Molecular unraveling of interspecies crosstalk

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Cover designed by Maciej Wojtas, an imagination of a fungal receptor for 3-oxo-C_{12} homoserine lactone molecule against the background of *Candida albicans* cells stained with calcofluor white photographed with a fluorescent microscope.

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university of groningen

Molecular unraveling of interspecies crosstalk

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Chapter 1

1.1. General introduction

Microbial spy games and host response; roles of a *Pseudomonas aeruginosa* small molecule in communication with other species

Lucja M. Jarosz, Ekaterina S. Ovchinnikova, Michael M. Meijler and Bastiaan P. Krom, PloS Pathogens **2011**; 7: e1002312.

ABSTRACT

Gathering and sharing of information is extremely important in the human society. Especially in times of war, the ability to obtain, encrypt and share information can make the difference between victory and defeat. Microbes produce and secrete species-specific molecules that allow others of the same, but also of other species, to detect the number of individuals present (known as quorum sensing (QS)). Species-specific molecules resemble encrypted messages, but like all encryption, it can be deciphered by others for which the signal is not intended. Infections related to burn wounds, cystic fibrosis and periodontal diseases consist most commonly of the bacteria *Pseudomonas aeruginosa* and *Staphylococcus aureus* and the fungus *Candida albicans,* and represent niches with an active host-response. We will provide five facts about how a *P. aeruginosa* QS molecule, $3OC_{12}HSL$, plays a pivotal role in this triangle of interspecies interactions and how $3OC_{12}HSL$ elicited microbial behavior has consequences for the host-response.

INTRODUCTION

Gathering and sharing of information is extremely important in human society. Especially in times of war, the difference between victory and defeat can depend on the ability to obtain, encrypt and share information, and sophisticated systems have been developed for exactly this purpose. Similarly, in their constant battles with competitors and the host immune system, (opportunistic) microbial pathogens have developed sophisticated cell-cell communication systems termed quorum sensing (QS) that allow exchange of critical information. In return, competing microbes, as well as the host immune system, have developed means to intercept and decode these messages. The information obtained by this molecular espionage is used for their benefit, either to win the war (microbe-against-microbe), or to prepare for an upcoming battle (microbe-against-immune system). To illustrate clinical importance of this microbial spy game, we will focus on the biological activity of a single bacterial QS molecule on surrounding microbes and the host immune system and its diverse "meaning" to different receivers. Infections related to burn wounds, cystic fibrosis and periodontal diseases consist most commonly of the bacteria *Pseudomonas aeruginosa* and *Staphylococcus aureus* and the fungus *Candida albicans,* and represent niches with an active host-response. Therefore, we will specifically provide five facts about_how the *P. aeruginosa* QS molecule, 3-oxo-dodecanoyl-L-homoserine lactone ($3OC_{12}HSL$), plays a pivotal role in this triangle of interspecies interactions and how microbial behavior elicited by $3OC_{12}HSL$ has consequences on the host-response.

Quorum sensing: sophisticated communication system

QS is a system that enables microbes to monitor population cell-density, through the production, secretion and sensing of small diffusible molecules (12). When such molecules reach a threshold concentration, microbial cells in the vicinity detect the signal and coordinately respond by modifying their gene expression; often genes associated with virulence and pathogenesis. Several different types of QS molecules have been described for a wide variety of microbial species.

In the Gram-negative pathogenic bacterium *P. aeruginosa*, the QS system is perhaps the most complex because several distinct QS sub-systems are

hierarchically intertwined at different stages (17) (Fig. 1). The best-studied of these systems is the LasI/R which consists of the LasI protein that catalyzes the synthesis of the diffusible molecule: $3OC_{12}HSL$ (7). Intracellular accumulation of $3OC_{12}HSL$ is sensed by the receptor LasR and induces expression of several virulence factors such as exotoxins, proteases and production of secondary metabolites. Significantly, LasR is also responsible for development and maturation of biofilms, which are communities of surface-adherent microbial species implicated in chronic and resistant infections such as burn-wounds, pneumonia in cystic fibrosis patients and periodontitis (5).

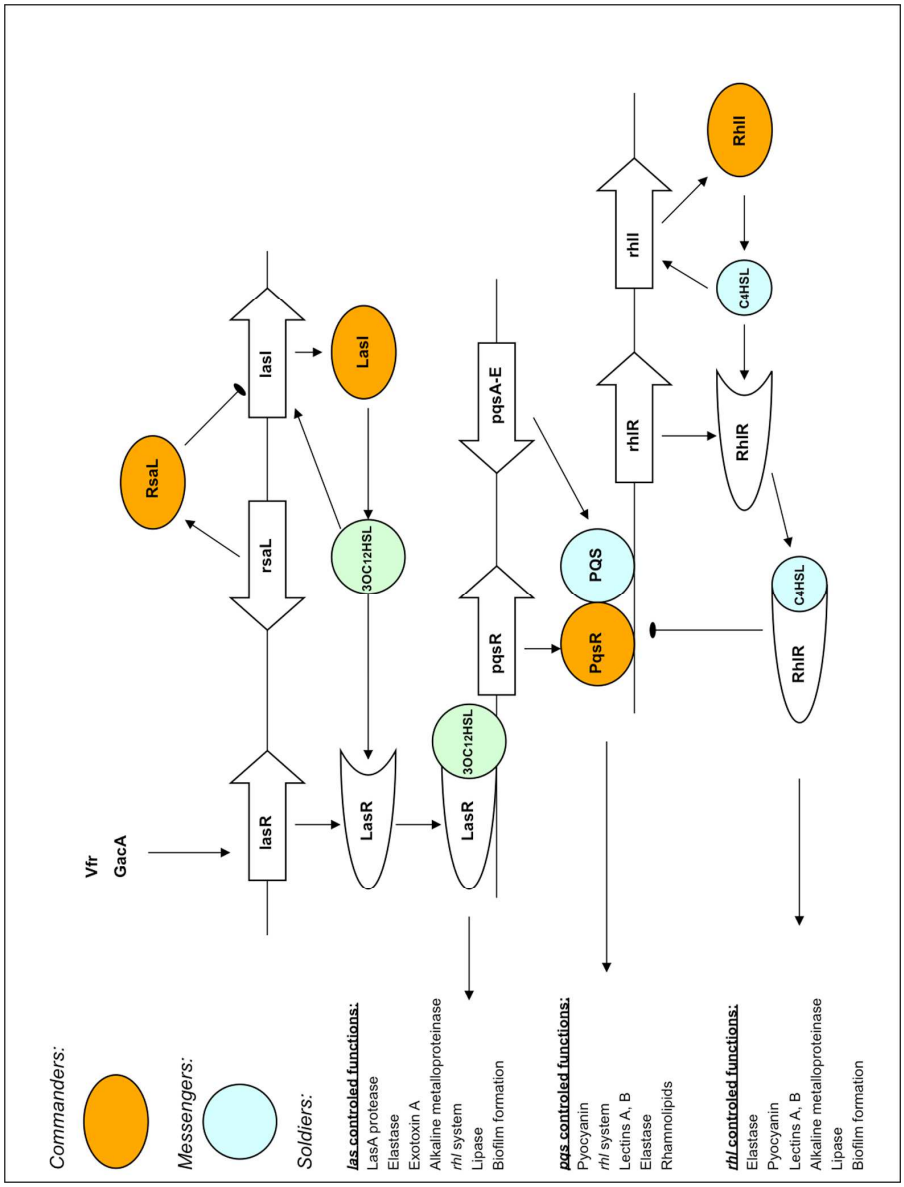

Fig. 1. Schematic overview of the complex QS systems present in *P. aeruginosa*. The commanders represent the major regulatory systems, while the messengers are the signaling molecules. The soldiers represent the various virulence factors that have a function in the interaction of *P. aeruginosa* with the host.

Host immune recognition of $3OC_{12}HSL$

$3OC_{12}HSL$ is secreted in considerable amounts by *P. aeruginosa* during growth and is therefore readily detected by the host. Several host cell-types, including macrophages and epithelial cells, have been shown to respond to synthetic $3OC_{12}HSL$ resulting in both a pro- and anti-inflammatory immune-modulatory response (15). For example, detection of $3OC_{12}HSL$ by corneal epithelial cells of the eye results in production of the macrophage attractant cytokine IL-6, creating a strong pro-inflammatory response (Fig. 2) (18). Although intercepting and deciphering microbial communication by epithelial and immune cells could be beneficial for the host, it is important to note that activation of the host immune system could also be beneficial for the pathogen, as over-stimulation of the inflammatory process results in extensive tissue damage. Moreover, $3OC_{12}HSL$ selectively diminishes the regulation of NF-κB signaling and attenuates TLR4-dependent innate immune responses, which potentially promote infection-persistence particularly in cystic fibrosis patients (11). The production of $3OC_{12}HSL$ by *P. aeruginosa* also suppresses the activation of immune cells and induces apoptosis in macrophages, thereby compromising host immune defenses.

Interspecies sensing: detection, hiding and early warning: *P. aeruginosa – S. aureus*

Similar to the host immune cells, competing bacterial species have developed ways to detect *P. aeruginosa* through secreted $3OC_{12}HSL$. Specifically, the bacterial pathogen *S. aureus* has been shown to respond to the presence of *P. aeruginosa* (13). An important staphylococcal surface protein, protein A, involved in *S. aureus* defense against the host immune system is up-regulated in response to $3OC_{12}HSL$ (Fig. 2). Binding of the Fc receptor on immunoglobulin G by Protein A prevents recognition of *S. aureus* by macrophages and neutrophils. This could represent a mechanism by which *S. aureus* prepares itself for an attack of the host immune system.

$3OC_{12}HSL$ induces down-regulation of the *sarA* and *agr* genes and consequently, several virulence factors such as hemolysin, exotoxin, fibronectin-binding protein and factors related to biofilm formation are down-regulated in *S.*

aureus in response to *P. aeruginosa* presence. This response of *S. aureus* to $3OC_{12}HSL$ is specific, as no response was observed for $3OC_4HSL$, nor for unsubstituted AHL's: $C_{12}HSL$ (13). Because there is also evidence for binding of $3OC_{12}HSL$ to a specific receptor in *S. aureus* it is conceivable that *S. aureus* sensing of *P. aeruginosa*, *via* detection of $3OC_{12}HSL$, could be interpreted as a sophisticated early warning for *S. aureus* of the presence of a competitor as well as an onset of the host immune response.

Inter-kingdom sensing: *P. aeruginosa – C. albicans*

C. albicans is a dimorphic fungus able to switch morphology between the yeast and hyphal forms (14), property crucial to its pathogenesis (Fig. 2). *P. aeruginosa* adheres to *C. albicans* hyphae but not to the yeast morphology, making only the hyphal morphology susceptible to killing by *P. aeruginosa* (2, 9). The bacterial factors involved in adhesion to hyphal cells such as the chitin-binding protein (CbpD) are under QS-regulatory control (Ovchinnikova *et al.* unpublished data). *Pseudomonas* $3OC_{12}HSL$ not only regulates the adhesion capabilities to *C. albicans* hyphae but also modulates *C. albicans* morphological switch by preventing the yeast-to-hypha transition (10) and *C. albicans* interprets $3OC_{12}HSL$ as a warning signal.

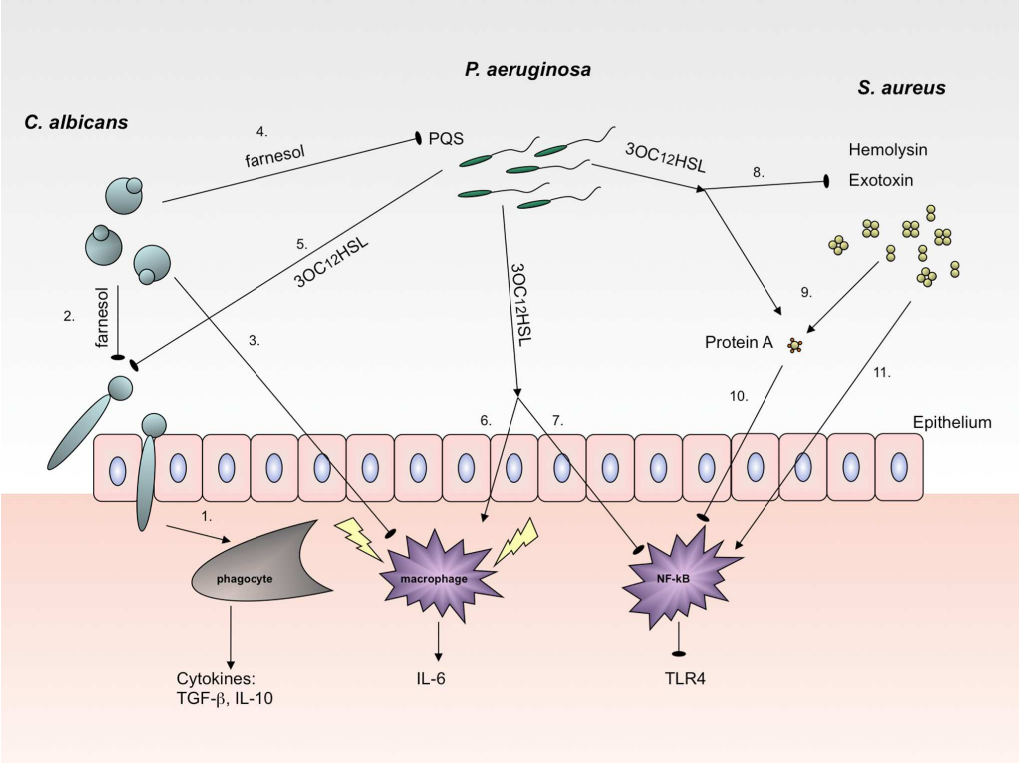

Fig. 2. Effect of $3OC_{12}HSL$ on *P. aeruginosa*, *S. aureus* and *C. albicans* that are relevant to immune recognition. Lines ending in arrows indicate induction and lines ending in circles indicate inhibition of the indicated process. *C. albicans*, in the hyphal morphology induces phagotycosis during inflammation (1.). Virulent hyphae can be inhibited and transformed to yeast cells by farnesol, the quorum sensing molecule secreted by *C. albicans* (2.). *Candida* yeast cells prevent macrophages induction (3.) and render *C. albicans* invisible for the immune system. *C. albicans* coexisting with *P. aeruginosa* exerts double-sided reaction; farnesol inhibits *Pseudomonas* quinolone signal production (4.), whereas $3OC_{12}HSL$ secreted by *P. aeruginosa* prevents *C. albicans* filamentation without changing the growth rate (5.). $3OC_{12}HSL$ sensed by the host induces a pro-inflammatory response by activation of macrophages (6.) but it can also give an anti-inflammatory reaction by selectively diminishing the regulation of NF-κB signaling and attenuating TLR4-dependent innate immune responses (7.). *P. aeruginosa* $3OC_{12}HSL$ influence *S. aureus* by inhibiting the growth, hemolysin and exotoxin production (8.) but inducing protein A expression (9.) which prevents recognition of *S. aureus* by macrophages and neutrophils (10.). *S. aureus* detected by the immune system trigger macrophages signaling pathways (11.).

Interfering with communication as potential therapeutic strategies

In light of the importance of information exchange, it is not surprising that different systems have developed that interfere with the successful exchange of information. Several mechanisms have been described in the literature, ranging from enzymatic degradation of the signaling molecule to competitive inhibition of receptor binding.

Enzymatic interference. Many bacteria possess genes that encode lactonases or acylases (1). These enzymes can deactivate $3OC_{12}HSL$ and in turn interfere with communication. In fungi, AHL hydrolyzing activity has been observed in *Ascomycetes* and *Basidiomycetes* (16). Although only C_6HSL and $3OC_6HSL$ have been shown to be hydrolyzed by these fungi, this example illustrates that fungi have developed systems to interfere with bacterial QS-mediated communication. Similarly, humans have also developed the ability to hydrolyze AHLs *via* a class of enzymes called paraoxonases (commonly referred to as PONs) (1). In a *Drosophila* infection model it was shown that human PONs are protective against *P. aeruginosa* infections.

Non-enzymatic interference. C. albicans secretes farnesol, a QS molecule similar in structure to $3OC_{12}HSL$, which at low cell density inhibits *Pseudomonas* quinolone signal (PQS) production (3) required for the expression of several virulence factors (Fig. 2) (6). At higher concentration, this molecule can suppress the effect of farnesol on PqsR activity (3). Recently, it was reported that farnesol has a stimulating effect on PQS production in a *P. aeruginosa lasR* mutant, indicating that there is a specific target for that interaction (4) . *P. aeruginosa lasR* deficient mutants arise frequently during chronic infection likely due to selective advantages in growth, death and lysis over wild-type cells (8) what suggests that $3OC_{12}HSL$ might be dispensable for *P. aeruginosa* virulence. This phenomenon may also reflect the negative effects of the host response to $3OC_{12}HSL$ on *P. aeruginosa* survival.

In summary, in addition to playing a pivotal role in virulence the secreted *P. aeruginosa* QS molecule $3OC_{12}HSL$ modulates the host immune-response to *P. aeruginosa* infection and influences the virulence of other opportunistic pathogens. In conclusion, the identification of quorum sensing as a microbial

strategy to control virulence by means of extracellular signal molecules has identified these molecules as potential drug targets for blocking pathogenicity.

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1.2. AIM AND SCOPE OF THIS THESIS

In light of the complicated interactions between bacteria and eukaryotes we hypothesized that specialized systems have developed to mediate interspecies communication. Therefore, the aim of this thesis was two-fold: 1) to identify additional, non-HSL bacterial quorum sensing molecules that affect *Candida albicans* biology and 2) to develop a new approach based on biomimetic molecules, to demonstrate the presence and identify specific receptors for bacterial quorum sensing molecules.

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Chapter 2

A rapid screening method for affectors of growth and germination in *Candida albicans* using a real-time PCR thermocycler

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ABSTRACT

Candida albicans is a polymorphic opportunistic pathogen. The switch from the yeast to hypha morphology is involved in virulence and biofilm formation. For this reason, compounds that affect the yeast to hypha transition have traditionally been subject of many studies. Traditional microscopy-based analysis of compounds is elaborate and testing of larger numbers of compounds that affect yeast to hypha transition would benefit from a fast and efficient method for activity screening. In this study we propose a rapid screening method using a real time PCR thermocycler in combination with *C. albicans* GFP reporter strains. Using P_{ACT1}-GFP and P_{HWP1}-GFP as reporters for growth and hypha formation, respectively, we show that for a wide range of known compounds the effect on growth and hypha formation can be assessed in a quantitative fashion within 3 h after inoculation. Importantly, not only repression of hypha formation, but also enhancement of hypha formation can be assessed as demonstrated with the quorum sensing molecule tyrosol.

INTRODUCTION

Candida albicans is a polymorphic, opportunistic human pathogen that can cause infections in immunocompromised individuals. Candidaemia, especially as nosocomial infection, has significant mortality rates despite the availability of antifungal treatments (31). The yeast to hypha switching is important in the virulence process (35) and biofilm formation (20, 29). For this reason, compounds that affect the yeast to hypha transition have traditionally been subject of many studies.

The yeast to hypha transition can be quantified using standard microscopy-based approaches; however these tend to be subject to observer-bias. Alternatively, observer-independent approaches such as gene-expression studies have been used. Several techniques are currently available for studying hypha-related gene expression; Northern blotting, DNA microarray, qPCR and more. While these techniques all have their own advantages, most are very elaborate and do not allow real-time analysis of gene expression. Green fluorescent protein (GFP) has been widely used as a quantitative reporter of gene expression in prokaryotes as well as eukaryotes (6). Since codon optimized GFP became available for *C. albicans* (7), GFP has been used as reporter for real-time gene expression within individual cells both *in vitro* and *in vivo* models (2, 3, 6, 9, 33).

Actin (encoded by *ACT1*) is constitutively expressed in all morphologies and commonly used as reference gene in real-time RT-PCR analyses of gene expression. In contrast, Hyphal Wall Protein 1 (encoded by *HWP1*) is an abundant protein that is only present on hyphal cell surfaces but it is neither expressed in the yeast nor in the pseudohyphal morphology (35). Importantly, *HWP1* expression is regulated on the transcriptional level (35). The promoter of *HWP1* has been used to create GFP reporter constructs as a powerful tool to follow and visualize hyphae formation while investigating mechanisms that control morphology and pathogenicity (18, 23, 34).

The aim of the present study was to determine GFP expression in an iCycler and use this as a sensitive, rapid, high-throughput and observer-independent approach to analyze compounds that affect growth and hypha formation. For this purpose we used a set of reporter strains containing fusions of

GFP with the promoters of *ACT1* (2) or *HWP1* (34) to evaluate the effect of known inhibitors and inducers of *C. albicans* growth and germination.

MATERIALS AND METHODS

Growth conditions

C. albicans was plated onto yeast nitrogen base pH 7.0 (YNB; BD Becton, Dickinson and Company, Sparks) supplemented with 0.5% glucose and 1.5% (w/v) agar from a 15% (v/v) glycerol stock maintained at -80°C. Cultures were incubated for 48 h at 30°C and plates were stored at 4°C never more than 1 week. For precultures, 5 ml YNB broth was inoculated and incubated overnight at 30°C while shaking at 150 rpm.

Strains used in that study

C. albicans SC5314, (13) and CAI4-P *ura3/URA3* (kind gift from Dr B. Distel) were used as non-fluorescent controls. HB12 (provided by Dr P. Sundstrom) is a CAI-4 derived strain expressing *GFP* under control of the *HWP1* promoter and has been described previously (34). P_{ACT1}-*GFP* was a kind gift from Dr C. Barelle (2) and was introduced into CAI4 (*ura3/ura3*) using electroporation (19). The pattern of *GFP*-expression in strains carrying P_{ACT1}-GFP and P_{HWP1}-GFP was verified using fluorescence microscopy (Leica DM4000B). Cells were observed with a 40x objective using a filter sets for GFP. Cells carrying P_{HWP1}-*GFP* showed *GFP*-expression exclusively in hyphae (Fig. 1A and B). In contrast, cells carrying P_{ACT1}-*GFP* showed similar level of fluorescence, irrespective of growth morphology (Fig. 1C and D). The yeast to hypha transition for both GFP expressing strains was determined to be comparable to the SC5314 and CAI4-P strain based on microscopic quantification of the percentage of yeast cells that form germ tubes under hyphae inducing condition (not shown).

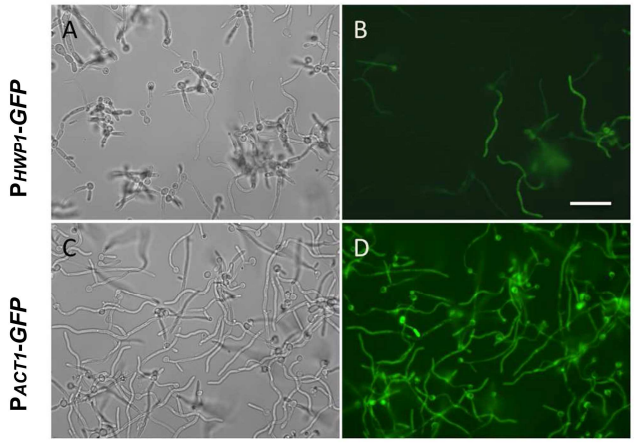

Fig. 1. Light and fluorescence microscopic images of *C. albicans* grown under hyphal-inducing condition. P_{HWP1}-*GFP* strain on phase contrast (A) with representation of hyphae, pseudohyphae and yeasts, whereas when using fluorescence microscopy (B), only true hyphae are visualized. In contrast, *C. albicans* P_{ACT1}-*GFP*, visualized with phase contrast (C) and fluorescence microscopy (D) both show all cell morphologies. Bar indicates 40 μm for all images.

Growth in iCycler

Cells from a preculture were harvested by centrifugation 7000 x *g* for 7 min, washed once with sterile phosphate buffered saline (PBS; 10 mM potassium phosphate, 150 mM NaCl, pH 7) and resuspended in YNB medium to an optical density measured at 600nm (OD_{600}) of 1. Serial dilutions in YNB were made in sterile Eppendorf tubes to obtain cell suspensions of the final OD_{600} used in experiments. Duplicate cells suspensions were placed in PCR tubes to a final volume of 100 μl. For testing the auto-fluorescence of compounds, serial dilutions of the test-compounds were made containing medium without cells. MyiQ2 two-color real-time PCR detection system, iCycler thermal platform (Bio-rad) was programmed to maintain constant temperature at 37°C. The fluorescence intensity was measured at time 0 and every 15 min for a total of 4.5 to 6 h.

Data analysis

The iQ™5 optical system software, version 2.1, used to control the MyiQ2 system, was set to analysis mode with subtracted background. Raw data were copied to an Excel file and the following calculations were made: baseline values at time 0 were

subtracted from the values at all subsequent time points, and the averages and standard deviations were calculated for duplicate samples. Additionally, a line chart was created for fluorescence intensity (AU) versus time (h) and depending on the obtained pattern of fluorescence increase for a specific period of time, that time period was isolated for further analysis; this involved fitting a linear trend line and slope calculations. Where applicable, auto-fluorescence of test-compounds was corrected for by subtracting the fluorescence intensity without cells (auto-fluorescence) for each individual concentration.

