concentration of 3-oxo-C_{12}-HSL, there was no consistent effect with increasing concentrations of ajoene (Fig. 5).

Ajoene treatment renders *in vitro* biofilms rhamnolipid deficient and thereby prevents the killing of PMNs. The effect of ajoene on rhamnolipid production prompted us to investigate whether this would in fact inhibit lysis of PMNs. *P. aeruginosa* biofilms were grown for 4 days in either the presence or absence of 100 μg/ml ajoene. When freshly isolated PMNs were subsequently introduced into the flow chambers of the biofilms grown in the absence of ajoene, PI staining indicated extensive necrosis of the PMNs (Fig. 6A). In contrast, when the biofilms were grown in the presence of ajoene prior to PMN exposure, no necrosis of the PMNs was observed (Fig. 6B).

Ajoene enhanced tobramycin effect on *P. aeruginosa* biofilm. We have previously published the results of an *in vitro* treatment study of biofilms grown in the presence of QSI bioactivities, including those associated with garlic extract and furanone C-30, where QS inhibition was found to greatly enhance the antimicrobial effect of tobramycin (8, 33). Biofilms of a *P. aeruginosa* strain were grown in either the presence or absence of 100 μg/ml ajoene. At day 3, the biofilms were treated with 10 μg/ml tobramycin for 24 h. A pilot study on biofilms grown in the absence of ajoene indicated that treatment with 10 μg/ml, 100 μg/ml, or 340 μg/ml tobramycin showed no difference in the extent of killing, as judged from live-dead staining and inspection by means of confocal scanning laser microscopy. Our analysis showed a more than 90% killing of cells when the biofilms were grown in the presence of ajoene and subsequently treated with 10 μg/ml tobramycin (Fig.

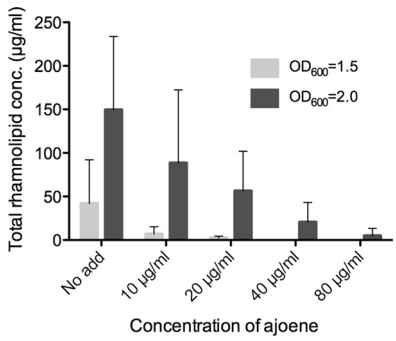

FIG 4 Total rhamnolipid concentration in untreated (no added ajoene [no add]) and ajoene-treated planktonic *P. aeruginosa*. The cultures were grown in medium supplemented with 10 μg/ml, 20 μg/ml, 40 μg/ml, and 80 μg/ml of ajoene (rhamnolipid is below the detection level for the 40-μg/ml and 80-μg/ml ajoene treatments at an OD of 1.5). Samples were retrieved at an OD_{600} of 1.5 (light gray bars) and at an OD_{600} of 2.0 (dark gray bars). Data represent the average of three individual experiments. Error bars are means ± SDs.

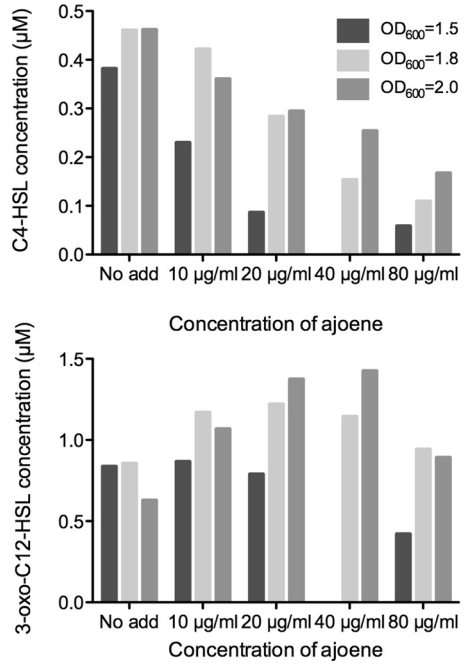

FIG 5 Concentrations of C_4-HSL and 3-oxo-C_{12}-HSL in untreated (no added ajoene [no add]) and ajoene-treated planktonic cultures of *P. aeruginosa*. The cultures were grown in medium supplemented with 10 μg/ml, 20 μg/ml, 40 μg/ml, and 80 μg/ml of ajoene. Samples were retrieved at an OD_{600} of 1.5 (dark gray bars), at an OD_{600} of 1.8 (light gray bars), and at an OD_{600} of 2.0 (medium gray bars).

7A). The synergistic effect was also evaluated on the clinical isolate from a patient with CF, CF438 (the first isolate from a child diagnosed with CF), which possesses functional QS systems, and once again, extensive killing of the biofilm was recorded (Fig. 7B).

Antimicrobial effects *in vivo*. We performed three individual *in vivo* treatment experiments with ajoene in a pulmonary infection model in mice using 25 μg ajoene g^{-1} BW. When combining the experiments, a significant difference was seen on day 3 ($P < 0.002$), with a more than 500-fold difference in clearance recorded between the groups (Fig. 8). Experiments with a *lasR rhlR* double mutant showed that this is the maximum obtainable difference in clearance that can be obtained in this infection model (6).

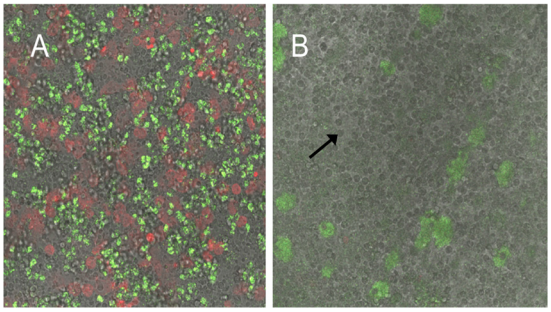

FIG 6 Combined fluorescence and light microscopic investigations at day 4 of biofilms of *P. aeruginosa* exposed to PMNs (arrow) for 180 min at 37°C and then subsequently stained with the DNA stain PI. (A) Biofilm grown without ajoene in the medium; (B) biofilm grown in the presence of 100 μg/ml ajoene in the medium. Red fluorescence indicates lysed PMNs, and green fluorescence indicates the *P. aeruginosa* biofilm.

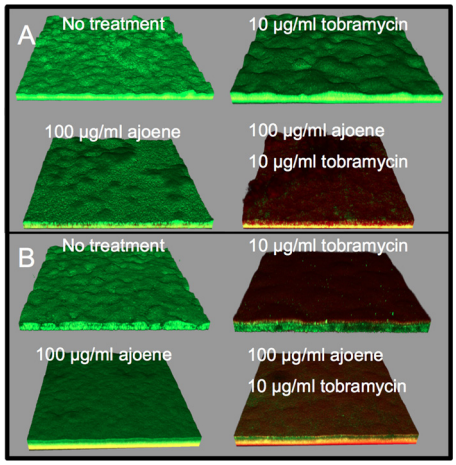

FIG 7 Biofilms of *P. aeruginosa* PAO1 (green) (A) and clinical *P. aeruginosa* isolate CF438 (green; stained with Syto 9) (B) at day 4 after no treatment, treatment with 100 μg/ml ajoene for 4 days, treatment with 10 μg/ml tobramycin for the final 24 h, or treatment with a combination of tobramycin-ajoene. Dead cells are stained with the DNA stain PI (red). The yellow color reflects a mixture of live and dead cells. The biofilms were visualized with a confocal scanning laser microscope.