Germ tubes assay

As a measure of filamentation, a germ tubes (GT) assay was performed in 12-well tissue culture polystyrene plates as described before (15, 18), with minor modifications. Briefly, each well of a 12-well plate was filled with 1 ml of YNB and inoculated with *C. albicans* strains to a final OD_{600} of 0.1 (corresponding to approximately $1x10^6$ cells/ml for these strains). When appropriate, synthetic compounds were added to defined concentration. The plates were incubated for 3.5 h at 37°C while shaking at 80 rpm. The morphological state of *C. albicans* was analyzed by microscopy using a 20 x objective on an inverted microscope (Olympus, Tokyo, Japan).

Fluorescence imaging

The fluorescence intensity at the final time point was validated using a bio-optical imaging system (IVIS Lumina II, Caliper LifeScience, Hopkinton, MA, USA). The samples were placed on the sample stage with a field of view of 7.5 x 7.5 cm. The excitation wavelength for detection of GFP was 465 nm, while a broad-band emission filter was used allowing fluorescence emission to be measured from 515 - 575 nm. The exposure time was set to 10 s. Fluorescence images were displayed on a pseudocolor scale that was overlaid on a gray-scale image. Average fluorescence radiances, R ($p/s/cm^2/sr$) over a user-defined region of interest (ROI) were determined for each image with the Living Image software package 3.1 (Caliper LifeScience, Hopkinton, MA, USA) which transforms electron counts on the CCD camera to an average fluorescence radiance, taking into account the current optical

parameters (area of the ROI, magnification, binning, diaphragm, exposure time and light collecting ability of the camera as calibrated with standard light sources).

Test-compounds

Farnesol (Sigma-Aldrich) was prepared as a 100 mM stock dissolved in methanol and kept at -20°C. *N*-(3-oxododecanoyl) homoserine lactone ($3OC_{12}HSL$) was a kind gift of Dr Meijler, (Department of Chemistry, Ben Gurion University of the Negev, Israel) and was dissolved in DMSO to a stock concentration of 25 mM and was stored at -20°C. Fluconazole (Sigma-Aldrich) was dissolved in DMSO (40 mM stock solution) and was stored at -20°C. Tyrosol (Fluka) was prepared as 100 mM stock in sterile demineralized water and kept at 4°C for a maximum of 1 week. Fetal bovine serum (Sigma-Aldrich) was stored at -20°C in aliquots. N-acetyl-D-glucosamine (GlcNAc) (Sigma-Aldrich) was dissolved to a 0.4 M stock in demineralized water and was stored at -20°C. Depending of the solvent, equal amounts of DMSO or methanol was used in controls.

RESULTS

Set-up of the assay

To find the optimal condition for performing the assay, two different media was compared. Tryptic soy broth (TSB) showed high levels of auto-fluorescence and a relatively low % of germinating yeast cells (data not shown). In contrast YNB did not show significant auto-fluorescence and high percentage of germinating yeast cells. Therefore YNB was selected for all further experiments.

As a non-*GFP*-expressing control, *C. albicans* CAI4-P and SC5314 were grown in the iCycler, but no fluorescence increase in time was observed (data not shown). In contrast, *C. albicans* carrying the P_{ACT1}-*GFP* fusion showed immediate increasing fluorescence intensity in time for OD_{600} equal to 1, 0.5 and 0.25 (Fig. 2A). The strongest increase of fluorescence signal over time was detected at the highest inoculation density (Fig. 2B). In order to allow analysis of both induction and inhibition of growth, for all further experiments with this strain OD_{600} equal 0.5 was used.

For *C. albicans* carrying the P_{HWP1}-*GFP* fusion a lag-phase of approximately 1 h before a detectable increase in fluorescence in time was observed (Fig. 2C). In contrast to the curves obtained for *C. albicans* carrying the P_{ACT1}-*GFP* fusion, here the graphs increased linearly over almost the complete assay period. The strongest increase of fluorescence signal over time was detected in OD_{600} equal 0.06 (Fig. 2D) and, in contrast to *C. albicans* carrying the P_{ACT1}-*GFP* fusion, not at the highest inoculation density. This illustrates the inoculum size effect first described by Odds in 1978 (26). At an inoculation density above 0.5 x 10^6 cells/ml, inhibition of hypha formation occurs. To allow detection of decreasing and increasing rate of fluorescence in time, an inoculation density OD_{600} equal 0.03 was selected.

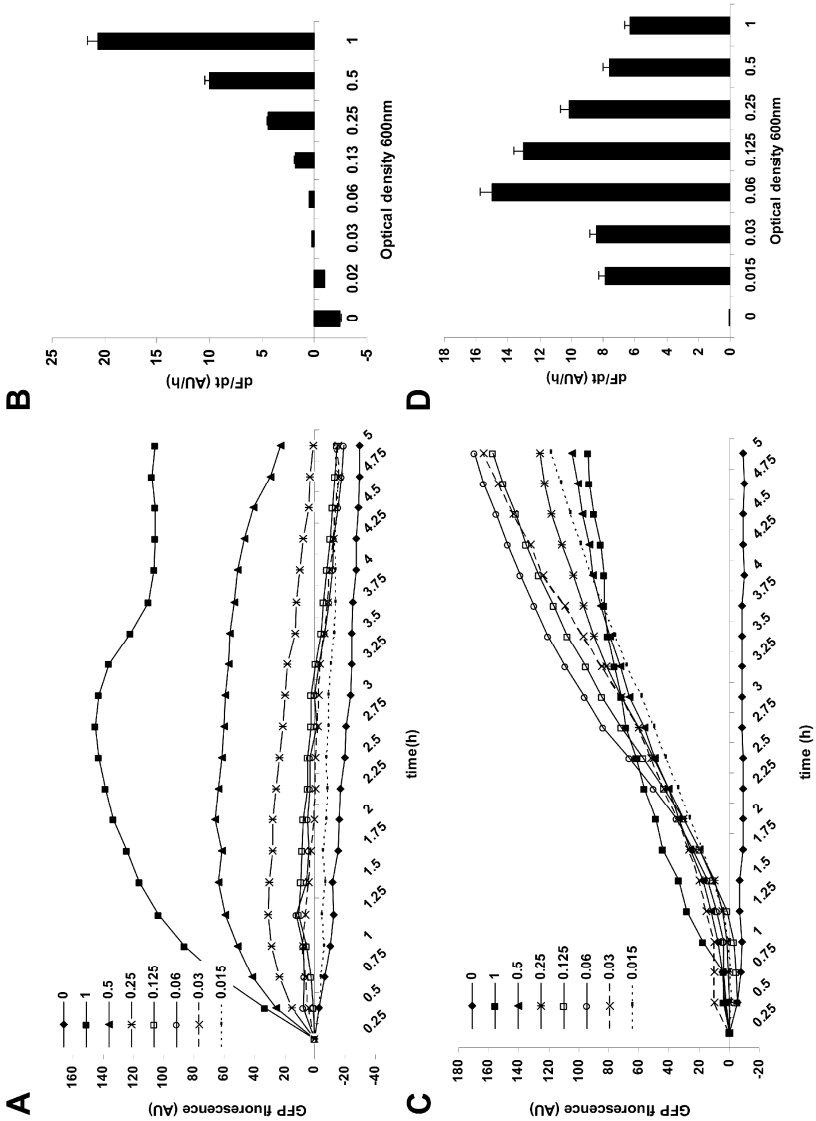

Fig. 2. Expression of GFP in time is dependent on inoculation density. *C. albicans* P_{ACT1}*-GFP* (A) and (B); P_{HWP1}*-GFP* (C) and (D) were inoculated to different densities (OD_{600}). The line charts (A) and (C) represent fluorescence intensity over time as measured using an iCycler. Subsequent analysis of the maximum rate of fluorescence intensity increase (dF/dt) at different inoculation densities illustrates a different behavior for *C. albicans* P_{ACT1}*-GFP* (B) when compared with *C. albicans* P_{HWP1}*-GFP* (D).

Validation of the assay

In order to validate the fluorescence levels as determined using the iCycler, fluorescence levels were also determined using the IVIS. PCR tubes with *C. albicans* carrying P_{ACT1}-*GFP* grown with fluconazole were imaged on IVIS Lumina II (Fig. 3A). The obtained fluorescence was comparable with those obtained using the iCycler (Fig. 3B).

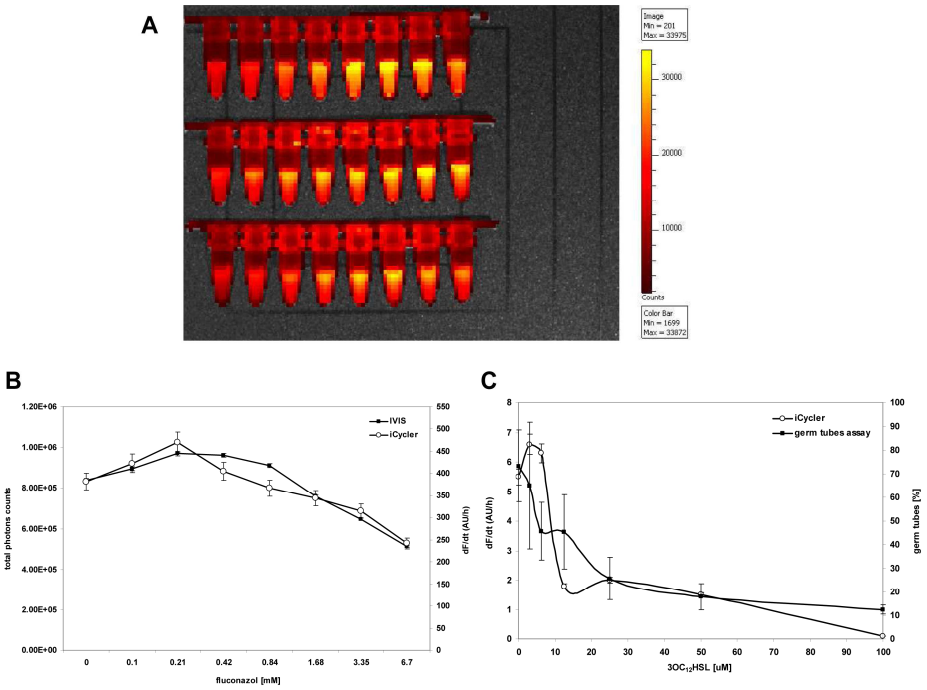

Fig. 3. (A) Fluorescence image taken using an IVIS Lumina II. Row 1 represents samples of *C. albicans* carrying the P_{ACT1}-*GFP* fusion with an inoculation density of OD_{600}=0.5, row 2 OD_{600}=0.06 and row 3 OD_{600}=0.03. A 2-fold serial dilution of fluconazole was prepared starting at 6.7 mM (left). A no-fluconazole control is included (right). (B) Comparison of the GFP-derived fluorescence signal obtained with an iCycler and IVIS from the same cultures grown for 5 h with fluconazole showing a good correlation between both methods. (C) Validation of HWP1-controlled GFP expression with GT formation. The GFP-derived fluorescence signal obtained from an iCycler for *C. albicans* P_{HWP1}-*GFP* strain was correlated with the % germ tubes for SC5314 in response to increasing concentrations of the hypha formation inhibitor $3OC_{12}HSL$ (in μM).

To further validate the iCycler based assay, we determined the correlation between P_{HWP1}-dictated *GFP* expression and traditional germ-tube quantification. Using $3OC_{12}HSL$ to inhibit hypheanation of SC5314 and *C. albicans* carrying the P_{HWP1}-

GFP, a good correlation was observed between fluorescence-detection in the ICycler and % GT formation using microscopic analysis (Fig. 3C).

Screening of compounds that inhibit growth

Fluconazole is a commonly used antifungal that inhibits growth (30). *C. albicans* carrying the P_{ACT1}-*GFP* fusion was grown for 5 h in iCycler with addition of a range of fluconazole concentrations as a growth inhibitor. Up to 0.4 mM there was no difference in rate of fluorescence intensity increase observed. At 0.8 to 3.3 mM there was a striking acceleration of the rate of fluorescence intensity increase (Fig. 4A, white bars). This is possibly due to induction of the general stress response. This is also observed for the strain carrying the P_{HWP1}-*GFP* fusion, be it at lower fluconazole concentrations (Fig. 4A, grey bars). At 1.6 mM fluconazole and higher there was no increase in fluorescence intensity observed. This data indicates that hypha formation is inhibited by fluconazole at lower concentrations than growth as reported previously (36).

Screening of compounds that affect hypha formation

Inhibitors. Farnesol, the signaling molecule secreted by *C. albicans* responsible for the observed inoculum size effect inhibits hyphae formation (17). *C. albicans* P_{HWP1}-*GFP* grown with farnesol showed a concentration dependent decrease of *GFP*-expression starting at 25 μM with no *GFP*-expression being detected at 200 μM (Fig. 4B). These concentrations have been found by others to be produced by *C. albicans* (17, 37) and inhibit hyphaenation (8, 22, 28). In contrast, *C. albicans* P_{ACT1}-*GFP* grown in the same conditions did not show an inhibition of *GFP*-expression compared with the control (data not shown). This seems in contradiction with a previous report that showed farnesol induced apoptosis and concentrations of 40 μM and above (32). However, it should be noted that in the current assay only short incubation times are being used, while induction of apoptosis is a time-dependent, slow process that might not be detectable using this approach.

$3OC_{12}HSL$ is a quorum sensing molecule produced by *Pseudomonas aeruginosa* (27). $3OC_{12}HSL$ is sensed by *C. albicans* and has a suppressing effect on

C. albicans hyphaenation (15). *C. albicans* P_{HWP1}-*GFP* grown with $3OC_{12}HSL$ showed a decrease in GFP fluorescence starting at 12.5 μM (Fig. 4C). At 100 μM there was no detectable increase in fluorescence.

Inducers. Serum, one of the best-known inducers of hyphaenation, was added to *C. albicans* P_{HWP1}-*GFP* up to 2%. Higher amounts of serum could not be used due to increased background fluorescence of the serum, however serum up to 2% has been used in previous reports to induce hypha formation (12). An increase of fluorescence in time was observed especially for 1 and 2 % serum (Fig. 4D). Similarly, for the amino sugar, N-acetyl-D-glucosamine, a potent inducer of hyphae growth (4), a 2-fold induction of *GFP*-expression was observed at a 4 mM concentration (data not shown). This is in line with previously published concentrations used for hypha induction (21).

Tyrosol has been identified as a second quorum sensing molecule produced by *C. albicans*, which, in contrast to farnesol, stimulates germ tubes formation (5). This inducing effect was observed in the early lag phase, and to our knowledge, no reports have corroborated the initial finding. *C. albicans* P_{HWP1}-*GFP* grown with tyrosol revealed an increase of fluorescence signal in the very early stage of growth (between 0.25 and 0.5 h), for two concentrations: 0.06 and 0.125 mM (Fig. 4E). Although initially judge as an artifact, this result was highly reproducible, both in timing and in extent of *GFP* induction (Fig. 4F). It should be noted that the effect disappeared within 1 h after inoculation, which could explain the elusive nature of this observation.

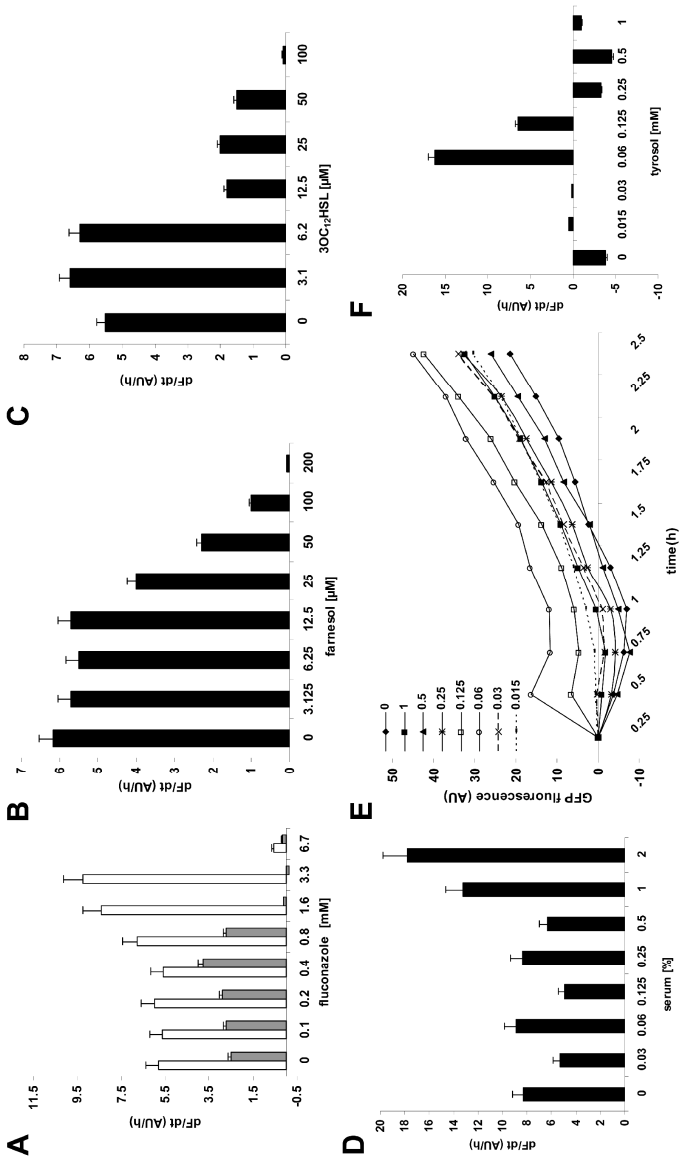

Fig. 4. Panel A: The maximum rate of fluorescence intensity increase (dF/dt) in *C. albicans* P_{ACT1}-*GFP* (white bars) and P_{HWP1}-*GFP* (grey bars) in the presence of increasing concentrations of fluconazole. Panel B and C: Effect of inhibitors of germination on the maximum rate of fluorescence increase (dF/dt) measured using the iCycler. *C. albicans* P_{HWP1}-*GFP* was grown with serial dilutions of (B) farnesol, (C) $3OC_{12}HSL$. Panel D-F: Effect of inducers of germination on the maximum rate of fluorescence increase (dF/dt). *C. albicans* P_{HWP1}-*GFP* was grown with serial dilutions of (D) serum and (E, F) tyrosol and the maximum rate of fluorescence increase was calculated. (E) shows the increase of GFP-derived fluorescence for the first 3 h, illustrating the very rapid and time dependent nature of the observed effect.

DISCUSSION

C. albicans constitutes a grave risk for various patient populations such as those who are immunocompromised and underweight newborns and these populations are growing. The yeast to hypha transition is an important virulence related process and affectors of this process have received a lot of scientific attention. The process of testing and evaluating compounds that inhibit the yeast to hypha transition is demanding, time consuming and expensive; therefore it is important to establish a fast, efficient and inexpensive assay for compound screening. GFP reporter systems are broadly applied in *C. albicans* as a method for assessing gene expression (2). In this study we have developed a fast, efficient and low-cost screening method for *C. albicans* based on promoter-*GFP* fusions. We have selected the promoters of *ACT1* and *HWP1* for analysis.

Actin is an essential protein with many biological functions like assembling cell cytoskeleton (14) expressed both in yeast and hypha at constitutive levels. It is commonly used as household gene in real-time RT-PCR analyses due to its stable expression (24, 32). Here we have used *C. albicans* carrying P_{ACT1}-*GFP* as a reporter for growth. Analysis of the linear part of the fluorescence curve (Fig. 2B) showed that the fluorescence-intensity increase was linearly related to the inoculation density. Growth in the presence of increasing concentrations of fluconazole (Fig. 4A) revealed an interesting effect, increased *ACT1* expression at sub-MIC concentrations. Two possible mechanisms could explain this observed increase in GFP fluorescence at sub-MIC fluconazole: 1) increased protein expression related to the general stress response. This is often seen in biofilm-related drug-susceptibility (25), but has to our knowledge never been reported for *C. albicans*. It is unlikely that this is specific induction of *ACT1* expression as this phenomenon was also observed for *C. albicans* carrying P_{HWP1}-*GFP*. 2) Alternatively, due to the general stress response the fluorescence intensity of GFP could increase. A similar process has been observed for bio-luminescence in *Staphylococcus aureus* (11). Whatever the mechanism, these results illustrate the importance of performing serial dilutions of the test compound after identification in an initial screen.

The ability to change its growth morphology from yeast to hypha is a well-studied virulence factor and has been the subject of many studies. *HWP1* is

expressed during hyphaenation (35), therefore we selected the promoter *GFP* fusion with this gene to screen for inhibitors and inducers of hypha formation, since *GFP*-expression correlated very well with hypha formation (Fig. 3C). Using this strain we visualized the inoculum size effect (Fig. 2D) as described by Odds in 1978 (26). Hypha formation is inhibited at high cell density and we could show that farnesol, the effector molecule of the inoculum size effect (16), readily inhibited hypha formation. Another inhibitor of hypha formation, $3OC_{12}HSL$ also suppresses *GFP* expression at low concentrations (12.5 μM) in contrast to the nearly 10-fold higher concentrations that have been reported before (10, 15). Using two well-know inducers of hypha formation, serum and N-acetyl-D-glucosamine, we showed that our assay also allows for analysis of compounds that accelerate hypha formation (Fig. 4D).

To prove the strength of this new approach we tested the ability of another *C. albicans* quorum sensing molecule, tyrosol, to accelerate hypha formation (1, 5). Tyrosol has been described to enhance hypha formation; however, this effect has remained elusive to other investigators, possibly due to its strong time-dependent nature. In our GFP-based assay we could show that addition of tyrosol does accelerate hypha formation, but this effect is very transient and is undetectable 1 h after of incubation.

Taken together, data provided in this study show that it is feasible to quantify a GFP derived fluorescence signal within living cells using a universally available device such as an iCycler. A fast induction or repression of GFP fluorescence reflects the ongoing process inside the cell allowing for signal quantification. The most commonly used assays to test inhibition of growth are related to MIC assessment that takes at least 24 h, if not 48 h, before results can be reliably read. The main benefit of our real time GFP expression analysis over the standard MIC assay is the short time it takes for the read-out. In the described new approach it is feasible to determine growth inhibition by new compounds within 1 h after inoculation.

Current systems for studying hyphal formation use microscopy and are observer dependent potentially resulting in artifacts due to subjective analysis (wishful thinking). This is especially relevant for microscopic analysis of hypha

formation, as hyphae end pseudohyphae are not easily distinguished by eye. Alternatively, mRNA expression followed by Northern blotting or qPCR analysis is performed. However, this approach is time consuming, expensive and requires relatively large numbers of cells meaning larger culture volumes at the expense of larger amounts of valuable test-compound. The advantage of our method is that it is observer independent, inexpensive and can be performed in 96 or 384 well plates to minimize compound usage. In combination with automation (robotics) it is suitable for increased screening throughput. An additional benefit to our approach is the frequent presence of an iCycler (or similar equipment) in molecular biology laboratories, which would make the purchase of a dedicated microtiter plate reader obsolete.

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Chapter 3

Streptococcus mutans competence stimulating peptide inhibits *Candida albicans* hypha formation

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ABSTRACT

The oral cavity is colonized by microorganisms growing in biofilms, in which interspecies interactions takes place. *Streptococcus mutans* grows in biofilms on enamel surfaces and is considered one of the main etiologic agents of human dental caries. *Candida albicans* is also commonly found in the human oral cavity where it interacts with *S. mutans*. *C. albicans* is a polymorphic fungus and the yeast-to-hypha transition is involved in virulence and biofilm formation. The aim of this study was to investigate inter-kingdom communication between *C. albicans* and *S. mutans*, based on production of secreted molecules. *S. mutans* UA159 inhibited *C. albicans* germ-tube (GT) formation in co-cultures, even when physically separated from *C. albicans*. Only *S. mutans* spent media collected in the early exponential phase (4 h-old cultures) inhibited GT formation of *C. albicans*. During this phase, *S. mutans* UA159 produces a quorum sensing molecule, competence stimulating peptide (CSP). The role of CSP in inhibiting GT formation was confirmed using synthetic CSP and a *comC* deletion strain of *S. mutans* UA159, which lacks the ability to produce CSP. Other *S. mutans* strains and other *Streptococcus spp*. also inhibited GT formation but to different extents, possibly reflecting differences in CSP amino acids sequences among *Streptococci spp*. or differences in CSP accumulation in the media. In conclusion, CSP, a *S. mutans* quorum sensing molecule secreted during the early stages of growth, inhibits the *C. albicans* morphological switch.

INTRODUCTION

The oral cavity is colonized by many different microbial species, where most reside in biofilms. Because of its multispecies nature, the oral microbial community is one of the best biofilm models for studying interspecies interactions (17). The Gram-positive bacterium *Streptococcus mutans* shows a high prevalence in dental biofilms and it is considered to be the major etiologic agent involved in human dental caries (21). The fungal species *Candida albicans* constitutes a minor part of the total microbial flora (19) and can be isolated as a commensal from the oral cavity of 50% to 60% of healthy adults (33). However, in immunocompromised individuals (for example due to HIV-infection or as a result of chemotherapy) and elderly patients, this fungus often leads to candidiasis (24). *C. albicans* is a polymorphic fungus that can exist in three morphotypes: budding yeast, pseudohypha and true hypha (5). The morphological switch from yeast to hyphal cells is important in many processes, such as virulence (22) and biofilm formation (10, 18) and therefore the subject of many studies.