Treatment of mice infected with clinical CF isolates. In CF patients, the appearance of the mucoid phenotype is considered a clinical marker for the onset of the chronic infection, which correlates with a poor prognosis for the infected CF patients. To test the efficacy of ajoene treatment on a QS-proficient mucoid *P. aeruginosa* strain, we adopted a clinical isolate from a patient who had had a chronic infection with *P. aeruginosa* for 16 years (44, 72). After administration of 25 μg/g ajoene, we noted an induction of abscesses, which resulted in an increase in the bacterial load in the lungs. This phenomenon was not observed with the placebo group. The experiment was performed twice with similar results. We then repeated the experiment with 12.5-μg/g and 6.25-μg/g ajoene treatments. The concentration of 12.5 μg/g gave the best results with respect to bacterial clearance and apparent well-being of the mice, but the clearance in the treatment group was not significantly different from that in the placebo group ($P < 0.5$). Treatment with 6.25 μg/g of ajoene did not have any significant effect on clearance compared with the placebo group (data not shown). An isogenic, nonmucoid derivative of the clinical isolate was also investigated in a treatment study using 12.5 μg/g of ajoene. This isolate cleared very rapidly from the lungs of the mice. Therefore, the experiment was evaluated at day 1 after infection. The difference between the treated group and the placebo group showed a nonsignificant ($P < 0.1$) 2-orders-of-magnitude reduction (data not shown). Both isolates have a functional QS system (44). To investigate the efficacy of ajoene on an early clinical *P. aeruginosa* isolate, an experiment with the first isolate of *P. aeruginosa* from a CF patient was conducted by treating the infected mice with 12.5 μg/g of ajoene. The experiment was evaluated at day 1, since the strain is highly virulent and therefore the mice would not survive for a 3-day period. There was a significant 20-fold difference ($P < 0.05$) between the treated group and the placebo group (data not shown). The isolate tested positive for a functional QS system (data not shown).

Stability of ajoene under experimental conditions. To test the stability in the presence of bacteria and whether ajoene could in-

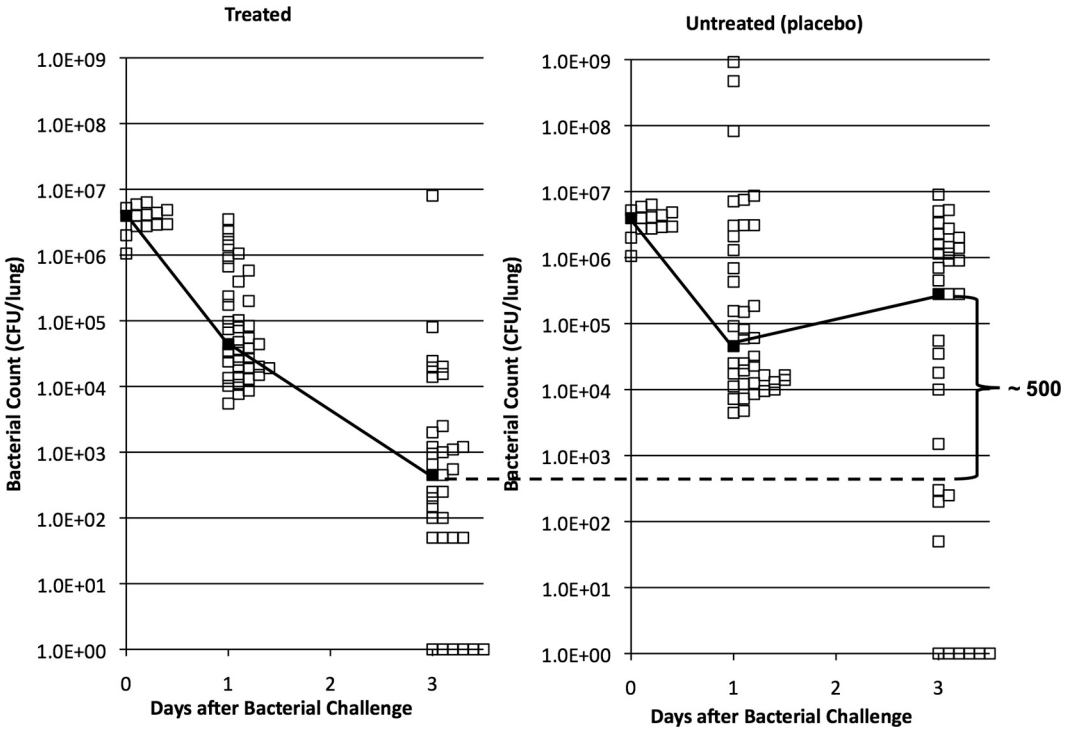

FIG 8 Combined results of three separate experiments of ajoene treatment versus no treatment (placebo) using the mouse model of pulmonary infection. The BALB/c mice were intratracheally challenged (at day 0) with alginate beads containing 1.5×10^8 CFU/ml *P. aeruginosa*. The two groups of mice were either untreated (placebo) or treated with ajoene at 25 μg/g BW once a day. The mice were given 2 days of prophylactic treatment or placebo. Mice were sacrificed on day 1 or day 3 postinfection, and the contents of bacteria in the lungs were determined. The median values are indicated with filled black squares. The statistical significance of the difference in clearance was tested by a Mann-Whitney U test (analysis of nonparametric data), and *P* values for the difference at day 1 and day 3 were 0.9 and 0.002, respectively.

teract directly with the QS signal molecules, 50 μg/ml of ajoene was incubated overnight at 37°C and 4°C with *P. aeruginosa* (PAO1) or a QS-deficient Δ*lasI rhlI* or Δ*lasR rhlR* mutant and pure C_4-HSL, pure 3-oxo-C_{12}-HSL, or no addition. Following the incubations, ajoene activity in the supernatant was assessed by means of a *lasB-gfp* dose-response bioassay. The activity in the supernatant disappeared after incubation with bacteria at 37°C overnight, whereas there was no decrease in ajoene activity when incubated with bacteria at 4°C or incubated in the absence of bacteria at 4°C and 37°C. There was no change in ajoene activity when incubated with pure C_4-HSL and 3-oxo-C_{12}-HSL, which indicates that ajoene is not likely to chemically react with the signal molecules and cause their inactivation (data not shown).

Effect of BSA on the bioactivity of ajoene. Because of putative annihilating effects of serum proteins on the bioactivity of ajoene, concentrations ranging from 12.5 μg/ml to 50 μg/ml of ajoene were incubated with bovine serum albumin (BSA). There was no notable reduction in the QSI activity of ajoene over the range of concentrations tested with serum albumin at concentrations up to 50 mg/ml, i.e., the concentration which corresponds to the content in adult serum (see Fig. S2 in the supplemental material).

Effects of ajoene on human lung epithelial cells. A549 human lung epithelial cells were employed as a model system to quantify potential apoptosis-inducing effects. According to cytometric determination of cleaved PARP as a marker, ajoene promotes apoptosis with a 50% effective concentration (EC_{50}) of about 100 μM (23 μg/ml). The PARP-activating effect of ajoene on lung epithelial cells was about 10-fold weaker than that of the positive control, tetrandrine, a natural compound used in Chinese medicine for the treatment of lung disorders such as silicosis (12). Consistent with these findings, ajoene inhibited proliferation of A549 cells with an IC_{50} of about 100 μM and is about 1 order of magnitude less potent in impairment of proliferation than the control compound, tetrandrine. General cytotoxicity in terms of induced cytolysis was determined by the release of LDH and, hence, LDH activity from damaged A549 cells. Ajoene induced LDH release in a dose-dependent manner, with an EC_{50} of 200 μM. It was found to be approximately 10-fold less cytotoxic than the established furanone QS inhibitor compound C-30 (see Fig. S3A to D in the supplemental material). Taken together, results from three independent assays coherently characterize ajoene as a compound with relatively weak interference with the cell physiology of the lung epithelium.