Bacteria and yeasts are often found together *in vivo* and there is growing evidence that interspecies, and even interkingdom interactions occur within these populations (7). These interactions can be mediated through signaling molecules (40) as recently described for the interaction between *C. albicans* and *Pseudomonas aeruginosa*, an opportunistic bacterial pathogen (15). N-3-oxo-C12 homoserine lactone, a signaling molecule involved in bacterial quorum sensing, completely represses *C. albicans* hypha formation without altering growth rate. Although many Gram-negative bacteria produce homoserine lactones (HSL) with shorter acyl chains (e.g. C4-HSL), inhibition of *C. albicans* hypha formation is specifically caused by long-chained HSL molecules. In addition, related, non-HSL, molecules with an long acyl chains, such as dodecanol and farnesol, also inhibit hypha formation of *C. albicans* (8).

A recent report described the co-culturing of *C. albicans* and *S. mutans* in model oral biofilms on hydroxyapatite (26). It was shown that *S. mutans* increased growth of *C. albicans* by stimulating co-adhesion while simultaneously suppressing formation of hyphae. *S. mutans* is a Gram-positive bacterium and does not produce

HSL type molecules and the nature of the interaction with *C. albicans* is presently unknown. In this study, the interaction between *S. mutans* and *C. albicans* was investigated by studying the effect of secreted molecules of *S. mutans* on *C. albicans* hypha formation.

MATERIALS AND METHODS

Strains and growth conditions

All bacterial and fungal strains used in this study are shown in Table 1. *C. albicans* was plated onto yeast nitrogen base pH 7.0 (YNB; BD Becton, Dickinson and Company, Sparks) supplemented with 0.5% glucose and 1.5% (w/v) agar from a 15% (v/v) glycerol stock maintained at -80°C, and incubated 48 h at 30°C. *Streptococcus spp.* were cultured on blood agar except for the *S. mutans* UA159 *ΔcomC* mutant which was cultured on Brain–heart infusion (BHI; OXOID, Basingstoke, UK) agar plates supplemented with erythromycin (10 μg/ml). YNB broth was used to grow *C. albicans* overnight at 30°C while shaking at 120 rpm. Cultures of *Streptococcus spp.* were grown statically in BHI broth overnight in 5% CO_2 at 37°C, supplemented with erythromycin (10 μg/ml) when appropriate. To obtain spent medium samples, *Streptococcus spp.* were grown in 200 ml BHI and 10 ml of medium was taken after 4, 6, 8 and 24 h, centrifuged at 10,000 x *g* for 5 min, pH was checked and if lower than 7, was set to 7, filter sterilized and stored at -20°C. Co-cultures of *C. albicans* and *S. mutans* were grown in medium containing 70% YNB and 30% BHI (v/v). Where indicated, spent medium replaced the 30% fresh BHI fraction. Competence stimulating peptide (CSP) with an amino acid sequence of NH_2-SGSLSTFFRLFNRSFTQALGK-COOH (2), with a purity > 95%, was purchased from Ansynth Service B.V., Roosendaal, The Netherlands. A 12 mM stock of synthetic CSP was prepared by dissolving the peptide in demineralized water and stored at -20°C.

Strains	Description	Source or reference
C. albicans SC5314		(12)
C. albicans HB12		(31)
S. mutans ATCC25175		ATCC
S. mitis ATCC33399		ATCC
S. mutans NS	Clinical isolate	UMCG Groningen
S. salivarius HB		UMCG Groningen
S. sanguis ATCC10556		ATCC
S. sobrinus ATCC33478		ATCC
S. oralis J22		From Kolenbrander, NIH, Bethesda
S. gordonii 10558	Clinical isolate	ACTA Amsterdam
S. mutans UA159	Wild-type	(1)
S. mutans UA159 *ΔcomC*	Mutant lacking in CSP production	This study

TABLE 1. Strains used in this study.

Construction of the *S. mutans* UA159 *ΔcomC* strain

To delete the *comC* gene, we used a PCR ligation mutagenesis method (20): The flanking regions of the *comC* gene were amplified from *S. mutans* UA159 genomic DNA with PCR primer pairs *comC*uf/*comC*ur and *comC*df/*comC*dr (see Table 2 for details). The erythromycin resistance gene (*erm*^{r}) was amplified from an *ermAM* cassette using the primers *ermF*/*ermR*. These three PCR fragments were digested with the appropriate restriction enzymes and ligated. The ligation mix was used as a template for amplification of the knock-out construct using the primers *comC*uf/*comC*dr. The resulting PCR product was purified and transformed into *S. mutans* UA159. The *comC* knockout strain was obtained via double crossover,

resulting in replacement of major part of the chromosomal *comC* gene by *erm*^{r} gene. The insertion of the *erm*^{r} gene was verified by PCR. Lack of CSP production by the *comC* knockout strain was confirmed by its incapability of bacteriocin production in competition assays (36).

Primer name	Sequence (5'→ 3')
*comC*uf	GCTATCAGCTGCGCTGTT
*comC*ur (*Not*I)	**GCGGCCGC**ATTTTATATCTCCTTTTTTTGATTA
*comC*df (*Asc*I)	**GGCGCGCC**AAATAAGATAGGCTAACATT
*comC*dr	CATCAATTGCAGGATACC
ErmF (*Asc*I)	**GGCGCGCC**TCGTGCTGACTTGCACCATATC
ErmR (*Not*I)	**GCGGCCGC**TTACAAAAGCGACTCATAGAAT

TABLE 2. Primer sequences used for *S. mutans* UA159 *comC* construction. Sequences of primers are indicated from 5'-3' with the restriction sites, when applicable, indicated in bold.

Saliva preparation and coating

Freeze-dried saliva was prepared as described previously (35). Briefly, stimulated saliva (by chewing Parafilm) was collected from healthy volunteers on ice and centrifuged twice at 10,000 x *g* for 5 min at 10°C. Phenylmethylsulfonyl-fluoride was added to a final concentration of 1 mM to prevent protein degradation. Afterwards, the solution was again centrifuged at 10,000 x *g* for 5 min, dialyzed against demineralized water overnight at 4°C, and freeze dried before storage at -20°C. For experiments, lyophilized saliva was dissolved in adhesion buffer (1mM $CaCl_2$, 2 mM potassium phosphate, 50 mM KCl) at a concentration of 1.5 mg/ml. This solution was centrifuged at 10,000 x *g* for 5 min at 10°C, the supernatant was checked for the absence of bacteria by microscopy and subsequently used to coat 12-well tissue culture polystyrene plates (Costar®, Corning Inc.) overnight. Before for the start of experiments saliva was removed and the wells were washed once with adhesion buffer. All volunteers gave their informed consent to saliva donation

in accordance with the rules set out by the ethics committee at the University Medical Center Groningen.

Germ tubes assay

As a measure of filamentation a germ tubes (GT) assay was performed in 12-well tissue culture polystyrene plates as described before (15), with minor modifications. Saliva coated culture plates filled with 1 ml of mixed medium were inoculated with C. albicans SC5314 to a final OD_{600} of 0.1 (corresponding to 1x106 cells/ml). When appropriate, bacteria were added to 1x107 cells/ml. The plates were incubated for 3.5 h at 37oC while shaking at 80 rpm. The morphological state of C. albicans was analyzed by microscopy using a 20x objective lens on an inverted microscope (Olympus, Tokyo, Japan). For selected experiments, ThinCerts™ tissue culture polystyrene inserts (Greiner bio-one) with 0.4 μm pore size were used to physically separate yeasts from bacteria.

Fluorescent microscopy of GFP expression

C. albicans HB12 carrying green fluorescent protein (GFP) under control of the *HWP1* promoter (31) was grown with addition of 1 μM CSP in a GT assay. HB12 expresses GFP only in true hyphae, and not in pseudohyphae or yeast cells. Fluorescence microscopy was performed with a Leica DM4000B fluorescence microscope. Cells were observed with a 20x objective. For visualization of all cells, calcofluor white (Fluorescence Brightener 28, Sigma-Aldrich) staining was applied in a final concentration of 3.6 mM. Separate images were taken using filter sets for GFP and UV. Overlay images were created using the Leica Application Suite version 2.8.0.

Statistical analysis

Differences in % GT formation was tested using a Student's *t*-test with the threshold of $p<0.05$ for statistical significance.

RESULTS

Effect of saliva and *S. mutans* UA159 on *C. albicans* SC5314 GT formation

Upon induction of GT formation at 37°C, *C. albicans* showed a higher fraction of germinating cells in saliva coated wells (52%) compared to uncoated wells (20%) (Fig. 1). Co-culturing with *S. mutans* UA159 gave only 35% of all cells germinated. This inhibition of the fraction of germinating cells from 52% to 35% was not significant ($p=0.06$) in saliva-coated and uncoated wells (20% to 16%; $p=0.25$). When *C. albicans* and *S. mutans* UA159 were physically separated from each other by polystyrene inserts, GT formation was significantly inhibited in saliva-coated wells (from 52% to 17%; $p=0.007$) but not in uncoated wells (from 20% to 9%; $p=0.06$) indicating that physical contact is not involved in this inhibition of GT formation. The effect of *S. mutans* UA159 on *C. albicans* is best visualized after saliva-coating and hence these conditions were applied in all following experiments.

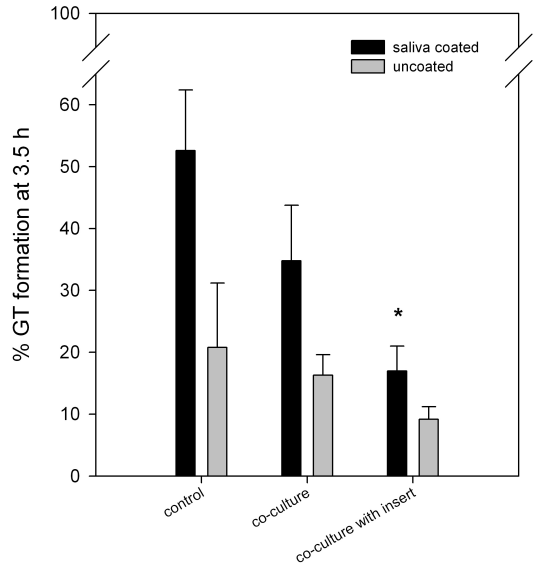

Fig. 1. Effect of different co-culturing techniques on GT formation of *C. albicans*. *C. albicans* was grown without (control) or in co-culture with *S. mutans* UA159, either without or with an insert physically separating both species. The morphology of *C. albicans* was determined after 3.5 h of growth. Wells were uncoated (grey bars) or coated with saliva (black bars). The results are averages of four independent experiments with at least 300 cells counted per sample for each experiment. * Indicates significant ($p<0.05$) difference compared to control.

Effect of spent medium of *S. mutans* on *C. albicans* GT formation

Spent medium obtained from *S. mutans* UA159 and *S. mutans ΔcomC* cultures at different phases during growth (4, 6, 8 and 24 h-old cultures) was tested for its ability to affect germination of *C. albicans*. Spent medium from 4 h-old culture of *S. mutans* UA159 inhibited *C. albicans* GT formation (from 50% to 8%), whereas spent medium of 6, 8 and 24 h-old cultures did not show any inhibition (Fig. 2A). CSP, a quorum-sensing molecule involved in stimulation of competence (27) encoded by *ComC* (14) is produced by *S. mutans* during the beginning of the exponential growth phase (25). Spent medium from *S. mutans ΔcomC* did not show any inhibition, whereas addition of 1μM CSP to 4, 6 and 8 h-old cultures inhibited GT to 4%, 38% and 31% respectively (Fig. 2A). It should be noted that the pH of the spent medium in the GT assay was always set to 7.

To chemically complement the *comC* mutation, synthetic CSP was added to 4 h-old spent medium of the *ΔcomC* strain at various concentrations. When 1 μM

CSP was added, the effect was very similar to 4 h-old wt spent medium showing GT formation of 8% (Fig. 2B). Together with reduction of CSP to 0.1 or 0.01 μM, the inhibitory effect was reduced (16% and 32% GT formation, respectively).

Effect of synthetic CSP on *C. albicans* GT formation

The effect of increasing concentrations of synthetic CSP, ranging from 0.1 to 5 μM is shown in Fig. 3A. Synthetic CSP at 0.1 μM significantly inhibited GT formation by 60% compared to the control. Using 1.0 μM CSP, inhibition of GT formation by 90% compared to the control was obtained, indicating that the inhibition of GT formation is dependent on the concentration of synthetic CSP added to the culture, however concentrations up to 5.0 μM did not enhance the effect. Synthetic CSP did not inhibit growth of *C. albicans* at the concentrations tested (data not shown). The effect of synthetic CSP on *C. albicans* GT formation was confirmed using *C. albicans* HB12, expressing GFP under control of the hyphae specific *HWP1* promoter. *HWP1* is expressed only in germinating cells and not in pseudohyphae or yeast cells. A decrease in the number of green fluorescent (germinating) cells and an increase in the number of pseudohyphae and yeast cells, compared to the control was observed in the presence of 1 μM CSP (Fig. 3 B, C).

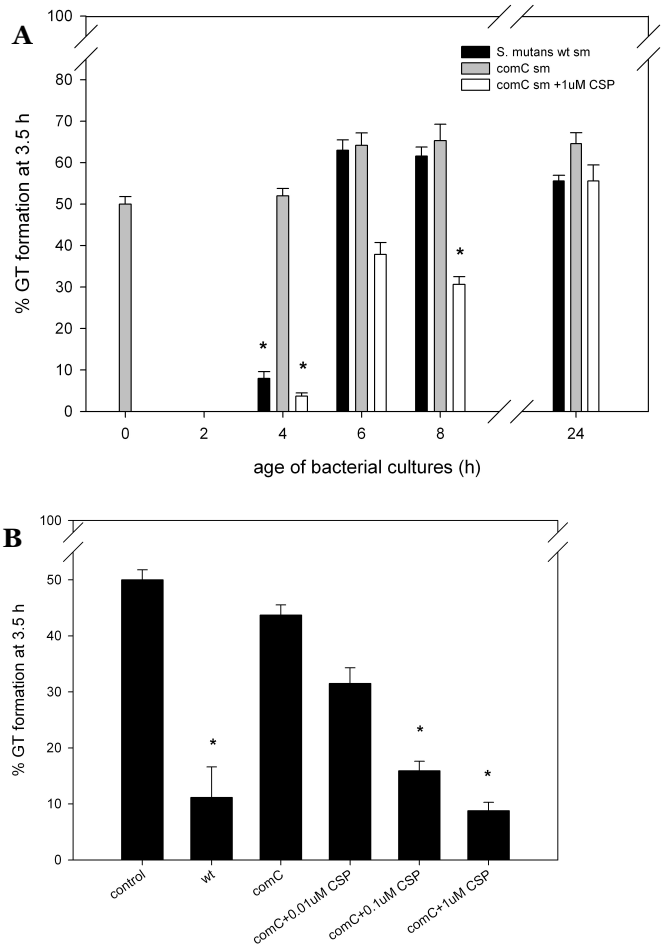

Fig. 2. Effect of spent medium from *S. mutans* UA159 and *S. mutans* UA159 *ΔcomC* cultures, from different growth phases on GT formation by *C. albicans* (Panel A). Spent medium of *S. mutans* UA159 (black bars) and *S. mutans* UA159 *ΔcomC* (grey bars), harvested at early exponential (4 h-old), mid exponential (6 h-old) early stationary (8 h-old) and late stationary phase show very different effects on GT formation. In addition, spent medium of *ΔcomC* (white bars) from all time points, was supplemented with 1μM CSP. Time point 0 depicts fresh medium (no bacterial growth) while 2 h-old medium was not tested. Effect of 4 h-old spent medium from *S. mutans ΔcomC* supplemented with various concentrations of synthetic CSP (Panel B). 1 μM CSP inhibited GT to similar level as the wild type spent medium. Decreased synthetic CSP concentration resulted in a dose-dependent behavior. The pH of all samples was set to 7 prior to the GT assay. The results are averages of two experiments with at least 300 cells counted per experiment. * Indicates significant ($p<0.05$) difference compared to control.

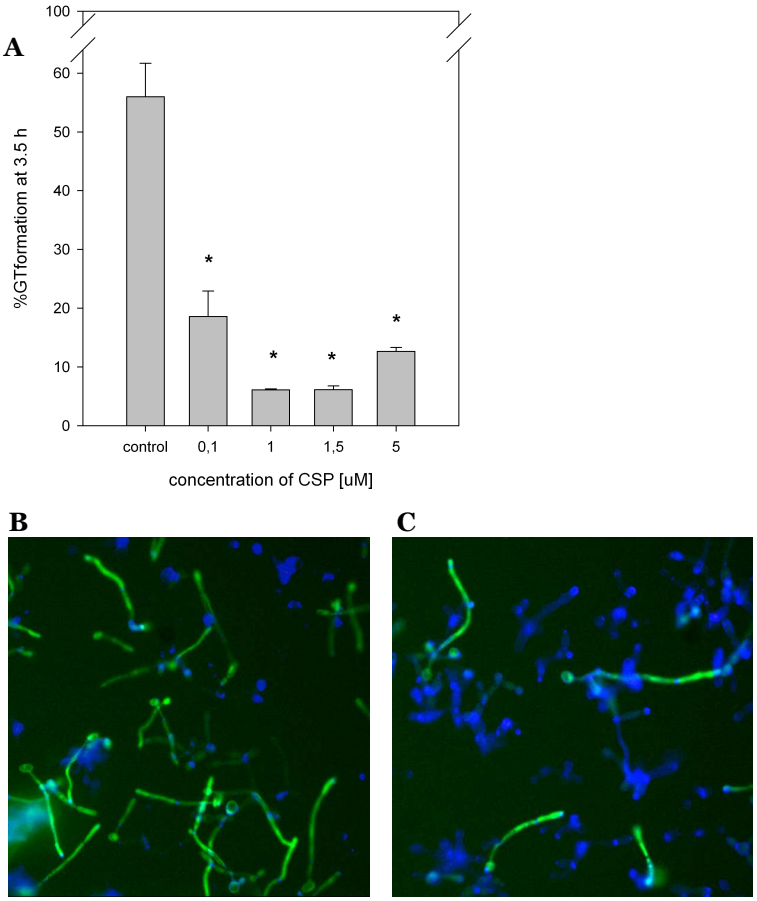

Fig. 3. Inhibition of GT formation by synthetic CSP is concentration dependent (panel A). The percentage GT formation decreases with increasing synthetic CSP concentration from 0.1 to 1.0 µM, concentrations up to 5.0 µM did not enhance the effect. The results are averages of two experiments with at least 300 cells counted per experiment. * Indicates significant ($p<0.05$) difference compared to control. Fluorescence microscopy of *C. albicans* HB12 carrying the *HWP1* promoter-GFP fusion grown without (panel B) or with 1 µM CSP (panel C). Germinating cells express *HWP1* (green) while calcofluor white stains the cell wall of all cells (blue) showing a simultaneous decrease of germinating cells and an increase of yeast cells and pseudohypha in the culture with 1 µM CSP compared to the control.

Effect of synthetic CSP on preformed GT

To investigate whether synthetic CSP can revert pre-existing hyphae back to yeast cells, *C. albicans* was grown under hyphae inducing conditions for 2 h, after which 1 μM of CSP was added followed by an additional culturing at hyphae inducing conditions for 2 h (Fig. 4). After 2 h of growth, 62% of all cells had germinated. Addition of 1 μM of synthetic CSP resulted in 5% GT, compared to 40% in the untreated control. Our results show that synthetic CSP not only inhibits germination, but may also stimulate the hypha-to-yeast transition.

Effect of spent medium from other *Streptococcus* strains and species on *C. albicans* GT formation

The influence of other *S. mutans* strains and other *Streptococcus spp.* on *C. albicans* GT formation is shown in Fig. 5. All 4 h-old spent media from *S. mutans* strains tested, as well as *S. gordonii* 10558, *S. oralis* J22, *S. salivarius* HB and *S. sobrinus* ATCC33478, showed GT formation of approximately 20-30%. Four hours spent medium received from *S. mitis* ATCC33399 and *S. sanguis* ATCC10556 showed GT formation of 74% and 45% respectively. None of the 24 h-old spent medium, corrected for pH, showed inhibition.

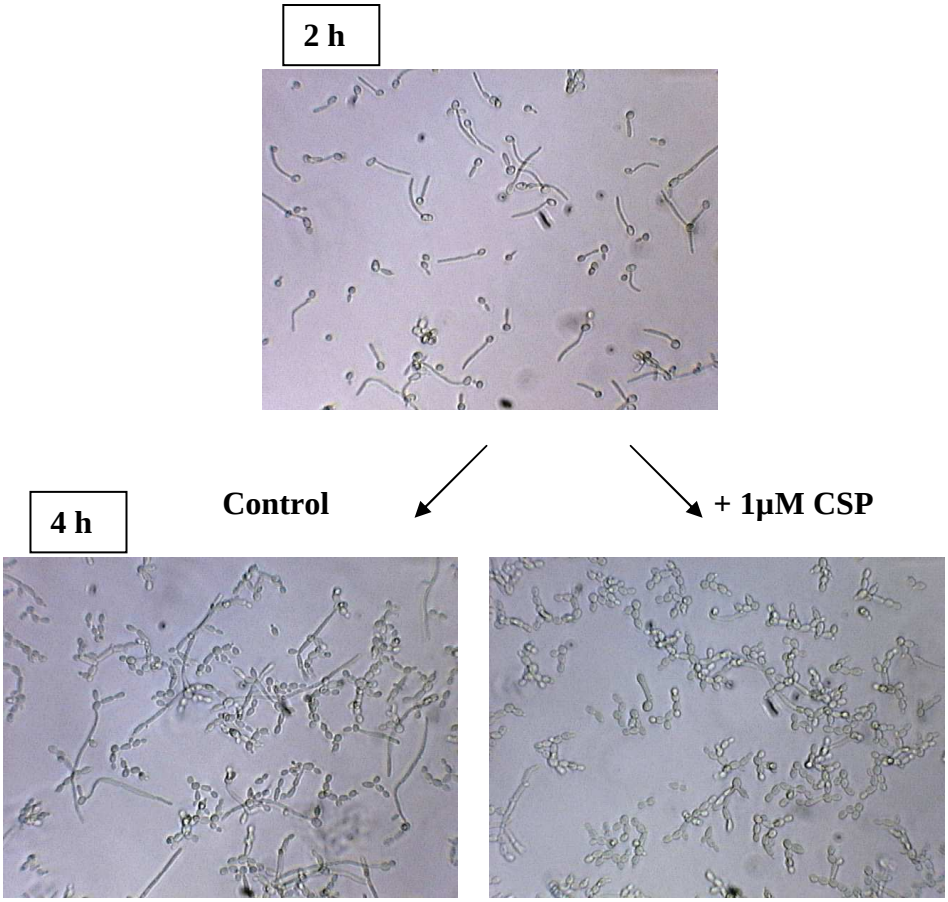

Fig. 4. Effect of synthetic CSP on germinating cells. The top panel shows GT formation after 2 h of incubation. The bottom right panel shows lack of germination 2 h after the addition of 1 µM synthetic CSP as compared to a control without synthetic CSP (bottom left panel).

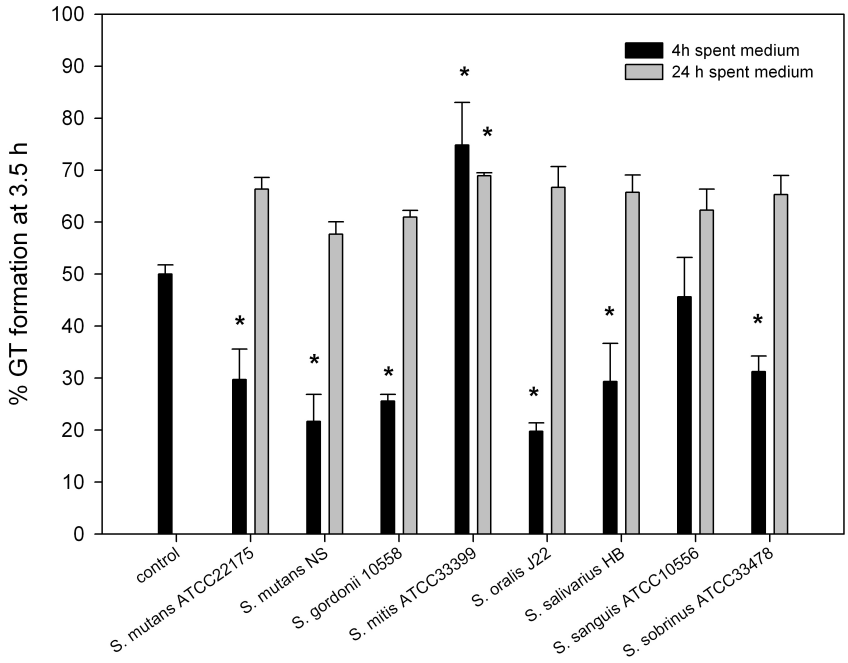

Fig. 5. Effect of spent medium of several *Streptococcus spp*. on GT formation of *C. albicans*. Spent medium of various *Streptococcus spp*. grown for 4 h (black bars) or 24 h (grey bars). The pH of all samples was set to 7 prior to the GT assay. The results are averages of two experiments with at least 300 cells counted per experiment. * Indicates significant ($p<0.05$) difference compared to control.