DISCUSSION

Intriguingly, worldwide emerging problems of infection control parallel a marked slowdown in the development of new antibiotics. Many pharmaceutical companies no longer have antibiotic drugs in the pipeline or research activities in the field. There are both strategic and economic reasons for this, but there have been profound scientific difficulties for the pharmaceutical industry in this context. One major limiting factor is that the study of free-living, planktonic bacteria has provided the basis for our general understanding of microbial life and, in particular, infectious diseases. Consequently, in the traditional design of antibiotic drugs, it is not appreciated that the biofilm habitat may dominate in

chronic infections just as it does in the environment. Antimicrobial treatment of biofilms is a challenge, in particular when it comes to heterogeneity, which is likely one of the special features that provide biofilm bacteria with such remarkable resilience.

The archetypical biofilm disease has for a long time been considered to be *P. aeruginosa* infection in CF patients. Furthermore, a recent investigation by us pointed out that the early colonizers of children with CF have functional QS systems. The first system lost is the Las system, but the strains are still able to express rhamnolipid and other PQS- and C_4-HSL-controlled genes (7). Therefore, strategies that help in disabling the protective mechanisms of *P. aeruginosa*, in particular, the rhamnolipid shield and eDNA production, are likely to be employed as a worthwhile addition to conventional antimicrobial chemotherapy. Different approaches can be used to identify and harness the QS inhibitors obtained from natural sources. Extraction of natural products has provided several positive QSI molecules, among them the *Delisea pulchra* furanones (32) and patulin from *Penicillium coprobium* (59). It is interesting that sulfur-containing compounds appear to be a new class of molecules capable of inhibiting QS (3, 5). Li et al. (40) found two sulfur-containing compounds from an *in silico*-based virtual screening to target the AI-2 QS system from *Vibrio harveyi*, and several analogues were identified to exert bioactivity (52). These molecules contain a sulfone group, in contrast to ajoene, which contains disulfide and sulfinyl groups. For a more detailed description of identified QSIs, see the work of Galloway et al. (29).

We have shown that approximately 80 μg/ml (341.6 μM) ajoene efficiently switches off the expression of the *rhlA-gfp* fusion in our dose-response bioassays and extensively downregulates *rhlA* gene expression, which corresponds to the decrease in concentration of rhamnolipid directly measured by HPLC. The gene *rhlA* encodes a rhamnosyltransferase which catalyzes a glycosyl transfer reaction in rhamnolipid synthesis. Rhamnolipids are glycolipids that have strong surfactant abilities. One rhamnolipid in particular, rhamnolipid B, is one of the two more abundant rhamnolipids and has been shown to cause necrosis of PMNs (36). Previously published data with a Δ*rhlA* mutant (71) (which is defective only in rhamnolipid synthesis) make us strongly believe that rhamnolipid is responsible for the lytic killing of the PMNs, also *in vivo*. We have shown that ajoene treatment of *in vitro* biofilms prevents the killing of PMNs. These experiments furthermore suggest that ajoene treatment is capable of attenuating the production of rhamnolipids. In addition, ajoene treatment is capable of rescuing the PMNs and is likely able to restore the action of the PMNs. The same results were observed with a Δ*rhlA* mutant (71): almost no necrotic PMNs were detected when exposed to biofilms of the rhamnolipid-deficient mutant.

One important issue in the treatment of bacterial biofilm infections is the lowered effectiveness of administered antibiotics. An infection in the airways of CF patients results in high concentrations of anionic polyelectrolytes like DNA (14) released from lysed inflammatory cells, such as the PMNs and bacteria. It has been shown that anionic polyelectrolytes, in particular, DNA, bind to and reduce the activity of cationic antibiotics like tobramycin (74, 78), which can lead to a decrease in the biological availability of tobramycin to as low as 5% of the existing dose (43). This suggests that by blocking the production of eDNA, it is possible to attenuate the otherwise subsequent inactivation of tobramycin. We have shown *in vitro* that addition of 100 μg/ml ajoene to a biofilm followed by addition of 10 μg/ml tobramycin kills more than 90% of the biofilm bacteria, whereas the presence of only tobramycin or ajoene had no effect. It is documented that the release of bacterial eDNA is controlled by QS (2), which, taking into consideration and combined with our results, points to a possible attenuation of the release of eDNA by ajoene.

This synergistic effect is also relevant *in vivo*. It has been demonstrated that treatment of an *in vivo P. aeruginosa* foreign-body biofilm infection with a combination of a QSI and tobramycin likewise showed a synergistic clearing effect on the bacteria (16). The results could be obtained with the use of either of the QSIs: furanone C-30, ajoene, or horseradish juice extract. In addition, rhamnolipid-mediated lysis of attacking PMNs may significantly contribute to the tobramycin annihilating effects *in vivo*. Our data indicate that this chain of neutralizing events may be obstructed by treatment with QSIs, including ajoene. A recent study showed similar promising results with treatment of a *Burkholderia cenocepacia* infection with a combination of tobramycin and the QSI baicalin hydrate in a mouse pulmonary model of infection (13). Several published papers by our group have demonstrated that QS deficiency (either by mutation or by QSI treatments) leads to faster clearing of the bacteria than of bacteria with functional QS in a mouse model of pulmonary infection (6, 8, 33). In this study, ajoene was administered prophylactically and was continued after infection. Enumeration by plate counts showed a significant difference between the treated group and the control group on day 3. This is concordant with the results obtained by Bjarnsholt et al. (8) in a study in which raw garlic extract was used as treatment. In addition, in a study by Harjai et al. (31), where garlic extract was given to mice orally, mice showed significantly reduced renal bacterial content of *P. aeruginosa* at day 5 postinfection.

Three different clinical isolates were tested in the lung model to demonstrate the efficacy of ajoene toward different isolates retrieved from CF patients. Ajoene treatment of a QS-proficient mucoid strain obtained from a CF patient chronically infected for 16 years and the isogenic nonmucoid strain did not have any significant effect compared to that on the placebo group, whereas ajoene treatment of a first CF isolate showed a significant difference. This increased susceptibility of the early clinical isolate to the QSI induced by ajoene is in line with our demonstration of the predominance of intact QS in early isolates from CF patients (7). These data were obtained at 1 day postinfection because of difficulties in keeping the mice in a proper healthy condition. Furthermore, the ajoene concentration was 2-fold lower than that in studies with wild type. These modifications (which were taken to comply with ethical constraints) in the experimental procedure might be the reason for the lower effectiveness of ajoene that we obtained with the clinical strains compared to wild type. When comparing the present studies with ajoene to earlier studies with garlic extracts, the present study offers a convincing indication of ajoene being the major active component in garlic able to reduce a *P. aeruginosa* infection. Two other sulfur-containing molecules previously isolated by us from garlic were found to possess QS-inhibitory activity toward the *Vibrio fischeri* LuxR QS system but not against the *P. aeruginosa* QS systems (53).

The question is whether it would be possible to obtain the promising treatment results in clinical trials performed on patients suffering from *P. aeruginosa* infections. The ajoene content in garlic is found to be concentrations of up to 172 μg/g of *E*-ajoene and 476 μg/g of *Z*-ajoene, as judged from rice oil-heated (80°C) freshly prepared garlic extracts (46). To match this rela-

tively low herbal content of ajoene with the dosages required for the present animal treatments, individuals would be required to intake approximately 5 kg of raw garlic per day. Despite this, a recently published pilot study investigating the effect of garlic capsules orally administered to CF patients reported a nonsignificant but nevertheless reduced decline in lung function in 1 s (FEV_1) in the treated group compared with the corresponding placebo group (65). The exact amount of ajoene present in the capsules was not determined. However, water extracts made directly on the content of the capsules showed bioactivity directed against our *lasB-gfp* reporter (data not shown). It therefore remains unknown if ajoene was present in biologically relevant amounts or whether the capsules contained ajoene-enhancing components that would increase the effects of small amounts of ajoene. In fact, upon subsequent purification close to 100% purity, as determined by LC-diode array detection-MS, we found that synthesized ajoene actually lost activity in the *in vivo* infectious models (data not shown). For example, fresh garlic extract shows a much more pronounced effect than synthesized ajoene on the transcriptome of *P. aeruginosa* (58). In contrast to our garlic extracts used previously, our transcriptome analysis revealed that synthetic ajoene affected only a few but nevertheless important number of QS-controlled genes, including *lasA*, *chiC*, and *rhlAB*, whereas *lasB* was not downregulated more than 5-fold. The effect on primarily QS-controlled genes and the small amount of genes affected suggest that synthetic ajoene, in contrast to our previous garlic extracts, inhibits expression of only a minor part of the QS regulon. In comparison, with more than 80% of the QS-controlled genes being downregulated by furanone C-30 treatment and no effect on signal generation, it is unlikely that ajoene targets both LasR and RhlR.