DISCUSSION

S. mutans can affect GT formation of *C. albicans* in co-cultures even if the bacteria and the fungi are physically separated. In addition, filter sterilized spent medium of *S. mutans* inhibited GT formation. These results indicate that *S. mutans* secretes one or more diffusible molecules that affect *C. albicans* hypha formation. Inhibition of GT formation occurs through several mechanisms, one of which involves the secreted quorum sensing molecule CSP. This is supported by several lines of evidence: *i*) a strong inhibitory effect of 4 h-old spent medium on GT formation (CSP is described to be produced specifically in the early exponential phase (25)). *ii*) synthetic CSP inhibits GT formation in a concentration dependent manner *iii*) a mutant of *S. mutans*, that is unable to produce CSP, showed reduced

inhibition of GT formation compared to the wild-type strain, but could be restored to wild-type levels of GT inhibition by the addition of synthetic CSP.

Saliva was present in all experiments to better resemble the conditions in the oral cavity, where saliva affects the behavior of microorganisms. In the present study, it was shown that saliva induces GT formation of *C. albicans*. CSP did not affect GT formation induced by serum (data not shown), which could indicate that saliva is a weaker inducer of hypha formation than serum. This is in line with a previous report showing that contact-stimulated hypha formation in the presence of serum was more pronounced than in the presence of saliva (23). Biofilms of *S. mutans* and *C. albicans*, grown under comparable conditions on hydroxyapatite, polymethylmetacrylate and soft denture liner discs, showed that the presence of *S. mutans* leads to suppression of hypha formation in *C. albicans* without affecting the extent of biofilm formation (26). The extent of *C. albicans* biofilm formation, when grown in 96-well tissue-culture polystyrene plates with saliva or serum coating, was not significantly affected by synthetic CSP (data not shown). A more detailed study on the effect of CSP and other secreted molecules of *S. mutans* are needed to obtain a deeper understanding of the mechanism governing this interaction in mixed-species biofilms.

Another quorum sensing molecule secreted by *Streptococcus spp.*, autoinducer 2 (AI-2) (39), has been implicated in interaction within mixed *C. albicans* biofilms. The presence of *S. gordonii* cells appeared to induce more extensive hypha formation, but not a *luxS* mutant (3). There was no significant effect of exogenous DPD (precursor to AI-2) on hypha formation. However, because many different bacterial species produce slightly different AI-2 derivatives (9), the role of AI-2 in *S. mutans* - *C. albicans* interaction should be carefully investigated in the future.

Maximal inhibition of GT formation (Fig. 3 A) was realized by the addition of 1 μM synthetic CSP. This CSP concentration is commonly used to induce competence and it has been speculated to be a physiologically relevant concentration (29, 30). Several attempts to determine CSP concentrations in the spent media used in the current study were unsuccessful. We also note that there are no reports in the literature on this, although many groups have studied CSP

related phenomena. In a paper where a reporter gene was used there is also no CSP concentration mentioned (32). Unfortunately there is only speculation on the physiological concentrations of CSP produced by *S. mutans* (28). Based on our chemical complementation of the *ΔcomC* strain, and a comparison between the response of *C. albicans* to spent medium and synthetic CSP lead us to predict that the natural concentration of CSP can reach the 1 μM order of magnitude. Synthetic CSP is not toxic to *C. albicans* as it does not inhibit growth nor does it induces expression of genes within the general stress response (11) such as *CTA1* and *HSP12* (data not shown).

The suppressing effect on *C. albicans* GT formation was only obtained with 4 h-old *S. mutans* UA159 spent medium, coinciding with maximal CSP production (25). The pH of the medium is one of the factors that is known to affect GT formation in *C. albicans* (4), and *S. mutans* is known to produce significant amounts of acids during growth (13). GT formation was significantly inhibited with spent medium from 8 and 24 h-old cultures of *S. mutans* UA159 and *ΔcomC* caused by acidification (pH 4.8, data not shown), whereas this inhibition disappeared when the spent medium was set to pH 7. However, after 4 h the pH of the spent medium had not yet changed and was still 7 (data not shown), therefore pH is not causing the inhibitory effect on GT formation. The richness of the BHI medium affects GT-formation; addition of 10% or more BHI in YNB significantly inhibited GT-formation (data not shown). Exhaustion of BHI in the spent medium experiments would therefore stimulate GT-formation. Both 6 h-old and 8 h-old spent medium of *S. mutans* UA159 and *ΔcomC* induced GT formation slightly by 15%. This effect could be explained by exhaustion of BHI. Alternatively, the effect could be explained by production of an inducer of GT formation in both strains, possibly AI-2 as reported for *S. gordonii* (3). In addition, induction of GT formation using 6 and 8 h-old spent medium illustrates the reduction of CSP production in *S. mutans* UA159 as described previously (34). Spent medium of 4 h-old cultures from *S. mutans* ATCC22175, *S. mutans* NS and several other streptococcal strains also inhibited GT formation be it to different extents. This could indicate some level of redundancy toward CSP sequences recognized by *C. albicans* as different streptococcal strains and species produce CSP with different

amino acid sequences (38). Interestingly, induction of GT was found for *S. mitis* ATCC33399 what could indicate the production of GT inducing factors. 24 h-old cultures of all species did not show inhibition of GT formation.

A number of C12 acyl chain containing molecules have been described that affect the morphological transition of *C. albicans*. Farnesol is produced and sensed by *C. albicans* (16) to levels as high as 58 μM (37). N-3-oxo-C12 HSL, produced by *P. aeruginosa* (15) and dodecanol (8) that mimics farnesol, were the first bacterial signaling molecules described to inhibit the yeast-to-hypha transition in *C. albicans*. Recently, cis-2-dodecenoic acid, (BDSF) produced by *Burkholderia cenacepacia* (6) was shown to inhibit the morphological transition at much lower concentrations than farnesol and N-3-oxo-C12 HSL. Because of the structural similarities, it is possibly that regulation of morphological transitions occurs through a shared pathway. CSP of *S. mutans* is a small, 22 amino acids containing peptide, structurally unrelated to the bacterial signaling molecules known to affect the *C. albicans* yeast-to-hypha transition. The discovery of *S. mutans* CSP as a signaling molecule involved in interactions with *C. albicans*, adds a structurally unrelated molecule to the list of known bacterial signaling molecules affecting *C. albicans*.

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Chapter 4

Aggregatibacter actinomycetemcomitans inhibition of *Candida albicans* biofilm formation depends on *luxS*

Manuscript under preparation

ABSTRACT

Aggregatibacter actinomycetemcomitans, a Gram-negative oral bacterium, produces a quorum-sensing molecule called autoinducer-2 (AI-2) synthesized by LuxS. AI-2 plays an important role in expression of virulence traits as well as ecological role in regulating the intra- and the inter-species oral microflora. *Candida albicans* is a polymorphic fungus that normally resides in the oral cavity as a commensal. Both of these organisms are also opportunistic pathogens of the human oral cavity, causing aggressive periodontitis and oral candidiasis, respectively. The aim of the present study was to investigate the role of AI-2 based signaling on the interactions between *C. albicans* and *A. actinomycetemcomitans*.

This study shows that *A. actinomycetemcomitans* and its spent medium affect *C. albicans* biofilm formation. *C. albicans* biofilms increased significantly when co-cultured with *A. actinomycetemcomitans luxS*, which lacksAI-2 production, compared to its parent strain. Spent medium of the wild-type as well as addition of synthetic DPD to spent medium of the *luxS* strain could restore the phenotype of the *luxS* strain to wild-type levels. Addition of synthetic DPD significantly inhibited hypha formation of *C. albicans*.

AI-2 of *A. actinomycetemcomitans*, synthesized by LuxS, is secreted in early stages of growth and inhibits *C. albicans* biofilm formation through reduction of hypha-formation. Identifying the molecular mechanisms underlying the interaction between bacteria and fungus may provide important insight into oral microbial pathogenesis.

INTRODUCTION

Aggregatibacter actinomycetemcomitans is a non-motile, Gram-negative coccobacillus, which can be found in oral environments. In addition to being a commonly isolated commensal, it is also the principal cause of aggressive periodontal disease (26). *A. actinomycetemcomitans* uses chemical signals to sense cell density and alter gene expression and virulence factors (23). This process of communication is known as quorum sensing (QS) and the only identified cell–cell signaling molecule in *A. actinomycetemcomitans* is autoinducer 2 (AI-2). AI-2, regarded as a general interspecies concentration dependent-signal, is synthesized in the cytoplasm by LuxS as a precursor, 4,5,-dihydroxy-2,3-pentanedione (DPD), which spontaneously undergoes cyclization and is secreted into the medium where it accumulates. Interestingly, DPD can be converted into several specific structures that can be recognized by different species (21). For instance, *Vibrio harveyi* produces an unusual furanosyl borate diester ((3aS,6S,6aR)-2,2,6,6a-tetrahydroxy-3a-methyltetrahydrofuro [3,2-d][1,3,2] dioxaborol-2-uide), while *Salmonella typhimurium* recognizes (2R,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran.

In addition to *A. actinomycetemcomitans*, the polymorphic yeast *Candida albicans* is one of the most commonly isolated fungi from the oral cavity (5, 22). In healthy individuals, *C. albicans* normally grow as a commensal mostly in the unicellular yeast, but in immune-compromised individuals this species is also capable of producing multicellular filamentous forms of growth, a pathogenic morphology. The morphological transition from the yeast-to-hyphal mode of growth is influenced by many factors, including pH, nutrient availability, temperature and the presence of small diffusible molecules such as QS molecules, which stimulate or repress the filamentation pathway as a function of cell density (14, 17). Recently, the interaction of bacteria with *C. albicans* through QS molecules has received a lot of attention (29). *Pseudomonas aeruginosa* was shown to inhibit hyphal formation through its QS molecule 3-oxo-C12-homoserine lactone (15). In contrast, the *C. albicans* QS molecule farnesol impacts virulence factor expression of *P. aeruginosa* (7). In the oral cavity two Gram-positive bacteria have been shown to affect *C. albicans* biofilm formation through QS molecules. *Streptococcus*

mutans was shown to inhibit hyphal formation (18, 35) but did not affect *C. albicans* biofilm formation (24). Inhibition of hyphal formation was mediated through the *S. mutans* QS molecule CSP (18) and the fatty acid signaling molecule trans-2-decenoic acid (35). In addition, *S. gordonii* was shown to secrete AI-2, which was shown to repress *C. albicans* QS by inhibiting the action of farnesol (2). In the oral cavity a multitude of bacterial species co-exist with *C. albicans*, both Gram-negative and Gram-positive (40). In contrast to the limited information on the effect of Gram-positive oral bacteria on *C. albicans*, no information is available on the effect of QS molecules of Gram-negative oral bacteria. Therefore, in the present study, it was our aim to investigate the effect of AI-2 produced by LuxS of the Gram-negative oral bacterium *A. actinomycetemcomitans* on biofilm formation of *C. albicans*.

MATERIALS AND METHODS

Microbial strains and growth conditions

A. actinomycetemcomitans spp. were routinely cultured for 18 h at 37°C under microaerobic condition containing 10% CO_2 on trypticase soy agar or broth containing 0.6% yeast extract (TSB-YE/Difco) as described previously (8). For *A. actinomycetemcomitans luxS*, the medium was supplemented with kanamycin (30 μg/ml). When appropriate, standardized cell suspensions were prepared with an optical density measured at 655 nm of 2.1 x 10^8 cells/ml and by serial dilution plating on agar medium followed by counting of colony-forming units (CFU's). *Escherichia coli* strain JM107 was routinely grown in Luria-Bertani broth at 37°C with constant aeration. For solid medium, 15 g agar per liter was added to the liquid medium. When required, ampicillin (100 μg/ml) or kanamycin (30 μg/ml) was added to the medium. The pBluescript II SK (+) was a kind gift from Dr Pieter Smooker, Department of Biotechnology and Environmental Biology, RMIT University, Australia. *C. albicans* strain ATCC 10231 was taken from stock cultures frozen in 15% glycerol at -80°C and subcultured twice onto yeast peptone agar plates with 2% glucose (YPD), prepared according to manufacturer's instructions with pH set to 7.0 using 1M KOH. Cultures were grown at 30°C for 48 h.

Spent medium preparation

Spent medium of *A. actinomycetemcomitans* cultures were prepared according to Jarosz *et al.* (18), with slight modifications. Briefly, cultures were grown statically in microaerobic condition at 37°C in 200 ml TSB-YE. After 4, 6, 8, and 24 hour, spent medium (10 ml) was taken, centrifuged (10,000 x *g* for 5 min) and sterilized through 0.22-µm filters. The protein concentration in each spent medium was measured using Bradford method and diluted in PBS to yield 10 and 100 µg/ml concentrations and used immediately or stored for short periods of time at -20° C.

Construction of *A. actinomycetemcomitans luxS* mutant

In this study, we used *A. actinomycetemcomitans* (serotype b) that was isolated in our periodontal clinic from periodontitis patients, with their consent. The isolated bacteria were identified by means of their morphocellular (Gram stain), morphocolonial (characteristic star-positive colonies on agar plate), and tight adherence to surfaces when grown in broth (30). The positive colonies were confirmed and serotyped by a PCR method (32), and bacterial chromosomal DNA was isolated using Genomic DNA purification Kit (Fermentas). All primers for plasmid construct were designed using *A. actinomycetemcomitans* data base (www.Oralgene.lanl.com). In order to create a *luxS* defective mutant, a suicide vector was constructed using the neighbor-joining technique as previously described for *Campylobacter jejuni* (1). Firstly, a 229-bp DNA fragment containing part of the upstream sequence adjacent to *luxS* was PCR-amplified using the primer *EcoR*I-L1 (ACGAATTCAATCCACCGCACTT, forward) and primer *BamH*I-L2 (TCGGATCCAAGTTTTCTTGTTAGG, reverse). The PCR product was cloned into pBluescript in the forward direction via the *EcoR*I and *BamH*I sites, and was confirmed by restriction enzyme analysis. The resultant construct (pBl-L1) was subsequently introduced into *E. coli* JM 107 by using a Rapid DNA and Transform Kit (Fermentas). Positive clones were selected on LB agar supplemented with ampicillin, X-Gal and IPTG, based on the blue and white phenotypes. Secondly, a 409-bp DNA fragment containing the downstream flanking region of *luxS* was amplified using primers *BamH*1-L3 (CATGGATCCGAAGAAGCACATCAA, forward) and *Xba*I-L4 (ATCTAGAGCAAGTTGCTCGTAA, reverse). The amplified fragment

was inserted into pBl-L1 between *Bam*HI and *Xba*I sites of the multiple cloning site. The resulted intermediate plasmid (pBl-L2) was then cut at the unique *BamH*1 site and a 1.4-kbp fragment containing a kanamycin cassette was ligated into the plasmid to obtain the constructed suicide plasmid, pBLkm^r.

Natural transformation

A biphasic system for *A. actinomycetemcomitans* transformation was performed as described previously (36). Transformation was done by incubating a suspension of 10^8 CFU/ml of *A. actinomycetemcomitans* (fresh cells from 24 h growth on agar medium) at 37^{o}C under microaerobic conditions for 3 h. Subsequently, 10 μg of the suicide vector was added and cells were incubated for 3 h at 37^{o}C. Cells were then harvested and plated on agar medium supplemented with kanamycin and incubated at 37^{o}C, under microaerobic conditions for the next 2 days to select for transformants. Homologous recombination and disruption of *luxS* by integrating the kanamycin resistance gene in *A. actinomycetemcomitans* genome, was confirmed using PCR analysis with primers flanking the target site (*Eco*RI-L1 and *Xba*I-L4).

Synthesis of (*S*)-4,5,-dihydroxy-2,3-pentanedione (DPD)

DPD was synthesized following a procedure published by Ganin *et al.* (12). Lyophilized DPD was dissolved in DMSO at 10 mM stock concentrations and stored at -20°C until required.

Biofilm formation and co-culture of *C. albicans* and *A. actinomycetemcomitans*

Quantification of *A. actinomycetemcomitans* biofilms was achieved by staining with Crystal Violet (CV). *A. actinomycetemcomitans* strains (5 μl) were used to inoculated wells of 96-well (flat-bottom) cell culture plates (Sumitomo Bakelite Co., Ltd, Tokyo, Japan) containing 95 μl TSB-YE in each well. After, 18 h of incubation under microaerobic condition, the culture medium containing planktonic cells was removed and the wells were carefully washed with 200 μl of distilled water. The adherent bacteria were stained with 50 μL of 0.1% Crystal Violet for 15 min at room

temperature. After rinsing twice with 200 µl of distilled water, the dye bound to the biofilms was extracted with 200 µl of 99% ethanol for 20 min. The extracted dye was then quantified by measuring the absorbance at 655 nm with a microplate reader (Model 3550, Bio-Rad Laboratories, Hercules, CA, USA).

Biofilm formation of *C. albicans* was analyzed as previously reported with slight modifications (20). Briefly, yeast nitrogen base pH 7, supplemented with 50 mM glucose (YNB) was used to grow *C. albicans* cells at 30°C while shaking at 150 rpm. After overnight growth the cells were harvested, washed twice in sterile PBS and then suspended at an optical density at 600 nm (OD_{600}) of 0.2 (approximately 2 X 10^6 cells/ml) in YNB. These cell suspensions were then added to microtiter 96-well plates (Falcon), which were coated with fetal bovine serum (Sigma). The wells were washed once with 200 µl PBS and incubated with 100 µl cell suspension for 90 min at 37°C. Non-adherent cells were removed by washing twice with 200 µl PBS. Subsequently, biofilm formation was propagated by adding 200 µl per well of YNB (pH7) containing 50 mM glucose. Mixed species biofilms of *C. albicans* (2 X 10^6 cell/ml) and *A. actinomycetemcomitans* (2.1 X 10^7cfu/ml) were grown in medium containing 70% YNB and 30% TSB-YE (vol/vol). Where indicated, the 30% fresh TSB-YE fraction was replaced by spent medium as described previously (18). In addition, when indicated, sterile spent medium from the *A. actinomycetemcomitans* was added to YNB at 10 µg/ml and 100 µg/ml protein concentration. After 24 and 48 h of growth, biofilm formation on the well of microtiter plates were washed once with PBS, and metabolic activity of the biofilms was quantified using (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) (MTT) as described (20). The MTT assay could be used to analyze *C. albicans* biofilms in mixed-species biofilms because *A. actinomycetemcomitans* does not reduce MTT to its insoluble formazan salt. All assays were carried out on three separate occasions with duplicate determinations on each occasion. Microtiter wells containing only YNB broth but no cells were used as negative controls.

Inhibition of hypha formation

Hypha formation was assayed as described previously (18) with minor modifications. Briefly, 12-well tissue culture polystyrene plates (Costar; Corning, Inc.) were filled with 1 ml 70% YNB/30% TSB medium pH 7 supplemented with 0.5% glucose. *C. albicans* SC5314 cells from overnight pre-culture were used to inoculated new cultures at a final optical density of 0.01 measured at 600nm (corresponding to $1x10^5$ cells/ml). When appropriate, synthetic DPD was added to the indicated concentrations. The plates were incubated for 3.5 hours at 37^oC while shaking at 80 rpm. The morphology of cells was analyzed using a 20x objective lens on an inverted microscope (Olympus, Tokyo, Japan). The experiment was repeated with two independent cultures and at least 100 cells were counted for each sample.

Statistical analyses

Differences between means were analyzed for statistical significance using two-tailed Student's t-tests. Differences were considered significant when $p \leq 0.05$ level.

RESULTS

Effects of *luxS* deletion on biofilm formation of *A. actinomycetemcomitans*

Deletion of *luxS* did not affect growth rate in planktonic cultures (data not shown). *A. actinomycetemcomitans luxS* formed significantly less biofilm compared to the wild type strain (Fig. 1). Spent medium of a 4 h-old culture of the wild type strain was able to rescue biofilm formation of the *luxS* strain at 100 µg protein/ml (Fig. 1, left panel). Medium of 6 h-old cultures also rescued the phenotype to a similar, but not medium of 8 and 24 h-old cultures (data not shown). Addition of synthetic DPD rescued this phenotype specifically at 100 nM DPD, but not at lower or higher concentrations (Fig. 1B, right panel).

Role of AI-2 on mixed species biofilms of *A. actinomycetemcomitans* and *C. albicans*

Compared to mono-species biofilm formation of *C. albicans*, mixed species biofilms of *C. albicans* with *A. actinomycetemcomitans* resulted in a reduction of more than 50% in metabolic activity after 24 h of culturing (Fig. 2). A similar decrease in biofilm formation was observed after 48 h of culturing (not shown). Mixed species biofilms of *C. albicans* with *A. actinomycetemcomitans luxS* had no significant effect compared to mono-species biofilms.

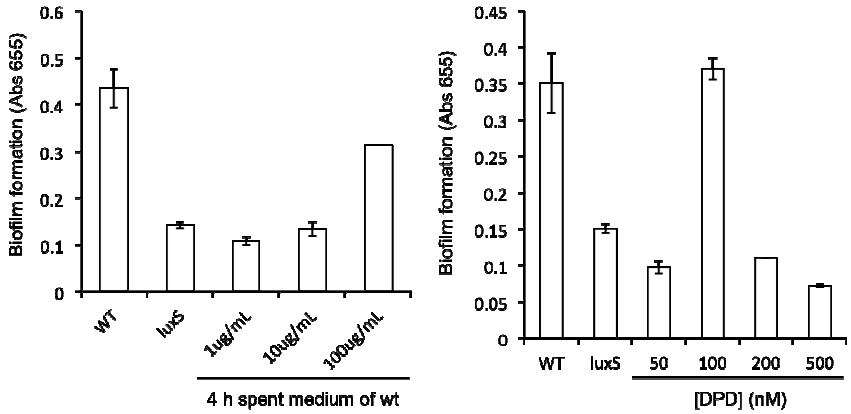

Fig. 1. AI-2 plays a role in biofilm formation of *A. actinomycetemcomitans*. Biofilm formation of *A. actinomycetemcomitans luxS* is restored to near wild-type levels by the addition of sterile spent medium (left panel) as well as by the addition of 100 nM synthetic DPD (right panel).

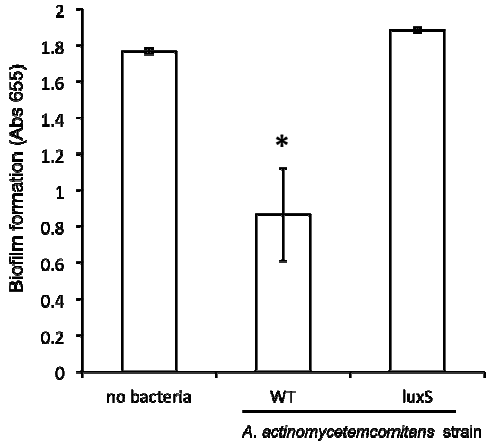

Fig. 2. The effect of co-culture with *A. actinomycetemcomitans* on *C. albicans* biofilm formation. *C. albicans* biofilms were grown without any bacteria or with *A. actinomycetemcomitans* wild-type or *luxS* mutant. Biofilm formation, quantified using the MTT assay, was measured after 24 h of growth. Data represent the mean and standard deviations of six biofilms grown on two separate occasions. * indicate statistically significant difference.

Effect of secreted factors on *C. albicans* biofilm formation

To further determine whether the inhibition effect was modulated by secreted compound of the bacteria, the spent medium of the wild type *A. actinomycetemcomitans* was used as a source of secreted molecules to complement *A. actinomycetemcomitans luxS*. When spent medium from 4 and 6 h-old wild-type cultures was added, *A. actinomycetemcomitans luxS* was able inhibited *C. albicans* biofilm formation, but this inhibition was not observed for spent medium derived from 8 and 24-h old cultures (Fig. 3).

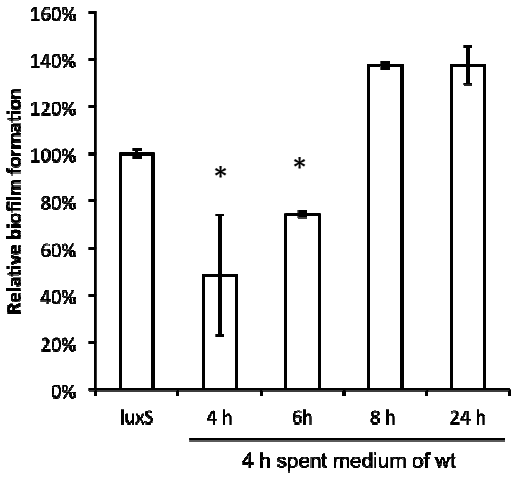

Fig. 3. The effect of spent medium on co-cultures between *C. albicans* and *A. actinomycetemcomitans luxS*. Spent medium of *A. actinomycetemcomitans* wild-type cultures grown for different times was added to the mixed species culture of *C. albicans* and *A. actinomycetemcomitans luxS*. Biofilm formation was quantified after 24 h of growth using the MTT assay. The relative biofilm formation compared to *C. albicans* + *A. actinomycetemcomitans luxS* without any spent medium was calculated. Data represent the mean and standard deviations of six biofilms grown on two separate occasions. * indicate statistically significant ($p \leq 0.05$) difference.