Sonnleitner et al. (66) have investigated the influence of the SM-like RNA-binding protein Hfq on the QS system. They documented a decrease in elastase, catalase, and pyocyanin production in an *hfq*-knockout mutant (66) and confirmed by transcriptome analysis that the effect on this subset of QS-regulated virulence factors was mediated by reduced expression of the corresponding genes (67). The authors suggested the following interactive path of Hfq and the QS system: Hfq binds to and stabilizes the regulatory RNA RsmY, which subsequently binds to the RsmA protein, which, in turn, negatively regulates RhlI messenger translation. Furthermore, they showed a decrease in the concentration of C_4-HSL in both a PAO1 Δ*rsmY* mutant and a PAO1 *hfq* mutant strain compared to the wild type (67), which corresponds to the results of our investigations, where the amount of C_4-HSL in cultures decreases with increasing ajoene concentrations. Furthermore, when comparing our transcriptome analysis with the transcriptome analysis of the PAO1 *hfq* mutant strain, there is a compelling correlation with genes which are significantly downregulated by ajoene treatment. It is, however, not trivial to compare the two studies. For example, the PAO1 *hfq* mutant strain showed a reduced growth rate compared to the wild type (66). Furthermore, the authors grew their samples in LB medium and isolated RNA from cultures grown to an OD_{600} of 2.5. In comparison, our samples, which were retrieved at an OD_{600} of 2.0, were grown in AB minimal medium supplemented with 0.5% Casamino Acids. Despite this, we suggest that either the Hfq protein or the RsmY RNA may constitute a possible target of ajoene.

The effects of ajoene on *lasB* transcription, monitored by RT-PCR and DNA array analysis, were only minor, in contrast to the observed effects on *lasB-gfp* expression. We see a strong reduction of fluorescence with the *lasB-gfp* reporter with increasing ajoene concentrations. The reporter strain is a translational fusion and reflects the reported effect of the small regulatory RNA molecules on posttranscriptional levels (as previously reported as a reduction in elastase production [66]). This is in support of our hypothesis that ajoene targets Hfq and the regulatory RNAs and that the effect of the lower parts of the QS hierarchy on transcription is mediated by a reduction in the C_4-HSL concentration. Furthermore, no effect on the transcription of *lasI* and *rhlI* is found on the transcriptomic analysis, which supports the view that the effect of ajoene on reducing RhlI expression is posttranscriptional. The much more pronounced effect of garlic extracts on a multitude of QS-controlled gene expression previously reported by us (58), taken together with our shortcomings in the extraction of hydrophilic compounds, suggests that garlic may in fact contain a multitude of QSI compounds or stabilizing agents that may act in synergy and thereby in concert cover a much larger spectrum of QS-controlled virulence gene expression. If true, quantities of garlic attainable in the food diet may in fact contribute to a natural prophylaxis against bacterial infections.

To address the question of whether synthesized ajoene constitutes a pharmaceutically relevant drug candidate, we investigated toxicity effects on human epithelium cells. Ajoene exerts proapoptotic, antiproliferative, and cytotoxic effects on A549 lung epithelial cells. The concentrations showing half-maximal effects in our assays were in the range of 23 to 46 μg/ml (100 to 200 μM). Compared to tetrandrine, a substance used as a lung therapeutic agent in Chinese medicine, ajoene is clearly less toxic (by a factor of 10) toward respiratory epithelial cells. Interestingly, ajoene is less toxic for A549 cells than for HL-60 leukemia cells, which already respond by intense apoptosis at concentrations of about 4.7 μg/ml (20 μM) (23). In comparison, the concentration that we used in the mouse experiments was 25 μg/g (107 μM). To more thoroughly evaluate the potential of ajoene as a putative component of future CF medicine, it will be important to compare its cytotoxic effects with those induced by antibiotics administered in the treatment of the CF syndrome.

In conclusion, we have demonstrated the use of synthetic ajoene to attenuate the virulence of *P. aeruginosa* by lowering expression of important QS-controlled virulence genes in *P. aeruginosa*. It is shown for the first time that successful antimicrobial treatments with the QS systems as targets can be obtained by inhibiting only a few but important virulence genes and not the entire QS regulon (mediated through LasR and RhlR). This new finding leads us to suggest that within the framework of QS inhibition as an antimicrobial strategy, small regulatory RNA molecules operating in the lower part of the QS hierarchy may constitute a new, functional antimicrobial drug target. At present, this possible novel antimicrobial target needs to be extensively pursued and confirmed by molecular approaches. Interestingly, small regulatory RNAs or microRNAs and their cognate targets are strongly implicated in cancer as either oncogenes or tumor and metastasis suppressors. Targeting of small regulatory RNAs to therapeutic antimicrobial ends would therefore parallel future developments in anticancer therapy, with cancer-specific microRNAs to be exploited not only to produce a direct anticancer effect but also to improve the response of tumor cells to conventional treatments (35, 69). Similarly, the biofilm-weakening properties of ajoene with respect to enhancing the effect of conventional antibiotics

such as tobramycin may become instrumental for the future development of combinatory treatments.

It is worth acknowledging that QS inhibition does not remove the *P. aeruginosa* ability to form a biofilm. However, all available data indicate that a QS-deficient biofilm is more fragile than a QS-proficient biofilm (6). Since, for example, the matrix component DNA is missing (matrix production is C_4-HSL-RhlR controlled), the biofilm is sensitive to shear forces and can slough off, depending on the hydrodynamic forces. In addition, since rhamnolipid is not formed, the biofilm becomes sensitive to the action of PMNs (because the PMNs are not killed when they get in contact with the biofilm). We have previously shown that *in vitro* biofilms of QS-deficient bacteria can be phagocytosed by freshly isolated PMNs, in contrast to QS-proficient biofilms (6). Central in our model for biofilm tolerance of PMNs is that rhamnolipid production forms a protective shield against the incoming PMNs, and we have several data supporting this (1, 36, 71). The use of QSIs should therefore greatly enhance the antimicrobial properties of the PMNs and allow them to efficiently eradicate biofilm-forming bacteria. Furthermore, rhamnolipid lyses the PMNs, which subsequently spill out their content of DNA, hydrolytic enzymes, and oxygen radicals. This creates an "evil circle," particularly with respect to tissue damage, increasing inflammation and induction of mutations in *P. aeruginosa* that result in the appearance of the mucoid phenotype, which significantly contributes to exacerbations. The key thing is that this should never happen, and a QS-inhibitory drug should prevent this from happening. Our animal experiments with clinical isolates suggest that late mucoid isolates are not sensitive to the blocking of QS-controlled phenotypes, whereas early isolates are likely to show sensitivity. Ajoene might be able to prevent initial adherence and colonization of *P. aeruginosa*, and this treatment strategy might prevent the chronic lung infection by mucoid strains of *P. aeruginosa* in CF patients. The decrease in infection in the mouse experiments, the removal of *in vitro* biofilms in a combinatorial experiment with tobramycin, and the initial toxicity test with ajoene suggest the potential of using ajoene as an antipathogenic drug for treatment of chronic *P. aeruginosa* infections in the future.

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