Effect of synthetic DPD on *C. albicans* hypha formation and biofilm growth

Synthetic DPD was added to *C. albicans* under hypha inducing conditions. DPD inhibited hypha formation at 100 nM and 1 μM with 30 and 70%, respectively (Fig. 4, left panel). When synthetic DPD was added to spent medium of *A. actinomycetemcomitans luxS* during *C. albicans* biofilm growth, a concentration

dependent inhibition of *C. albicans* biofilm formation was observed with the maximum inhibition reached at 100 nM DPD (Fig. 4, right panel).

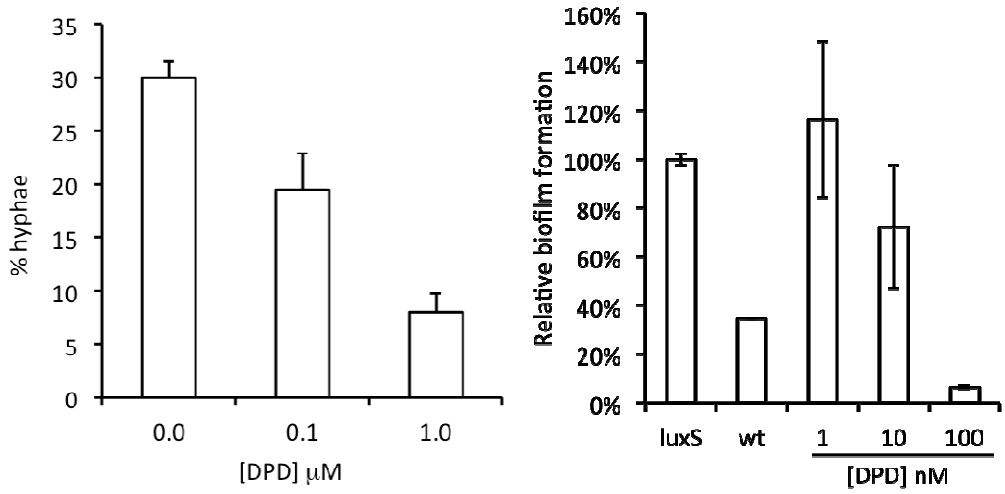

Fig. 4. Effect of synthetic DPD on *C. albicans*. Hypha-formation was induced by switching fresh cultures to 37°C for 3-4 h in the presence of the indicated concentration of synthetic DPD (left panel). Hyphae and yeast morphologies were counted and plotted as % of all cells. The results represent the mean of two independent experiments, each consisting of at least 100 cells per sample. Synthetic DPD added to spent medium of *A. actinomycetemcomitans luxS* inhibits *C. albicans* biofilm formation (right panel).

A. actinomycetemcomitans spent medium disrupts established *C. albicans* biofilm

To test the ability of *A. actinomycetemcomitans* to disrupt *C. albicans* biofilms, we challenged established biofilms (24 h old) of *C. albicans* with spent medium from *A. actinomycetemcomitans* WT and *luxS* cultures of increasing age. A significant decrease in viability was observed when *C. albicans* biofilms were exposed to spent medium of a 4 and 6 h-old cultures of the WT strain, but not to spent medium derived from the same aged cultures of the *luxS* strain (Fig. 5). Spent medium from 8 or 24 h-old cultures could not decrease viability of the preformed *C. albicans* biofilms.

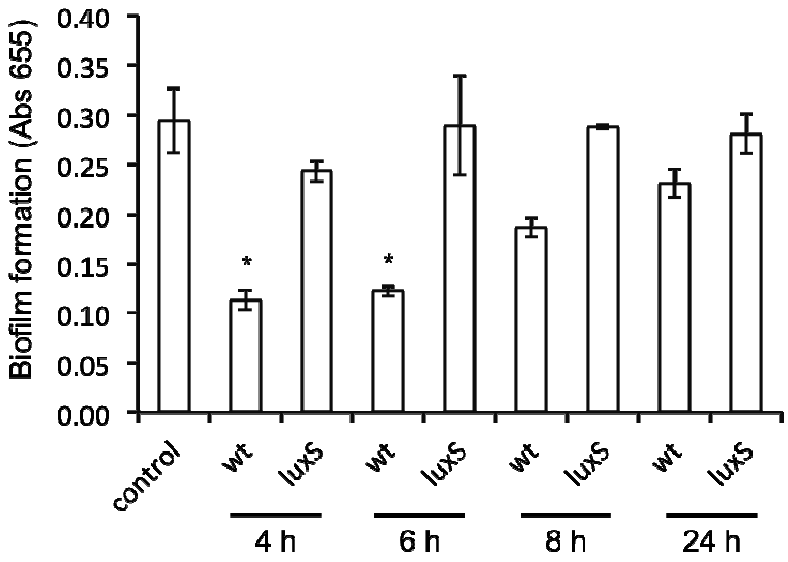

Fig. 5. Effect of spent medium of *A. actinomycetemcomitans* wt and *luxS* on preformed *C. albicans* biofilms. Biofilms of *C. albicans* were grown for 24 h after which they were exposed to spent medium of *A. actinomycetemcomitans* cultures of increasing age for an additional 24 h. Total amount of biofilm was determined using the MTT assay and results depict the averages of a total of 4 wells in 2 independent experiments. * indicate a significant ($p \leq 0.05$) difference compared to the control (*C. albicans* biofilms without addition of spent medium).

DISCUSSION

Several studies have reported on the isolation of *Candida* spp., from periodontal pockets of patients with periodontitis (19, 25, 33). Because biofilms are the most common mode of growth of *Candida* spp., as observed *in vivo*, and *A. actinomycetemcomitans* is related to severe periodontitis, we assumed that interspecies interaction occurs preferentially in mixed species biofilms including in the periodontal pocket.

AI-2 activity has been discovered in spent culture supernatants of many oral bacteria (3, 10, 18, 37). However, the function of AI-2 as a general bacterial signaling molecule is an issue that is yet to be resolved. Several studies have suggested that AI-2 is involved in biofilm formation (6, 11, 39). On the other hand, it has also been suggested that the main function of the LuxS enzyme is in the regulation of metabolic processes (16, 31, 38). Inactivation of *luxS* gene has also

been shown to cause phenotypic alterations, including biofilm formation and reduced colonization, in various experimental infection models (13, 34). Our data demonstrated that deletion of *luxS* of *A. actinomycetemcomitans* did not induce a growth defect (data not shown) in line with a previous report (28). Biofilm formation of the mutant strain was decreased as compared with its parent strain, thus confirming a previous report showing that AI-2 signal has a role in *A. actinomycetemcomitans* biofilm formation (28).

In this experiment, we did not test the spent medium in the *V. harveyi* model to monitor the AI-2 produced by *A. actinomycetemcomitans*. Instead, synthetic DPD was used to indicate that the *luxS* mutant phenotype is due to absence of the quorum sensing molecule (12, 38). We observed that the exogenous addition of AI-2/DPD to the growth medium restored the *luxS* phenotype in a dose-dependent manner, in contrast to *S. gordonii* (2), exogenously added DPD did partially compensate for the *luxS* mutation in *A. actinomycetemcomitans* in this study. This is because the differences in biofilm formation, between wild-type *A. actinomycetemcomitans* and its *luxS* mutant, occurred when DPD was added to the culture medium at maximum concentration (100 nM) (Fig. 1A). Thus, it cannot be excluded that the phenotype characteristic of *A. actinomycetemcomitans luxS* mutant is probably related to the metabolic role of *luxS* gene. Furthermore, introduction of wild-type *A. actinomycetemcomitans* spent medium restored the ability of *luxS* mutant strain to develop biofilm *in vitro* (Fig. 1B and C). This indicates, spent medium of wild-type *A. actinomycetemcomitans* have a similar chemical complementation effect with DPD.

Communication within oral bacteria involves two classes of signal molecules, competence-stimulating peptides (CSPs) synthesized by Gram-positive bacteria and AI-2 is a universal signal produced by both Gram-positive and Gram-negative bacteria (9, 27). Our finding that *luxS* of *A. actinomycetemcomitans* was involved in reducing the viability of *C. albicans* biofilm is intriguing because the both signaling molecules appear to have opposing effects on *C. albicans*hyphal formation. CSP from *S. mutans* inhibits the formation of hyphae (18), whereas *S. gordonii* AI-2 is involved in promoting the initiation of hyphae (2). Moreover, result from this study shows that spent medium of wild-type *A.*

actinomycetemcomitans has the ability to inhibit the hypha synthesized by *C. albicans*. On the contrary, the mutant strain, which is disabled to develop bacterial biofilm, showed marked increasing in the viability of *C. albicans* after 24 h in the present of wild type strain spent medium. These data indicate that the presence of secreted molecule regulated by *luxS* of *A. actinomycetemcomitans*, but not the bacteria cell, may offer biofilm sensitivity against *C. albicans* during time period used in our experiment. One reason for this could be the secreted molecule of the periodontopathogen interfering with biofilm physiology of *C. albicans*. Another explanation could be a nonspecific adherence mediated by *tad* fimbriae (28) may involve the physical interaction between *A. actinomycetemcomitans* and *C. albicans* filaments, a phenomenon that has been described for *Pseudomonas-Candida* interactions (15). Further studies are needed to explore the reasons for this finding.

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Chapter 5

Synthesis and validation of a probe to identify quorum sensing receptors

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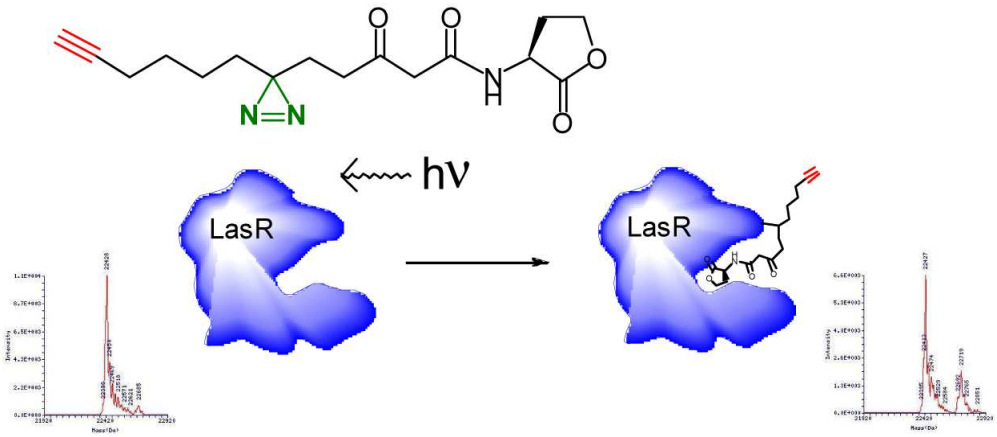
ABSTRACT

The synthesis and evaluation of a 'tag-free' probe to isolate and identify receptors for N-acyl homoserine lactones is described.



INTRODUCTION

The term "quorum sensing" (QS) is used to describe the mechanism that bacteria use to coordinate their behavior in a cell-density dependent manner (7). Using this mechanism, which is based on the exchange of small diffusible signaling molecules between cells, bacterial cultures can essentially function as multicellular organisms. Examples of QS-controlled behaviors are production of light (bioluminescence), virulence factor expression and biofilm formation. These processes are advantageous to a bacterial population only when they are carried out simultaneously by all its members (8).

During the past two decades a wealth of information regarding intercellular communication among bacteria have been uncovered. QS is mediated by secretion and recognition of small diffusible molecules, of which an example is the class of N-acyl homoserine lactones (AHLs), used by many known Gram-negative bacteria, such as the opportunistic pathogen *Pseudomonas aeruginosa*. The QS molecule N-(3-oxododecanoyl) homoserine lactone (3-oxo-C_{12}-HSL, C12 Fig. 1, **1**) has been indentified as the primary signal to enable QS in *P. aeruginosa* and this system involves the LasI (synthase) and LasR (receptor) proteins. This is part of a sophisticated strategy to survive under conditions that do not favor bacteria living solely as individual cells.

A growing number of reports indicate that bacterial AHL autoinducers can influence gene expression in eukaryotic cells. Most of these studies focus on the effects of the *P. aeruginosa* autoinducer C12 on the production of a number of cytokines, such as IFN-γ, by immune cells in vitro and in vivo (5, 12, 14, 15, 15, 18, 22-25). A recent study by Kravchenko *et al.* has uncovered a mechanism in which C12 selectively disrupts NF-κB signaling in activated mammalian cells (14, 15). These findings indicate *that P. aeruginosa* has developed means to detect host immune activation and to institute countermeasures (27). The resulting response in eukaryotes seems to point to a conserved down-regulation of the immune response, which is beneficial to the bacteria and may or may not be beneficial to the host. In another example of eukaryotic response to bacterial crosstalk, studies have shown that that the nematode *Caenorhabditis elegans* senses the presence and possibly

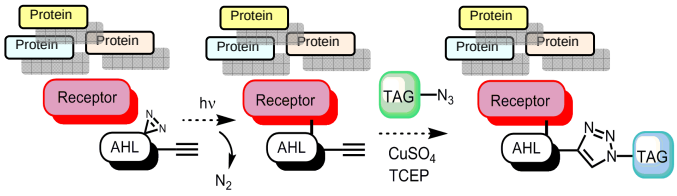
also the population size of *P. aeruginosa* by recognizing and responding to C12 (3, 28).

Recently, it has been shown that in many cases of *Candida albicans* infection, biofilm formation is involved. For both the infection process and biofilm formation, a morphological transition from yeast cells to filamentous cells (hyphae) is essential (16). Because of its importance, this morphological switch is tightly regulated. Recently, C12 was shown to repress hyphal formation (10). The mechanisms underlying the interactions between C12 and putative receptors in the mentioned eukaryotes are completely unknown. Jahoor *et al.* recently proposed that PPARγ is a mammalian C12 receptor, although no binding studies were reported (12); their observed effects of C12 on PPARγ activity could be the result of C12 binding to a different protein, ultimately resulting in downstream activity. We set out to isolate, identify and characterize eukaryotic receptors for C12, through a 'tag-free' activity-based protein profiling approach based on a highly selective copper (I) mediated azide-alkyne cycloaddition reaction (19, 21, 26). Our design scheme was based on the following findings: 1) It was shown previously that any modification in the lactone ring leads to diminished activity in mammalian cells (5); 2) small modifications in the alkyl chain did not cause a significant loss of activity.

RESULTS AND DISCUSSION

We therefore chose to design AHL analogs with only minimal structural deviations in the alkyl chain so as to increase the chances of recognition. In this approach, the probe is first conjugated to its receptor through irradiation of a functional moiety, such as diazirine. This smallest possible photoactive group that can be activated by light irradiation to generate a highly active carbene has been used successfully by others to label proteins (1, 6, 20). Then, following methodology developed by Cravatt and co-workers for the identification of unannotated enzymes (21), the probes, while attached to their target proteins will undergo reaction with their cycloaddition partner, which itself is attached to a tag / reporter group such as biotin or rhodamine (Scheme 1). This will then enable isolation of the probe-bound protein, followed by identification by mass spectrometry. By applying this general

scheme, we aim to develop a method to characterize the interaction between AHLs and unknown proteins that bind the AHLs.



Scheme 1. Labeling of unknown receptors with use of activatable bifunctional AHL-based probes. Addition of two minimally perturbing moieties to the homoserine lactone will allow for specific binding to an AHL receptor amidst a wide variety of proteins. Irradiation with UV light (360 nm) will result in a covalent bond between the probe and the receptor. A highly specific 1,3-dipolar cycloaddition reaction ('click') with a tag (or fluorophore) will then allow for isolation and identification of the receptor.

Probe **2** (Fig. 1, **2**) was prepared from 6-chlorohexyne as shown in Scheme 2. Following procedures described by Hodgson *et al.* (9), 4-oxononynoic acid **5** was prepared, and upon treatment with liquid ammonia the aziridine intermediate was obtained. Oxidation with iodine resulted in the formation of diazirine **6**, followed by homologation with mono tert-butyl malonate. Deprotection of **7** and coupling with homoserine lactone yielded diazirine alkynyl probe **2**.

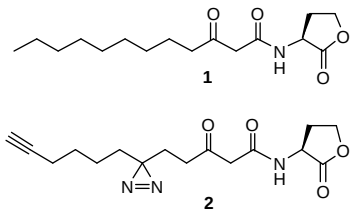
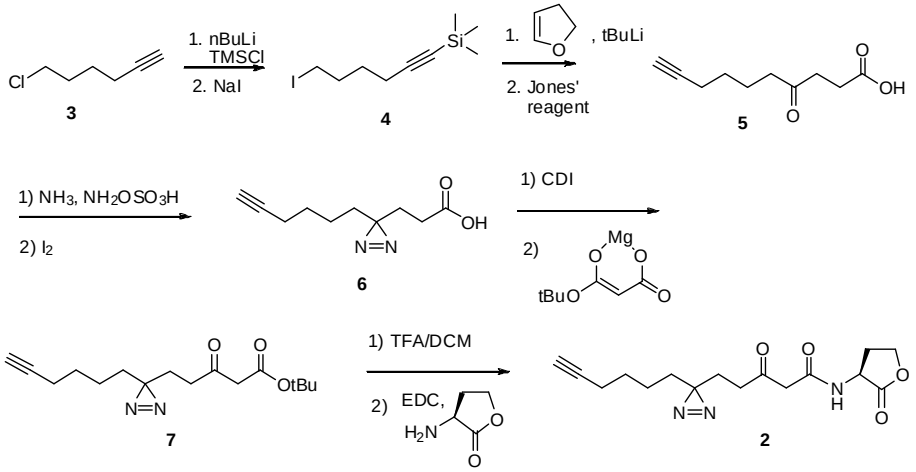


Fig. 1. Structures of 3-oxo-C_{12}-HSL (C12, **1**) and diazirine alkynyl probe **2**.



Scheme 2. Synthesis of 'tag-free' diazirine alkynyl AHL probe **2**.

Probe **2** was first tested for its ability to mimic C12 using *Escherichia coli* pSB1075 as reporter strain. Induction of luminescence, indicative of activation of the LasI/LasR QS system was observed (Fig. 2A). In addition, the induction of elastase, a virulence factor under control of the Las system was analyzed in a *P. aeruginosa lasI* mutant unable to synthesize C12. IC50 values ranged from 1 µM for C12 to 0.03 µM for probe **2** (Supplementary Fig. S1). We then verified whether **2** would mimic the activity of C12 in two eukaryotic systems. Phosphorylation of the kinase EIF2a in macrophages was induced by **2**, albeit slightly less than by C12 (Fig. 2B). Inhibition of germtube (GT) formation in *C. albicans* was inhibited by C12 and probe **2** similarly (Fig. 2C). These experiments indicate that probe **2** is capable of mimicking C12 in diverse microbial and eukaryotic systems.

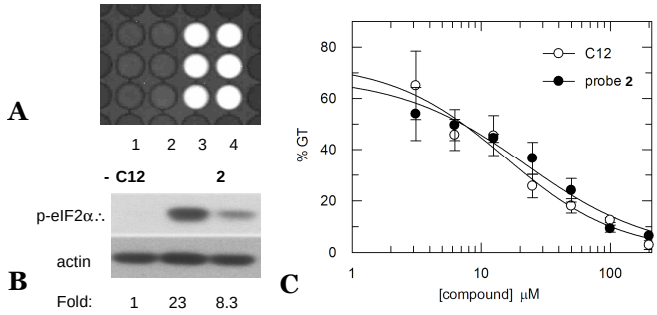


Fig. 2. A) Induction of bioluminescence by 5 μM C12 (lane 4, triplicates) and 5 μM probe **2** (lane 3) in a LasR-based AHL biosensor (*E. coli* pSB1075) strain. Luminescence was measured after 3 h growth. Lane 1 contains 0.5% DMSO in LB medium, lane 2 only LB medium. All wells were inoculated with a 1/100 diluted overnight culture; B) Biological activity of C12 and its diazirine alkynyl analog **2** in bone marrow-derived macrophages (BMDM). BMDM were treated with C12 or **2** for 30 min, and the cellular extracts were analyzed by Western blot for the phosphorylated form of eIF2a (p-eIF2a), as a biochemical marker of C12-mediated activation of mammalian cells. Western blot for actin was used as a control. Relative fold of p-eIF2a induction was estimated by densitometry. See (14) for experimental details; C) effects of C12 and probe **2** on germ tube formation in *C. albicans*. Calculated IC50: 15.3 μM (C12), 21.8 μM (**2**).

In order to determine accurate and optimized conditions for protein labeling by this probe and other diazirine based probes, we focused on labeling of LasR. Bottomley *et al.* published the first crystal structure of the ligand binding domain (LBD) of LasR (4), bound to C12, and following their protocol we overexpressed this protein in *E. coli*.

During expression of the protein in the presence of **2**, cells were irradiated for 15 min with a UV-lamp (360 nm), after which cell membranes were lysed and LasR-LBD was isolated through affinity purification. Proteins were analyzed by LC/MS as described by us previously (2), and while extracted protein that was expressed in the presence of C12 alone (20 μM) yielded a single peak (22,427 Da, Fig. 3A) corresponding to the expected mass of LasR-LBD (22,430 Da), the experiment in which LasR was expressed in the presence of probe **2** (20 μM) resulted in an additional peak with a mass difference of 292 Da (Fig. 3B, expected difference: 290 Da). In a third experiment in which LasR was expressed in the presence of both C12 and **2** (20 μM each) the additional peak had disappeared (Fig. 3C), showing that **2** competes with C12 for the same binding site, albeit with lower

affinity. From these experiments it follows that under the crosslinking conditions a significant fraction of LasR is labeled covalently and specifically.

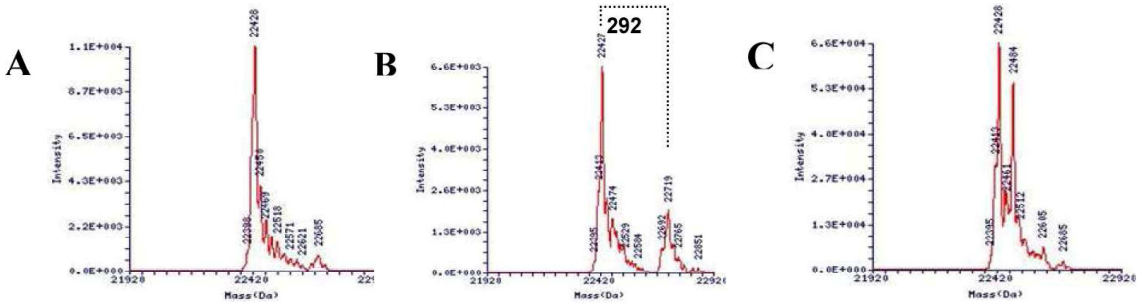


Fig. 3. Deconvoluted ESI mass spectra of crude LasR-LBD: A) overexpressed in *E. coli* in the presence of C12 (20 μM). Calculated mass: 22,430 Da. Observed: 22,428 Da, and several adducts - possibly the result of phosphorylation and/or methionine oxidation; B) overexpressed in the presence of diazirine probe **2** (20 μM) Calculated mass increase upon labeling with **2**: 290 Da. Observed increase: 292 Da; C) overexpressed in the presence of both probe **2** (20 μM) and C12 (20 μM). 22,484 Da is an unknown (possibly oxidized) adduct. All three cultures were irradiated with a UV lamp (360 nm) for 15 min to effect crosslinking of the probe, before lysis and affinity purification.

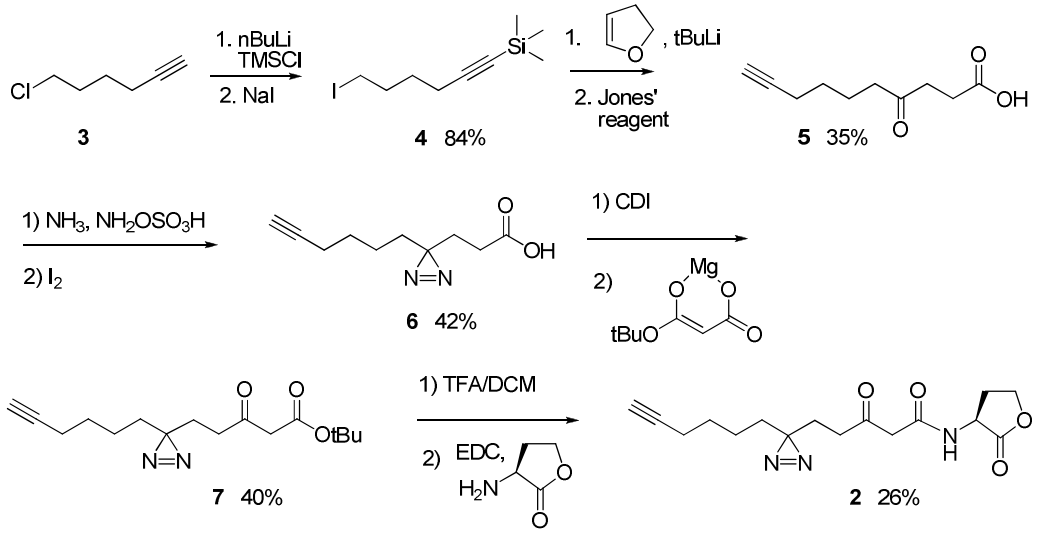
As the interaction between C12 and LasR is highly specific and small changes in the structure of C12 have been reported to lead to a significant loss in activity of C12 analogs, the specific labeling of LasR by probe **2** as observed in our experiments is encouraging in our attempts to isolate and identify putative receptors in eukaryotes that bind C12. We are currently using probe **2** in pursuing this target. As the exact location of the diazirine moiety may influence specific binding to C12-binding proteins in different organisms we are also currently expanding our toolset with C12-based probes with diazirine moieties at various locations in the alkyl chain.

SUPPORTING INFORMATION

General methods

All chemical reagents were purchased from Sigma-Aldrich or Acros and used without further purification. THF was distilled from sodium/benzophenone. Reactions were monitored by TLC using commercially available glass backed plates precoated with silica (0.25 mm, Merck 60 F254), that were commonly stained using potassium permanganate. Flash chromatography was performed using Merck 40-63 μm silica gel. ^{1}H and ^{13}C NMR spectra were acquired on a Bruker Avance DPX200 or alternatively on a Bruker Avance DMX500 spectrometer. ^{1}H and ^{13}C chemical shifts are reported with respect to TMS and spectra were calibrated on residual solvent signal.

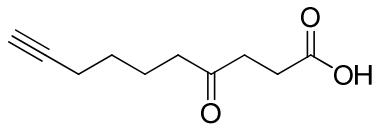
Synthesis of 5-(3-(hex-5-ynyl)-*3H*-diazirin-3-yl)-*N*-*((S)*-2-oxo-tetrahydrofuran -3-yl)-3- oxopentanamide 2



Synthetic procedures

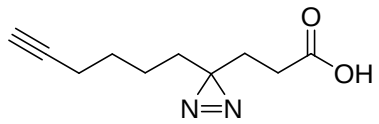
4-oxodec-9-ynoic acid 5

Prepared by a known procedure (7) via lithiation of 2,3-dihydrofuran and alkylation with TMS-protected alkyne iodide **4**, followed by direct oxidation of the crude hydroxynonynone using Jones' reagent (7).



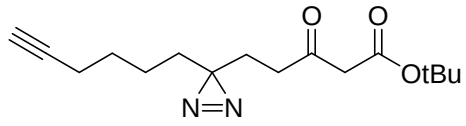
4-diazirine-9-decynoic acid 6

Prepared following procedures described by Husain *et al.* (11). Anhydrous ammonia (3.5 ml) was condensed into a round bottomed flask. 4-oxodec-9-ynoic acid **5** (0.54 g, 2.9 mmol) was added dissolved in small amount of anhydrous methanol. The mixture was stirred at -35-40 ^{o}C for 5 h. The solution was cooled with dry ice, and a solution of hydroxylamine-*O*-sulfonic acid (0.38 g, 3.3 mmol) in anhydrous methanol (2 ml) was added over a period of 30 min. The dry ice bath was removed, and the mixture was refluxed with stirring at -35 ^{o}C for 1 h. The mixture was allowed to warm slowly to room temperature and stirred overnight. The ammonia was then allowed to evaporate. The resulting slurry was filtered and the filter cake washed with several portions of methanol. The combined solution was concentrated *in vacuo*. The crude aziridine residue was dissolved in dichloromethane (1.7 ml) and treated with triethylamine (0.5 ml). A solution of iodine (0.5 g, 3.9 mmol) in dichloromethane (3 ml) was slowly added with stirring until the appearance of a persistent orange-brown color. The mixture was purified by flash chromatography on a column of silica gel using as eluent dichloromethane/ethyl acetate 4/1. Yield: 42%. ^{1}H NMR (200 MHz, CDCl3): δ = 2.20 – 2.12 (m, 4H); 1.95 (t, 1H, *J* = 2.62 Hz); 1.75 (t, 2H, *J* = 7.76 Hz); 1.55 – 1.39 (m, 4H); 1.31 – 1.14 (m, 2H). ^{13}C NMR (200 MHz, CDCl3): δ = 178.14; 83.83; 68.63; 32.18; 29.64; 28.22; 27.82; 27.75; 22.85; 18.12.



1-*tert*-butyl-3-oxo-6-diazirine-11-dodecynoate 7

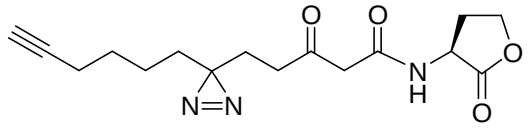
Prepared following a modified procedure based on reactions described by Kaufmann *et al.* (13). A: To a solution of 4-diazirine-9-decynoic acid **6** (0.22 g, 1.1 mmol) in dry THF (2.5 ml) uder argon, CDI (0.21 g, 1.3 mmol) was added at room temperature. The mixture was stirred at room temperature for 4 h. B: To a solution of mono *tert*-butyl malonate (0.2 g, 1.3 mmol) in dry THF (2.5 ml) at 0 ºC under argon, isopropyl magnesium chloride (2 M in THF, 1.2 ml, 2.4 mmol) was added dropwise. After 30 min at 0 ºC the solution was heated at 50 ºC for 30 min, then was cooled again to 0 ºC and solution A was added via cannula. The mixture was left warming to room temperature and was stirred for 16 h, then quenched with HCl 1 M (6 ml). The aqueous phase was extracted with ethyl acetate (3x12 ml). The organic layers were combined and washed with NaOH 1M, dried over $MgSO_4$ and concentrated under reduced pressure. The crude residue was purified by flash chromatography using as eluent hexane/ethyl acetate (0-10%). Yield: 40 %. ^{1}H NMR (500 MHz, CDCl3): δ = 3.32 (s, 2H); 2.30 (t, 2H, *J* = 7.45 Hz); 2.13 (dt, 2H, *J* = 7.02 Hz, *J* = 2.64 Hz); 1.94 (t, 1H, *J* = 2.64 Hz); 1.74 (t, 2H, *J* = 7.45 Hz); 1.54 (m, 2H); 1.47 (s, 9H); 1.41 (t, 2H, J = 8.00 Hz); 1.25 – 1.19 (m, 2H). ^{13}C NMR (500 MHz, CDCl3): δ =201.43; 166.12; 83.87; 82.17; 68.58; 50.54; 36.58; 32.48; 29.67; 28.26; 27.77; 26.34; 22.91; 18.14.



5-(3-(hex-5-ynyl)-*3H*-diazirin-3-yl)-*N*-*((S)*-2-oxo-tetrahydrofuran-3-yl)-3-oxopentanamide 2

Prepared following a modified procedure based on reactions described by Kaufmann *et al.* (13). The *tert*-butyl protecting group was removed by stirring 1-

tert-butyl-3-oxo-6-diazirine-11-dodecynoate in TFA/DCM 1:1 for 20 min. After solvent evaporation, the resulting 3-oxo-6-diazirine-11-dodecynoic acid (0.091 g, 0.385 mmol) was dissolved in 1,4-dioxane and EDCI (0.090 g, 0.467 mmol), HOBT (0.0453 g, 0.333 mmol) and homoserine lactone hydrobromide (0.061g, 0.333 mmol) were added alongside a few drops of water (3.33ml) at room temperature. Triethylamine (0.093ml, 0.667 mmol) was added and the solution was stired at room temperature for 3 h. The reaction mixture was diluted with ethyl acetate (30 ml), washed with water, dried over $MgSO_4$, the solvent was evaporated and the crude was purified by flash chromatography using as eluent ethyl acetate/hexane 4/1. Yield: 26%. ^{1}H NMR (500 MHz, CDCl3): δ = 7.49 (s, 1H); 4.57 (m, 1H); 4.48 (t, 1H, *J* = 8.95 Hz); 4.28 (m, 1H); 3.44 (s, 2H); 2.76 (m, 1H); 2.30 (t, 2H, *J* = 7.21 Hz); 2.24 (m, 1H); 2.15 (dt, 2H, *J* = 7.01 Hz, *J* = 2.60 Hz); 1.95 (t, 1H, *J* = 2.60 Hz); 1.75 (t, 2H, *J* = 7.21 Hz); 1.47 (m, 2H); 1.41 (t, 2H, *J* = 7.90 Hz); 1.22 (m, 2H). ^{13}C NMR (500 MHz, CDCl3): δ = 204.22; 174.80; 165.99; 83.91; 68.70; 65.96; 49.17; 48.42; 37.50; 32.50; 29.84; 27.995; 27.79; 26.15; 22.94; 18.19. ESI: C16H21N3O4 calc. (MH^+): 320.2; found: 320.1, 342.1 (MNa^+).



Biological evaluations

Elastase activity

Determined using the elastin Congo red (ECR) assay, as previously described (17, 29) with minor modifications. The supernatant of a *P. aeruginosa* Δ*lasI* strain (kindly provided by D. A. Hogan) grown in tryptic soy broth (TSB) for 5 h with various concentrations of C_{12} or probe **2** was collected by centrifugation. 20 mg of ECR (Sigma) was added to each tube containing 900 µl of ECR buffer (100mM Tris, 1mM $CaCl_2$, pH 7.5) and 100 µl of culture supernatant. The mixtures were incubated at 37°C for 18 h while shaking at 150 rpm after which the suspension was centrifuged at 10,000 x *g* for 15 min. The absorbance at 490 nm of the supernatants

was measured using a spectrophotometer. Background absorbance was subtracted and uninoculated TSB was used as a negative control.

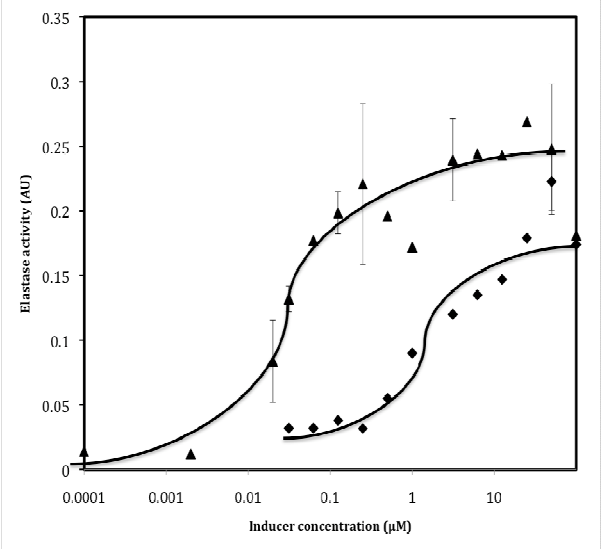


Fig. S1. Elastase activity induced by C12 (diamonds) and probe **2** (triangles) in *P. aeruginosa lasI*. Lines are drawn to guide the eye. IC50 values calculated for C12 = 1.0 µM and for probe **2** = 0.03 µM. The stronger induction of elastase activity by **2** compared with C12 is not understood by us, and is the subject of further investigations.

Activity test in biosensor *E. coli* pSB1075

The biosensor strain carrying a bioluminescence reporter plasmid (*lux*) was grown overnight in 5 ml LB medium with 50 µg/ml ampicillin at 37°C. The bacterial culture was subsequently diluted 100 times in LB medium. 200 µL of the bacterial suspension was added to wells of a 96-well microtiter plate. Conditions were tested as follow: 5 µM of probe **2** was added, 5 µM of 3-oxo-C_{12}-AHL (C12), LB medium or LB medium with 0.5% DMSO. The plate was incubated at 37°C, while shaking at 60 rpm for 3 h, followed by measurement of luminescence.

Effects of 3-oxo-C_{12}-AHL (C12) and probe 2 on germ tube formation in *C. albicans*

C. albicans SC5314 were grown overnight in 5 ml yeast nitrogen broth (YNB) medium at 30°C shaking (120 rpm). Cells were spun down at 14,000 x *g* for 5 min,

resuspended in saline phosphate buffer (PBS), pH 7.4, followed by OD600 measurement. The cell suspension was diluted in YNB to receive OD_{600} = 0.01 and added to 12 wells plate. 200, 100, 50, 25, 12.5, 6.25, 3.12 and 0 μM of probe **2** and C12 were added to the plates following by incubation at 37°C, shaking at 80 rpm for 3 h. Every sample was examined using an inverted light microscope – numbers of yeasts and hyphal cells were counted.

Expression of LasR-LBD

The expression of full length LasR was found to yield largely insoluble protein in the presence or absence of the native ligand C12 (4). Therefore expression was performed using a plasmid with the pETM-11 vector encoding for a shortened, His_6- tagged LasR construct, LasR-LBD (ligand binding domain), spanning Met-1 to Lys-173. The plasmid was transferred *E.coli* BL-21, and cells were plated on LB agar plates containing kanamycin (50 μg/ml). Proteins were expressed in the presence of either native C12, probe **2** or both and purified by Ni^{2+} affinity chromatography as described elsewhere (17). The purification process was monitored by SDS-PAGE electrophoresis and the molecular mass of the purified proteins was confirmed by LC/MS (Thermo-Finnigan LTQ). Following the procedure reported by Amara *et al.* (2), 1 ml of an overnight grown cell culture was used to inoculate 10 ml of rich LB medium containing kanamycin (50 μg/ml) and 10 μM of C12 (or 20 μM probe **2**, or 20 μM probe **2** + 20 μM C12). Cells were grown to an optical density at 600 nm (OD_{600}) of 0.4, after which expression was induced at 21°C by addition of 0.2 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG). After reaching an OD_{600} of 1.4 (approx. 6 h) cells were centrifuged at 4,000 x *g* at 4°C, washed and resuspended in PBS. Samples were was irradiated with a hand-held UV lamp (360 nm) for 20 min on ice. Cells were centrifuged and resuspended in lysis buffer containing 50 mM Tris-HCl at pH 8, 300 mM NaCl, 0.2 % (v/v) triton, 0.75 □g/ml DNAse-I, 0.05 mM $MgCl_2$, 0.01 mM $CaCl_2$, 5 mM imidazole and 0.01 % (v/v) protein inhibitor cocktail. Cells were incubated 60 min at 37 °C and centrifuged at 4,000 x *g*. The supernatants were purified using Ni-NTA spin colums following kit procedures by QIAGEN. SDS-PAGE was performed

using a NuPAGE Surelock Xcell, on NuPAGE Novex Bis-Tris Pre-Cast gels purchased from Invitrogen (NP0342).

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Chapter 6

Optimizing conditions for labeling and tagging proteins in *Pseudomonas aeruginosa* using a biomimetic 3OC12 HSL probe

ABSTRACT

In *Pseudomonas aeruginosa* LasR is the specific receptor for the autoinducing quorum sensing molecule, N-(3-oxo-dodecanoyl)-L-homoserine lactone ($3OC_{12}HSL$) but several other proteins are known to bind $3OC_{12}HSL$. We have recently designed and synthesized a $3OC_{12}HSL$ diazirine alkynyl probe in order to identify and purify $3OC_{12}HSL$ receptors. The aim of the present study was to optimize the conditions for labeling and tagging proteins in *P. aeruginosa lasI* mutant using a biomimetic $3OC_{12}HSL$ probe in combination with diverse azide tags using click-chemistry. We first optimized conditions for photoactivation of the probe by UV irradiation. Based on low background tagging, tetramethylrhodamine (TMR) was chosen as an optimal fluorescent tag. Multiple specific bands of the *P. aeruginosa* proteins were observed on a SDS-PAGE, which allowed a proof-of-principle experiment to further separate and identify all specifically labeled proteins using 2D electrophoresis followed by MS/MS. 2D imaging revealed a unique spot in accordance with LasR based on molecular weight and isoelectric point. In addition, QscR could be putatively identified based on molecular weight and isoelectric point.

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative bacterium and an opportunistic human pathogen. It is commonly isolated from burn wound infections or lethal infection related to cystic fibrosis (10, 15). This species has one of the most complicated and best-studied quorum sensing (QS) systems. QS is the process that allows bacteria to sense the density of the culture. The QS system of *P. aeruginosa* is arranged hierarchically in at least three different sub-systems; the *las*-, *rhl*- and *pqs*-system. The *las*-system is at the top of the cascade, positively regulating the *rhl*- and *pqs*-systems (4, 18). When the culture reaches a threshold density, the LasI synthase-produced signal N-(3-oxo-dodecanoyl)-L-homoserine lactone ($3OC_{12}HSL$) accumulates in the external medium. Due to active transport or diffusion, the intracellular concentration increases and binding of intracellular $3OC_{12}HSL$ to LasR activates this transcriptional regulator (6). The resulting LasR-$3OC_{12}HSL$ complex dimerizes (11) and activates gene expression of probably 250 different genes, many of which are virulence factors such as exotoxins, secondary metabolites and are involved in biofilm formation (17, 23).

It is generally accepted that LasR is the specific receptor for $3OC_{12}HSL$ (2, 6), however there are several other proteins in *P. aeruginosa* known to bind $3OC_{12}HSL$, e.g. PvdQ, RhlR and QscR. PvdQ is an N-acyl homoserine lactone acylase capable of binding $3OC_{12}HSL$ in an unusual substrate-binding pocket (1). Binding of $3OC_{12}HSL$ results in acyl-chain removal what effectively inactivates this molecule. PvdQ has therefore been proposed to act as a quorum quenching enzyme (9). Although RhlR has been shown to be the receptor for N-butanoyl-homoserine lactone (C_4HSL), RhlR can also bind $3OC_{12}HSL$ and function as an inhibitor of the Rhl-system (22). Binding of $3OC_{12}HSL$ to RhlR, instead of C_4HSL, induces dissociation of RhlR homodimers thereby preventing activation of gene expression (22). QscR is a homolog of LasR-RhlR proteins but does not have its own cognate HSL synthase (3). QscR responds to variety of acyl-HSLs including C_4HSL and $3OC_{12}HSL$. Binding of $3OC_{12}HSL$ to QscR is less avid than to LasR but the specificity of that binding is broader in comparison to LasR (14). Binding of C_4HSL or $3OC_{12}HSL$ to QscR induces a dissociation of the QscR oligomer (13) what repress

expression of several QS regulated genes, particularly *lasI* during the exponential growth phase (3).Therefore, in addition to the long-known $3OC_{12}HSL$ binding proteins, numerous additional proteins can bind this signal-molecule and many more remain to be discovered.

A novel approach to find and identify receptors for small molecules utilizes biomimetic probes. Biomimetic probes are synthetic molecules that mimic natural molecules in their mode of action but have a slightly changed chemical structure. We have recently designed and synthesized a biomimetic probe for $3OC_{12}HSL$ in order to identify and purify $3OC_{12}HSL$ receptors (5). The design of the probe was based on the observation that small modifications in the alkyl chain do not significantly change activity of $3OC_{12}HSL$ (8). The probe contained two functionalities, a diazirine to allow photoactivatable crosslinking to any protein in the direct vicinity of the probe, and an alkyne-moiety to allow subsequent labeling through click-chemistry (5, 19, 20). The probe was found to mimic natural $3OC_{12}HSL$ in bacteria (*Escherichia coli* and *P. aeruginosa*), in fungi (*Candida albicans*) and in macrophages. The clickable group on the biomimetic probe has the advantage that it is small and consequently does not impact the size of the probe too much, as would be the case for larger bulky labels such as biotin, fluorescein, His-tags and green fluorescent protein. In addition, the clickable group also allows the use of specific tags for specific purposes. For instance, azide-biotin allows downstream protein purification through streptavidin-based affinity chromatography while azide-fluorescein and azide tetramethylrhodamine allow fluorescent detection in protein PAGE applications (1D, 2D, native page etc.) or *in situ* localization in whole cells. The aim of the present study was to optimize the conditions for labeling and tagging proteins in *P. aeruginosa* using a biomimetic $3OC_{12}$ HSL probe.

MATERIALS AND METHODS

Fine chemicals

The $3OC_{12}HSL$ biomimetic probe was synthesized as described previously (5). Freezed-dried probe was dissolved in DMSO (Sigma) to stock concentrations of 10 mM and stored at -20°C until needed. To prevent pre-mature activation of the diazirine moiety, care was taken to shield the probe from light as much as practically possible. When appropriate, synthetic $3OC_{12}HSL$ was added from DMSO, stored at -20°C.

Liquid chromatography - mass spectrometry (LC-MS)

To optimize photo-activation conditions, water-diluted probe was exposed to UV light (360 nm) and loss of the diazirine moiety was analyzed using a LCQ Fleet mass spectrometer (Thermo Scientific) with an electrospray ionization (ESI) source. Spectra were collected in the positive ion mode and analyzed by Xcalibur software (Thermo Scientific).

Bacterial strains and growth conditions

P. aeruginosa PA14 *lasI* (a markerless *lasI* deletion mutant (8)), was routinely grown at 37°C on tryptone soya agar plates (TSB, OXOID, Basingstoke, England) inoculated form frozen glycerol stock kept at -80°C. This plate was kept at 4°C for maximum one week. Several colonies were taken to inoculate a 5 ml pre-culture in TSB, which was incubated overnight at 37°C while shaking at 150 rpm. Bacteria from this pre-culture were diluted 1:100 in 50 ml TSB to inoculate a main culture. Probe or HSL enantiomers were added at the appropriate concentration and the cultures were incubated, protected from light, for 5 h at 37°C while shaking at 150 rpm. Cells were harvested by centrifugation for 10 min 10,000 x *g* at 10°C (Beckman Coulter J-Lite), washed once with phosphate buffered saline (PBS: 10 mM potassium phosphate, 150 mM NaCl, pH 7), centrifuged again and resuspended in 5 ml PBS. These suspensions were placed in 6-well tissue culture polystyrene plates on ice and exposed to UV (360 nm) for 15 min after which time the bacteria were pelleted by centrifugation.

Protein extraction and fractionation

The pellets were snap-frozen in liquid N_2 and broken by grinding using mortar and pestle. The resulting powder was resuspended in 50 mM Tris-HCl buffer pH 8 with 25-times diluted protease inhibitor cocktail (Complete Protease Inhibitor Cocktail, Tablets, Roche, Germany). Intact cells and cell debris were removed by centrifugation at 6,500 x *g* for 10 min at 4°C and the supernatant (total protein extract) was placed in a sterile Eppendorf tube. The total protein extract was centrifuged at 30,000 x *g* for 45 min at 4°C. The supernatant (cytosolic protein fraction) was carefully removed and stored while the resulting pellet (membrane protein fraction) was resuspended in Tris-HCl buffer. Protein concentration of the three fractions was determined using Quant-iT™ Protein Assay Kit (Invitrogen) and measured on Qubit® fluorometer (Invitrogen). Samples were stored in -80 °C until use.

Click reaction

200 µg of each protein fraction was clicked using Click-iT® Protein Reaction Buffer Kit (Invitrogen) with azide and precipitated with methanol following the protocol provided by the manufacture. Dry protein pellets were stored in -20°C until use.

SDS-PAGE gel electrophoresis and fluorescence imaging

All dried pellets obtained after the click reaction were resuspended in 1 x Sample Buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue, 5% 2-mercaptoethanol or 350 mM DTT, demineralized water), kept at room temperature for 10 min followed by heating at 70°C for 10 min. Protein concentrations were measured as described above. A total of 50 µg of protein was loaded in each lane of a 12% polyacrylamide gel containing SDS. Each gel contained a lane with pre-stained marker (Kaleidoscope Prestained Standards, BIO-RAD) for reference. The gel was running for approximately 1 h at a constant potential of 150 V, rinsed once with demineralized water and either analyzed directly for fluorescence or processed for Western blotting. Direct in-gel imaging was performed using a Typhoon 9400 scanner (GE, Healthcare, Uppsala, Sweden) using the fluorescence mode, green laser and the PMT voltage was set at 750 V. The

excitation and emission wavelengths were set to 532 and 580 nm, respectively. After scanning the gel was stained with Coomassie brilliant blue to verify equal loading of samples.

Western blotting

After SDS-PAGE the proteins in the gel were electro-blotted onto PVDF Immobilon-P transfer membrane (Millipore) following the protocol for semi dry blotting (12). Electro-blotting was performed for 35 min at a constant current of 0.8 mA/cm^2. The membrane was carefully removed and blocked with 1x blocking buffer (Sigma) either over night at 4^{o}C or for 1 h at room temperature with mild shaking.

Odyssey infrared imaging

For antibody detection of the azide tagged proteins, the membrane was exposed to one- or two-step incubation with antibodies listed in Table 1. Eventually the membrane was exposed to IRDye antibody, prepared in 1:10000 dilution for 1 h at room temperature with mild shaking. The membrane was washed and subjected to detection using an Odyssey infrared imaging system (LI-COR).

Colorimetric detection

Prior to colorimetric detection of the azide tagged proteins, one- or two- incubation steps with one of the antibody listed in Table 1 was applied. Eventually the membrane was exposed to alkaline phosphatase (AP) antibody diluted in ratio 1:1000, for 1 h at room temperature with mild shaking. The 5-bromo-4-chloro-3-indolyl phosphate (BCIP®)/nitro blue tetrazolium (NBT) liquid substrate system (BCIP/NBT) (Sigma-Aldrich) was used as the substrate for alkaline phosphatase in accordance with the protocol provided by the manufacturer.

Antibody	Source	Dilution	Azide Dye	Application
IRDye® streptavidin 800CW	LI-COR	1:10000	biotin	Odyssey
anti-FITC-AP rabbit	Sigma-Aldrich	1:100	fluorescein	BCIP/NBT
anti-TMR rabbit	Invitrogen	1:1000	TMR	diverse
anti-rabbit goat AP	Invitrogen	1:1000	TMR	BCIP/NBT
IRDye® anti-rabbit goat	LI-COR	1:10000	TMR	Odyssey

Table 1. Antibodies used in the study and applications

2D gel electrophoresis

After the click-reaction, the pelleted proteins were resuspended in 100 µl of 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% (w/v) DTT and 0.5% (v/v) ampholytes (rehydration loading buffer). The protein concentration of the samples was determined using the 2D-quant kit (GE Healthcare) and the volume was adjusted with rehydration loading buffer in order to obtain a protein concentration of 0.5 mg/ml. The samples were loaded on IPG strips with pH range 4-7 (GE Healthcare) by the overnight rehydration method (7). Strips were focused in an Ettan IPGphor (GE Healthcare) with a 0-1000 V gradient for 3 h, stabilization at 1000 V for 1 h, a gradual increase from 1000 to 8000 V for 30 min and final focusing at 8000 V for 6 h (48000 Vhrs). Focused strips were equilibrated with 6 M urea, 50 mM Tris-HCl, pH 8.8, 30% (w/v) glycerol and 2% SDS (w/v) in two steps with initial reduction of cysteine disulfide bridges with 10 mg/ml DTT, followed by alkylation with 25 mg/ml iodoacetamide. Second dimension SDS-PAGE was performed in an Ettan DALTtwelve (GE Healthcare) on 12.5% acrylamide gels cast on low fluorescence glass plates (GE Healthcare) coated with PlusOne™ Bind-Silane solution (*g*-methacryloxypropyltrimethoxysilane, GE Healthcare). The labeled proteins were visualized in-gel using a Typhoon 9400 scanner (GE Healthcare) with excitation wavelength and emission filter of 532 nm and 580 nm with band pass 30 nm. The speckles present in the images were removed by filtering in Image quant software (GE Healthcare). Images were analyzed and the pick lists for

protein spots were generated using the Differential In-gel analysis module in the Decyder Differential Analysis Software, version 6.5 (GE Healthcare).

Protein identification from 2D gel plugs

For identification, all the protein spots visible were picked into a 96 well plate using the Ettan spot picker (GE Healthcare) equipped with a 2 mm diameter picker head. The tryptic digestion of the protein spots and mass analysis was performed using a MALDI TOF/TOF 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA) as described before (16). The peptide mass profiling data were submitted to Mascot (Matrix Science) and searched against the *P. aeruginosa* database (http://www.pseudomonas.com (21)). The database was created by combining forward and reversed entries of the *P. aeruginosa* database (release version 31.08.07) plus extra sequences of porcine trypsin (P00761), human keratins (P35908, P35527, P13645, NP_006112) yielding a database with a total of 4905 entries. Peptide tolerance was set to 10 ppm and 0.8 Da for intact peptides and fragment ions, respectively, and allowing for 1 missed trypsin cleavage. Oxidation of methionine residues and deamidation of asparagine and glutamine were specified as variable modifications. The tandem mass spectrometry (MS/MS) based peptide and protein identifications were further validated with the program Scaffold (version Scaffold_2_04_00, Proteome Software Inc., Portland, OR). Protein identifications established at greater than 99.0% probability, based on at least 2 peptides identified independently by MS/MS with probability higher than 95%, were accepted.

RESULTS

In order to establish accurate conditions for $3OC_{12}HSL$-binding protein labeling, several steps, as the photoactivation-condition for the probe, specificity of the tag, in situ labeling condition and probe concentrations, were needed to be optimized.

Optimal conditions for photoactivation of the probe

The diazirine moiety is photoactivatable, meaning that upon irradiation with light of the appropriate wavelength, N_2 is released from the alkyl chain. During this decomposition carbine is created which is able to react with any available chemical group in its vicinity, also the amino acids of the receptor that binds the probe. The exposure-time and distance from the light source are crucial and therefore these parameters were optimized. Diazirine $3OC_{12}HSL$ probe, containing alkyne (10 mM in DMSO) was diluted in water and exposed to UV light for various times and at a distance of 0 cm (data not shown) and 2 cm from the UV lamp. A sample from each time point was analyzed with LC-MS to determine the amount of the probe that was left after the irradiation (Fig. 1).

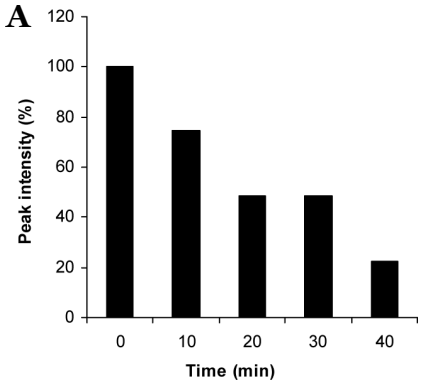
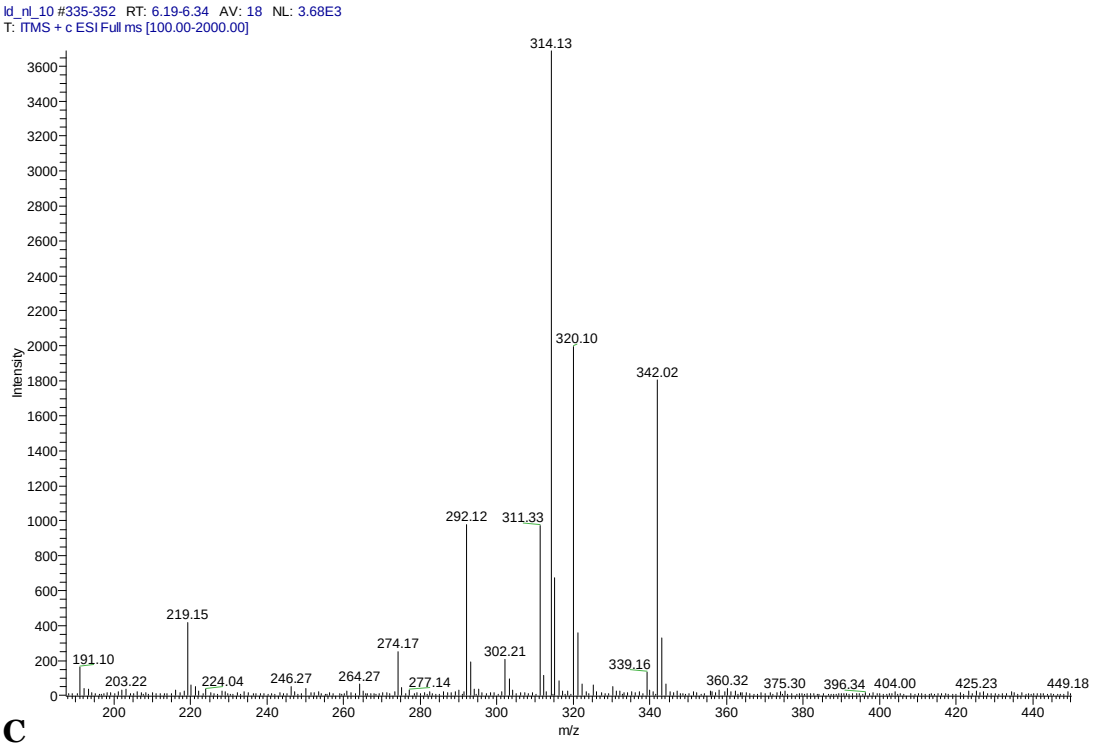
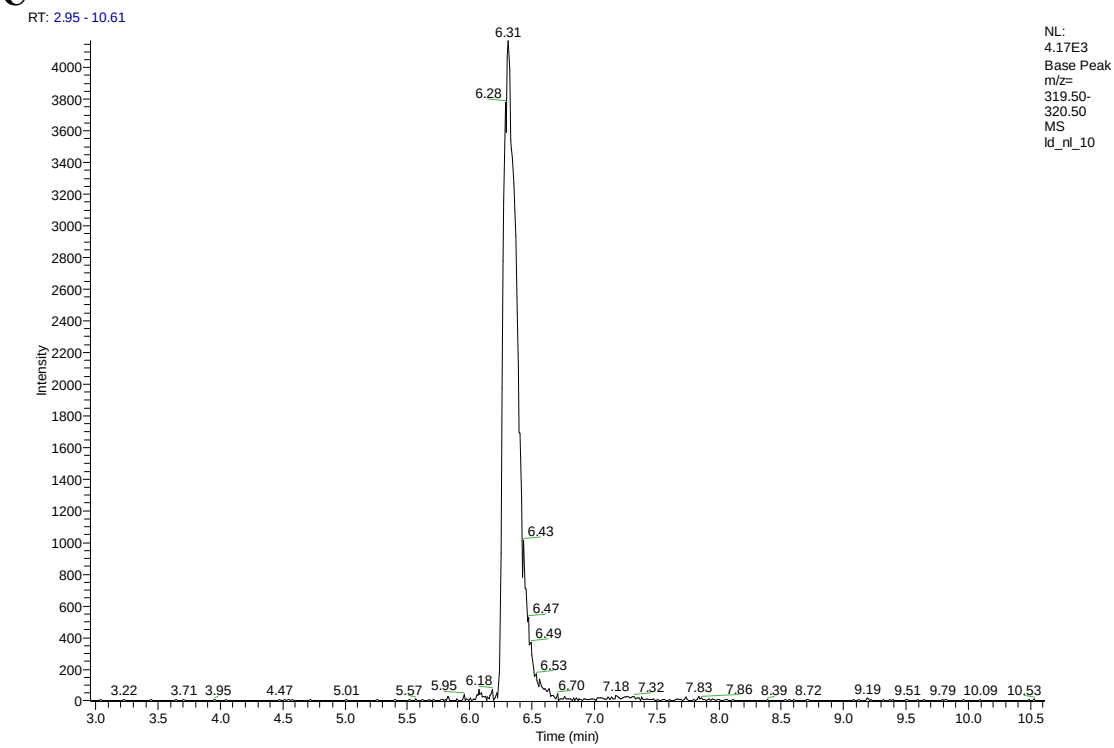


Fig. 1. (A) The relative peak intensity of the mass spectrum shows a decrease in mass of acyl-HSL diazarine probe due to its decomposition in response to UV irradiation over time. Graph depicts results from the distance of 2 cm (experiment was done once), (B) and (C) represent LC-MS results: (B) the corresponding mass spectrum where different masses are the fragments of the probe due to UV irradiation and (C) extracted ion chromatogram for the probe mass equal 320 Da.

B


ld_nl_10 #335-352 RT: 6.19-6.34 AV: 18 NL: 3.68E3
T: ITMS + c ESI Full ms [100.00-2000.00]
Intensity
m/z
314.13
320.10
342.02
292.12
311.33
219.15
274.17
302.21
191.10
203.22
224.04
246.27
264.27
277.14
339.16
360.32
375.30
396.34
404.00
425.23
449.18

C


RT: 2.95 - 10.61
NL:
4.17E3
Base Peak
m/z=
319.50-
320.50
MS
ld_nl_10
Intensity
Time (min)
6.31
6.28
6.43
6.47
6.49
6.53
6.18
6.70
3.22
3.71
3.95
4.47
5.01
5.57
5.95
7.18
7.32
7.83
7.86
8.39
8.72
9.19
9.51
9.79
10.09
10.53

Performed experiment revealed that the optimal time of UV exposure is 20 min with the distance of 2 cm chosen due to practical reasons; however distance of 0 cm was comparable. For further experiments done on *P. aeruginosa* cells, the time was shortened due to its potential harmful over-exposure effect on bacteria and proteins. It was set to 15 min and distance of 2 cm.

Selection of suitable fluorescent tags

Several tags are available in combination with the biomimetic probe; each can be used for one or more specific purposes (Table 2). However, for a tag to be suitable, it needs to meet certain criteria. Most importantly, it needs to show low background tagging in protein preparations. To test the background tagging of each individual tag in *P. aeruginosa*, they were clicked to a protein of *P. aeruginosa lasI* in the absence of the probe and visualized by fluorescence imaging and Western blot.

Azide Tag	Source/description	Potential application
Biotin-fluorescein	Conjugate of biotin and fluorescein M. Meijler	Direct in-gel imaging, Western blotting, and streptavidin-based purification
Biotin	Invitrogen	Western blotting, streptavidin-based purification, cellular localization using EM in combination with streptavidin-gold
Fluorescein	M. Meijler	Direct in-gel imaging, Western blotting
Tetramethylrhodamine (TMR)	Invitrogen	Direct in-gel imaging, Western blotting and magnetic dynabeads purification

Table 2. Clickable tags with their source and potential application

Biotin/Biotin-fluorescein

Azide-biotin-fluorescein is a multifunctional tag, allowing direct imaging in a gel (due to the fluorescein moiety), Western blot analysis with streptavidin-AP as well as streptavidin assisted affinity purification. Biotin-fluorescein tagged protein samples showed only a single non-specific fluorescent band upon fluorescence imaging (data not shown). In contrast, in the absence of the probe, many biotinylated proteins could be seen upon Western blot analysis detected using streptavidin (Fig. 2, lane 2). The presence of the probe did not result in a specific band appearing on the Western blot that could be competed away with $3OC_{12}HSL$ (not shown). Due to the large number of biotinylated proteins, biotin tagging with subsequent streptavidin purification does not seem feasible.

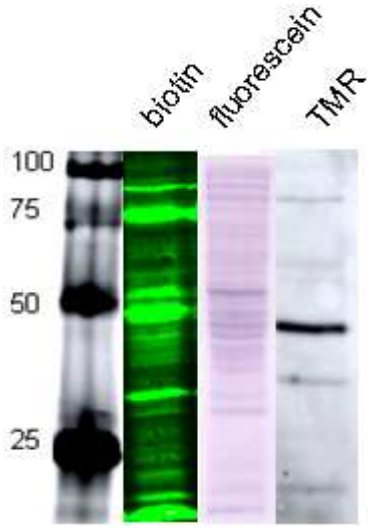


Fig. 2. *P. aeruginosa* cytosolic protein fraction clicked with 40 µM biotin, 100 µM fluorescein or 40 µM tetramethylrhodamine (TMR), respectively. Biotin was analyzed by Western blot using streptavidin-IRD and visualized on Odyssey Infrared Imaging system. Fluorescein was detected on Western blot membrane hybridized with anti-fluorescein AP antibody and detected with BCIP/NBT. TMR fluorescence was imaged in-gel using a Typhoon fluorescence scanner.

Fluorescein

Fluorescein as tag offers diverse applications such as in-gel imaging, Western blotting and colorimetric detection using BCIP/NBT. In contrast to the biological molecule biotin, fluorescein is not normally produced in microbes. Unexpectedly,

either direct fluorescence assisted detection (not shown) and Western-blot detection (Fig. 2, lane 3) of fluorescein resulted in a high background. Lowering the fluorescein concentration to 50 or 10 µM did not solve this. Apparently, fluorescein-azide reacts randomly with proteins during the click-reaction, therefore fluorescein was not suitable as tag for detection of labeled proteins.

Tetramethylrhodamine

Finally, tetramethylrhodamine (TMR) was investigated for its suitability as tag. TMR tagging of unlabeled proteins resulted in a minimal background upon direct in-gel imaging using a Typhoon scanner (Fig. 2, lane 4). Due to the availability of TMR-antibodies, application of TMR as a tag allows, in addition to direct in-gel imaging using the Typhoon scanner, Western blotting detection as well as partial purification using magnetic dynabeads, although this last method will need additional optimization and is not discussed in this chapter.

Tagging of labeled protein

Since tagging of unlabeled proteins with TMR in a sample containing all proteins yielded only minimal background, all subsequent experiments were performed with TMR as tag. Since LasR is a cytosolic protein, we continued our proof-of-principle study using the cytosolic protein fraction only. Tagging of probe-labeled proteins resulted in several fluorescent bands (Fig. 3). These bands were not detected in the sample labeled with natural $3OC_{12}HSL$.

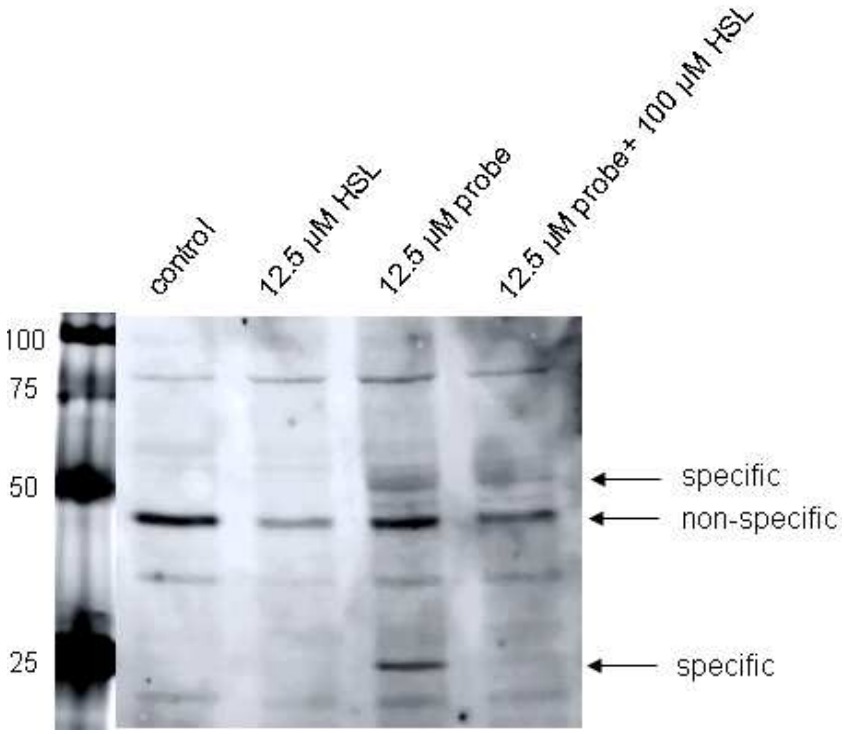


Fig. 3. SDS-PAGE gel of *P. aeruginosa lasI* cytosolic fraction labeled with the probe, natural $3OC_{12}HSL$, mixed of the probe and $3OC_{12}HSL$ tagged with TMR, scanned on Typhoon fluorescence scanner. The bottom band indicated by the arrow in the lane with the probe coincidences with the expected LasR signal.

One very prominent band was detectable at the expected position of the LasR receptor (molecular weight of approximately 26 kDa). Furthermore, some high-molecular weight specific bands were detectable, at the expected position of a LasR-dimer. Kiratisin *et al.* showed that LasR forms a homo-dimer upon binding of $3OC_{12}HSL$ (11). This specific band disappeared when the probe was mixed with 100 µM natural $3OC_{12}HSL$ due to competition of this two compounds for the binding site. It is evident that the probe binds to that protein, but when natural $3OC_{12}HSL$ at a 8 x higher concentration is mixed with the probe, it out-compete the probe from the binding site.

To investigate the effect of probe concentration on receptor binding, decreasing probe concentrations were tested. Upon addition of serial diluted probe concentrations, bands imagined putative LasR dimer revealed in the gel in a

concentration dependent manner (Fig. 4A). The band was present in each lane with the probe, but their abundance differed. Analysis of those bands using ImageJ showed increasing band density in accordance with increasing probe concentration (Fig. 4B).

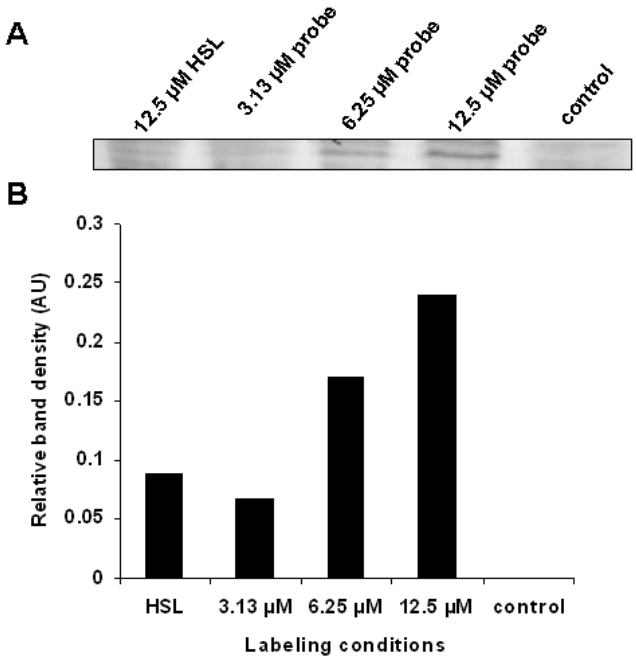


Fig. 4. (A) Putative LasR dimer labeled with increasing probe concentrations, tagged with TMR, scanned on Typhoon fluorescent scanner. (B) Relative band density received from SDS-PAGE gel, where various probe concentrations were compared with $3OC_{12}HSL$. The density of bands was analyzed using ImageJ.

2D gel electrophoresis

In addition to the suspected LasR protein, at least one more specific signal was observed, we attempted to identify these labeled proteins. The labeled cytoplasmic fraction was separated using 2D SDS-PAGE, followed by MALDI-TOF/TOF. Prior to applying the sample on isoelectric focusing (IPG) strips, the effect of addition of urea as dissociating agent was assessed. Increasing concentrations of urea were

added to the sample, and fluorescence was assessed (Fig. 5). The optimal concentration of urea was found to be 7 M.

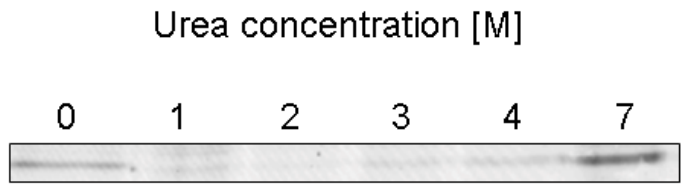


Fig. 5. Representation of *P. aeruginosa* PA14 *lasI* cytosolic proteins labeled with 12.5 µM probe and tagged with TMR, on an SDS-PAGE gel scanned on a Typhoon scanner. Protein samples were dissolved in various urea concentrations in order to choose the optimal concentration for 2D gel electrophoresis.

Subsequently, cytosolic proteins treated with 7 M urea were separated on a 2D gel (Fig. 6) after which the gel was scanned on the Typhoon scanner. Comparison of the gel in the pH range of 4-7 with a gel without probe, revealed the existence of several unique spots (encircled) represented by the proteins specifically labeled with the probe. Within these spots a putative LasR receptor was detected (no. 4) based on its calculated isoelectric point (*pI* = 6.4) and molecular weight (Mw = 27 kDa). The spot labeled no. 3 probably represent QscR as it runs at the calculated isoelectric point (*pI* = 5.7) and molecular weight (Mw = 27 kDa) and QscR has been described to bind $3OC_{12}HSL$ (3). The other unique spots represent unknown proteins that bind $3OC_{12}HSL$ (no. 1&2). Compared with control samples ($3OC_{12}HSL$ treated or untreated), some spots were determined as non-specifically TMR tagged. To further investigate that phenomenon, proteins were processed for identification.

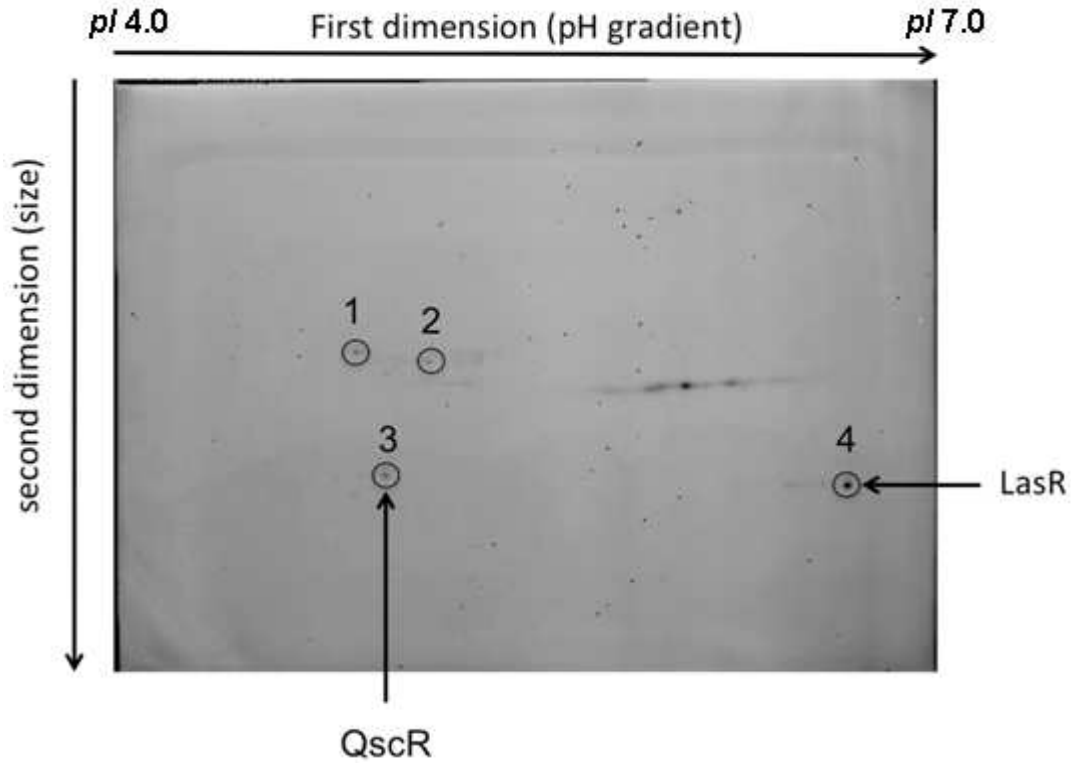


Fig. 6. 2D gel of *P. aeruginosa* PA14 *lasI* cytosolic fractions treated with 12.5 µM probe clicked with TMR, scanned on Typhoon scanner. The *pI* increases from 4.0 on the left side to 7.0 on the right and the molecular weight decreases from top-to-bottom. Numbers 1-4 indicate specifically labeled unknown proteins. No. 3 could represent the QscR protein (*pI*=5.7 and Mw=27 kDa) and no. 4 fits the properties of LasR (*pI*=6.4 and Mw=27 kDa). The other spots on this image (not encircled) are non-specifically labeled or tagged proteins also present in the control samples with natural $3OC_{12}HSL$ alone or untreated.

Protein identification from 2D gel plugs on MALDI TOF/TOF

Following separation by 2D electrophoresis and imaging using the Typhoon scanner, the proteins were prepared for identification. Analysis of the gel images using the Decyder 6.5 program allowed selection of 38 significant spots (indicated in Fig. 7). Of these, 35 were picked, trypsin digested and analyzed on MALDI TOF/TOF (Table 3). Unexpectedly, no known or putative HSL binding proteins could be identified.

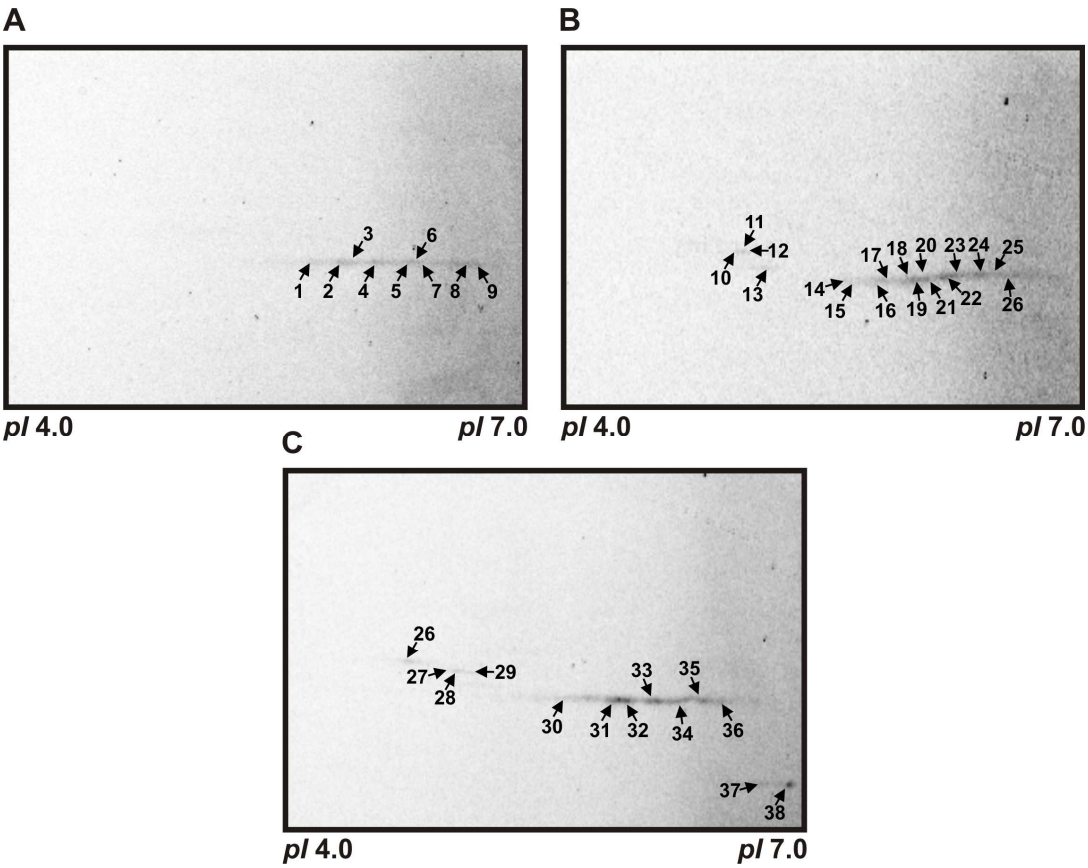


Fig. 7. Images of labeled and TMR tagged cytosolic proteins extracted from *P. aeruginosa* PA14 *lasI* after separation in a 2D gel. The extracted proteins were either labeled with 12.5 µM $3OC_{12}HSL$ (A), 100 µM $3OC_{12}HSL$ + 12.5 µM Probe (B) 12.5 µM Probe (C). High molecular weight proteins on top, and low molecular weight proteins on the bottom. The spots picked for identification were annotated by numbers; their protein description is given in Table 3.

Spot no. assigned on image	Protein identified
1	Alcohol dehydrogenase
2	Alcohol dehydrogenase
3	Alcohol dehydrogenase
4	Alcohol dehydrogenase
5	Alcohol dehydrogenase
6	Cytochrome c551
7	Alcohol dehydrogenase
8	Cytochrome c551
9	Cytochrome c551
10	DNA directed RNA polymerase/Ef-Tu
11	DNA directed RNA polymerase/Ef-Tu
12	Ef-Tu
13	NI
14	NI
15	Alcohol dehydrogenase
16	Cytochrome c551
17	Alcohol dehydrogenase
18	Alcohol dehydrogenase
19	Alcohol dehydrogenase
20	Alcohol dehydrogenase
21	Cytochrome c551
22	Cytochrome c551
23	Keratin
24	Cytochrome c551
25	Cytochrome c551
26	Trypsin
26	Ef-Tu
27	DNA directed RNA polymerase/Ef-Tu
28	DNA directed RNA polymerase/Ef-Tu
29	Ef-Tu
30	NI
31	Alcohol dehydrogenase
32	Alcohol dehydrogenase
33	Cytochrome c55
34	Trypsin
35	Cytochrome c55
36	Poly amine transport/Cytochrome c551
37	Electron transfer flavo protein
38	Succinate dehydrogenase

Table 3. List of identified proteins according to its assigned numbers in the 2D gels see Fig. 7. NI, not identified.

DISCUSSION

To facilitate discovery of $3OC_{12}$HSL binding proteins in a variety of organisms, we previously designed and synthesized a probe that mimics the function of natural $3OC_{12}$HSL (5). This biomimetic probe is biologically active in bacteria and eukaryotic cells and binds specifically to the $3OC_{12}$HSL receptor, LasR, when overexpressed in *E. coli*. In order to discover unknown $3OC_{12}$HSL binding proteins these should than be tagged using the alkyne moiety of the probe for which a variety of molecules for different purposes are available. The aim of the present study was to optimize the conditions for labeling and tagging proteins in *P. aeruginosa* using a biomimetic $3OC_{12}$ HSL probe. For this purpose we used *P. aeruginosa lasI*, unable to produce $3OC_{12}$HSL, to prevent unwanted competitive inhibition between $3OC_{12}$HSL and the probe.

A putative working scheme for identification of unknown $3OC_{12}$HSL binding proteins is as follows: 1) photoactivatable cross-linking between the protein and probe (using UV), 2) protein isolation, 3) tagging of labeled proteins, 4) separation using a SDS PAGE approach (1D, 2D, native etc.), 5) visualization using antibody detection or in-gel fluorescence imaging, followed ultimately by protein identification using techniques such as MALDI-TOF/TOF mass spectroscopy. In addition, affinity purification and in situ detection would be possible, but was not elaborated in this chapter. Here we focused on determination of efficient cross-linking parameters, tag-selection resulting ultimately in a proof-of-principle experiment including 2D PAGE followed by MALDI-TOF/TOF mass spectroscopy of labeled and tagged proteins in *P. aeruginosa* PA14 *lasI*.

Cross-linking parameters: for efficient photoactivation of the label, we had to take into consideration that too long UV exposure might result in possible damage to proteins. LC-MS experiments indicated that exposure of the label for 15-20 min at a distance of 2 cm, resulted in activation of the label and these conditions were subsequently used.

Tag-selection: a variety of azide tags was analyzed for suitability. The most important criterion tested was low background tagging. Biotin-fluorescein, biotin and fluorescein all showed considerable background and were deemed not suitable.

The optimal fluorescent tag for use in *P. aeruginosa* was TMR, due to its low background. Using TMR-azide we visualized the putative LasR receptor, with the molecular weight of 27 kDa on a SDS-PAGE. Presence of other hypothetical proteins able to bind $3OC_{12}HSL$ was also detected. One of these putative proteins could represent a LasR dimer, which is stable while activated by $3OC_{12}HSL$ binding (11). Titration of the probe revealed that the intensity of this band diminished with decreasing probe concentration, indicative for specificity of $3OC_{12}HSL$ dimerization. It is important to realize that this tag-selection is most likely species specific. However, it is fair to state that TMR would be our first-choice tag.

Proof-of-principle: The observation of multiple specific bands on a SDS-PAGE allowed a proof-of-principle experiment to further separate and identify all specifically labeled proteins in *P. aeruginosa* PA14 *lasI* using 2D electrophoresis followed by MS/MS. 2D imaging revealed a unique spot in accordance with LasR based on molecular weight and isoelectric point. In addition, three additional spots were also detected that were specifically labeled with our probe. Several additional $3OC_{12}HSL$ binding proteins are known in *P. aeruginosa*, for instance PvdQ, RhlR and QscR. Only the latter protein could be putatively identified based on molecular weight and isoelectric point. Protein identification using MALDI-TOF/TOF however did not result in any positive identification, neither for LasR nor for any of the other specifically labeled proteins. Most likely, these spots were visible due to the high sensitivity of the fluorescence scanner but protein identification could not confirm their identity due to low abundance of these proteins. These proteins remain unidentified and subsequent experiments are needed to accomplish that study.

The described method could for instance be improved by partial purification of labeled and tagged proteins. Possible application to use would be magnetic dynabeads with anti-methylrhodamine antibody on it. Proteins applied on 2D electrophoresis could yield a clear spot pattern with higher relative abundance of the labeled and tagged proteins. In addition, possible simple and direct purification of alkyne-probe-labeled proteins to, now commercially available azide beads seems a very feasible solution to the problem of low abundance (19).

Summarizing, the experiments and results described in this chapter are only the first steps in an exciting field. It is clear from our results that the acyl-HSL probe offers great potential for the future, leading to identification of $3OC_{12}HSL$ binding proteins playing an important role in the quorum sensing system of *P. aeruginosa* and $3OC_{12}HSL$ dependent interspecies communication.

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Chapter 7

Identification of the N-(3-oxo-dodecanoyl)-L-homoserine lactone receptor in *Candida albicans* using a biomimetic probe

ABSTRACT

Communication between *Pseudomonas aeruginosa*, a Gram-negative bacterium and the fungus *Candida albicans* is an example of interspecies (or inter-kingdom) communication mediated by quorum sensing molecules. N-(3-oxo-dodecanoyl)-L-homoserine lactone ($3OC_{12}HSL$) produced by *P. aeruginosa* represses the yeast-to-hypha transition. The ability of *C. albicans* to sense the presence of *P. aeruginosa* through $3OC_{12}HSL$ and actively react to it might be a sign that it has a specific receptor for $3OC_{12}HSL$. To identify that unknown receptor in *C. albicans* we designed and synthesized a diazirine probe which mimics natural $3OC_{12}HSL$. An optimized methodology was applied to label and tag $3OC_{12}HSL$ binding proteins in *C. albicans*. The presence of a receptor was detected by labeling with the probe and tagging with tetramethylrhodamine. This receptor is localized in the membrane and has a molecular weight of approximately 50 kDa. Binding of the probe was assessed to be specific but the apparent affinity was lower compared to natural $3OC_{12}HSL$. In conclusion, we for the first time show that *C. albicans* has a $3OC_{12}HSL$ binding protein located in the cytoplasmic membrane. Future research will focus on purification and identification of this receptor in *C. albicans*.

INTRODUCTION

Candida albicans, an opportunistic human fungal pathogen, constitutes a great risk for immunocompromised patients. For instance, it causes candidemia in AIDS/HIV patients, underweighted newborns, elderly people or intensive care unit patients. *C. albicans* is becoming increasingly resistant to commonly used antifungal agents; therefore there is an urgent need for new and improved antifungal drugs for the clinical management of candidiasis. *C. albicans* is a polymorphic fungus, existing in various cellular morphologies: yeast, hypha and pseudohypha. The transition from yeast to hypha is required for virulence and determined by many factors like pH, temperature and serum.

Interestingly, small secreted molecules from microbial origin also affect the morphology of *C. albicans* without modifying its growth rate. These molecules can be produced by *C. albicans*, for instance, farnesol, a 12-carbon sesquiterpene, released by *C. albicans* inhibits the yeast to hypha transition (9, 11). Alternatively, these molecules can be of bacterial origin; such as competence stimulating peptide of *Streptococcus mutans* (Chapter 3) or autoinducer 2 (AI-2) of *Aggregatibacter actinomycetemcomitans* (Chapter 4). N-(3-oxo-dodecanoyl)-L-homoserine lactone ($3OC_{12}$HSL) is a small molecule produced by *Pseudomonas aeruginosa* that is similar in structure to farnesol (12-carbon backbone). $3OC_{12}$HSL also represses yeast to hypha transformation (8). *P. aeruginosa*, a Gram-negative bacterium often exist in the same ecological niche with *C. albicans*, e.g. in burn wounds, cystic fibrosis infections, on intravenous catheters or in oral cavity, where this two microorganisms compete for a living space and nutrients. Communication between these two microorganisms is an example of interspecies (or inter-kingdom) communication mediated by quorum sensing (QS) molecules. QS is a common microbial system where the density of a population is monitored through the release and sensing of small diffusible molecules (13). These molecules accumulate in medium and upon reaching a threshold concentration, are sensed through a pathway of sensors-activators ultimately resulting in modulation of expression of QS-regulated genes.

Cross-talk between *C. albicans* and *P. aeruginosa* is very complex and involves a two-way communication. *P. aeruginosa* $3OC_{12}$HSL blocks yeast to hypha

transition (8) acting by a similar mechanism as farnesol (4) but through a different pathway (6). This is probably a defense mechanism of *C. albicans* (Chapter 1) as *P. aeruginosa* is able to attach to and kill filamentous, but not yeast cells of *C. albicans* (7). Additionally, farnesol released by *C. albicans* inhibits swarming motility in *P. aeruginosa* and causes down-regulation of *Pseudomonas* quinolone signal (PQS), limiting pyocyanin production (2). Pyocyanin is a well know virulence factor and is toxic to *C. albicans*.

In light of the ability of *C. albicans* to sense the presence of *P. aeruginosa* and actively react to it, we hypothesize that it has a specific receptor for $3OC_{12}HSL$. To identify the unknown receptor for $3OC_{12}HSL$ in *C. albicans* we designed and synthesized diazirine $3OC_{12}HSL$ probe which mimics natural $3OC_{12}HSL$ (Chapter 5). The activity of the probe was validated on *C. albicans* germ tubes formation and was comparable to natural $3OC_{12}HSL$. In a previous study (Chapter 6) we optimized the methodology to label and tag $3OC_{12}HSL$ binding proteins in *P. aeruginosa*. Here we used this methodology to show the presence of a specific $3OC_{12}HSL$ receptor in *C. albicans*.

MATERIALS AND METHODS

Fine chemicals

The $3OC_{12}$HSL biomimetic probe was synthesized as described previously (5). Freezed-dried probe was dissolved in DMSO (Sigma) to stock concentrations of 10 mM and stored at -20°C until needed. To prevent pre-mature activation of the diazirine moiety, care was taken to shield the probe from the light as much as practically possible. When appropriate, natural $3OC_{12}$HSL was added from a 10 mM DMSO stock, stored at -20°C.

Fungal strain and growth conditions

The *C. albicans* SC5314 was routinely grown on tryptone soya broth (TSB, OXOID, Basingstoke, England) agar plates for two days at 30°C form frozen glycerol stock kept at -80 °C. This plate was kept at 4 °C for a maximum of one week. Several colonies were taken to make 5 ml pre-culture in yeast nitrogen base pH 7, supplemented with 0.5% D-glucose (YNB), which was incubated at 30°C shaking at 150 rpm for 5 h. Pre-culture was used to inoculate the main 250 ml culture in YNB in ratio 1:100 which was grown overnight at 30 °C shaking at 150 rpm. The main culture was centrifuged at 10,000 x *g* for 10 min at 10°C (Beckman Coulter J-Lite), the cells were washed with sterile phosphate buffered saline (PBS: 10 mM potassium phosphate, 150 mM NaCl, pH 7), and divided into 10 ml cultures in fresh YNB. To these independent cultures either $3OC_{12}$HSL, probe or vehicle control (DMSO) at the appropriate concentrations were added as indicated. The cultures were incubated for 1.5 h at 37 °C while shaking at 150 rpm, protected from light. Cells were harvested by centrifugation, washed once with PBS, centrifuged again and resuspended in 5 ml PBS. Cultures were placed in 6-well tissue culture polystyrene plates on ice and exposed to UV light (wavelength) for 15 min and subsequently pelleted by centrifugation (Chapter 6).

Protein extraction

Total protein extracts: The cell pellets were snap-frozen in liquid N_2 and broken by grinding using mortar and pestle. The resulting powder was resuspended in 50 mM Tris-HCl buffer pH 8 with 25-times diluted protease inhibitor cocktail (Complete

Protease Inhibitor Cocktail, Tablets, Roche, Germany). Intact cells and cell debris were removed by centrifugation at 6,500 x *g* for 10 min at 4°C and the supernatant (total protein extract) was placed in a sterile Eppendorf tube.
Fractionation of proteins: The total protein extract was centrifuged at 30,000 x *g* for 45 min at 4°C. The supernatant (cytosolic protein fraction) was carefully removed and stored while the resulting pellet (membrane protein fraction) was resuspended in 50 mM Tris-HCl buffer pH 8. Protein concentrations were determined using Quant-iT™ Protein Assay Kit (Invitrogen) and measured on Qubit® fluorometer (Invitrogen). Samples were stored in -80 °C until use.

Click reaction

To tag labeled proteins, 200 µg of a protein fraction was clicked using Click-iT® Protein Reaction Buffer Kit (Invitrogen) with tetramethylrhodamine (TMR) azide and precipitated with methanol following the protocol provided by the manufacture (Chapter 6). Dry protein pellets were stored in -20°C until use.

SDS-PAGE gel electrophoresis and fluorescence imaging

The dried pellet obtained after the click reaction was resuspended in 1 x Sample Buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue, 5% 2-mercaptoethanol or 350 mM DTT, demineralized water), kept at room temperature for 10 min followed by heating at 70°C for 10 min. Protein concentrations were measured as described above. A total of 50 µg protein was loaded in each lane of a 12% polyacrylamide gel containing SDS. Each gel contained a lane with pre-stained marker (Kaleidoscope Prestained Standards, BIO-RAD) for reference. The gel was running for 1 h at a constant potential of 150 V. Finally, the gel was rinsed once with demineralized water and analyzed directly for fluorescence using a Typhoon 9400 scanner (GE, Healthcare, Uppsala, Sweden) using the fluorescence mode, green laser and the PMT voltage was set at 750 V. The excitation and emission wavelengths were set to 532 and 580 nm, respectively. After scanning the gel was stained with Coomassie brilliant blue to verify equal loading of samples.

RESULTS

Detection of the $3OC_{12}$HSL receptor in *C. albicans*

C. albicans cultures were exposed to the probe, $3OC_{12}$HSL and DMSO. Total protein fractions were compared: after tagging with TMR on SDS-PAGE gel electrophoresis. Comparison after in-gel scanning by Typhoon fluorescence imager revealed the presence of a high intensity band in the sample incubated with the probe that was absent in the control and $3OC_{12}$HSL samples (Fig. 1). This labeled protein has a molecular weight around 50 kDa.

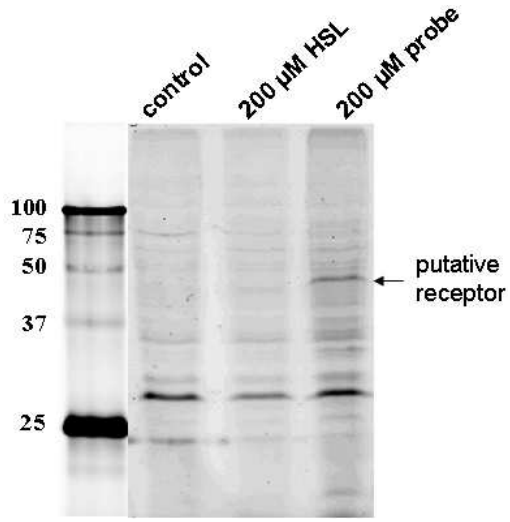

Fig. 1. Comparison of total protein fractions from *C. albicans* SC5314, incubated with DMSO (control), 200 µM natural $3OC_{12}$HSL or 200 µM probe. After photoactivatable cross-linking, total protein isolates were separated with SDS-PAGE gel electrophoresis and scanned on Typhoon fluorescence scanner.

Localization of the receptor for $3OC_{12}$HSL in *C. albicans*

In order to gain insight into the location of the $3OC_{12}$HSL receptor in *C. albicans*, the total protein fraction labeled with the probe was fractionated into a membrane and cytosolic fraction. These three protein fractions were tagged with TMR followed by separation on SDS-PAGE and direct in-gel scanning by Typhoon fluorescence imager. The prominent band at 50 kDa was observed in the total protein fraction and in the membrane fraction, but not in the cytosolic fraction

(Fig. 2). Therefore, this experiment demonstrated that the $3OC_{12}HSL$ receptor in *C. albicans* is a membrane protein, or is at least membrane associated.

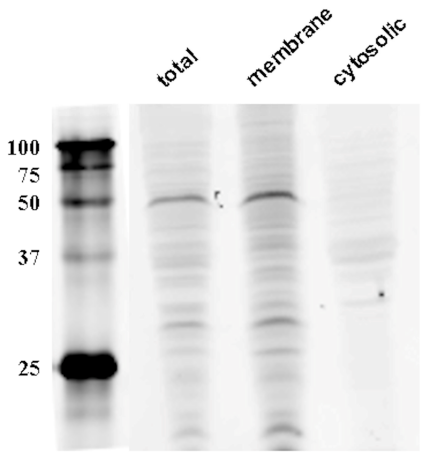

Fig. 2. Different *C. albicans* protein fractions labeled with probe and tagged with TMR after separation using SDS-PAGE, imaged on Typhoon fluorescence scanner. The presence of a 50 kDa fluorescent band in the total and membrane fraction, but not the cytosolic fraction indicates a membrane localization of the putative $3OC_{12}HSL$ receptor. Protein samples were labeled with 200 μM probe.

Specificity of binding

To confirm that the probe binds to the same protein as natural $3OC_{12}HSL$ a competitive inhibition experiment was performed. Increasing concentrations of $3OC_{12}HSL$ were added to *C. albicans* SC5314 culture in the presence of a constant probe concentration (200 μM). After SDS-PAGE, the fluorescent band at 50 kDa showed a decrease in intensity with increasing $3OC_{12}HSL$ concentrations (Fig. 3). The probe and $3OC_{12}HSL$ compete for the same binding site on the same receptor.

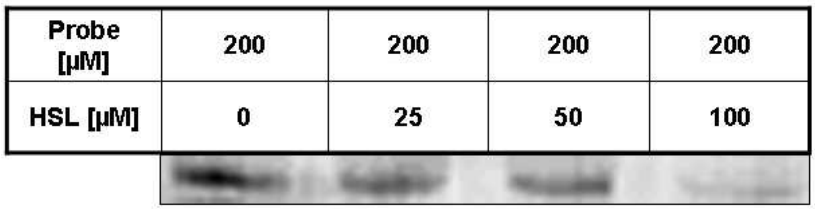

Fig. 3. Competition of the probe and natural $3OC_{12}$HSL for the same binding site. Representation of the receptor in *C. albicans* SC5314 for $3OC_{12}$HSL bounded with 200 µM probe and an increasing natural $3OC_{12}$HSL concentration (µM). Image depicts SDS gel scanned on Typhoon.

Probe titration

In order to determine the effect of the probe concentration on binding to the receptor *C. albicans* was exposed to a range of probe concentrations: 25, 50, 100, 200 and 400 µM. Analysis of the 50 kDa band-intensity showed an increasing with increasing probe concentration (Fig. 4). At concentrations lower than 100 µM the band was undetectable.

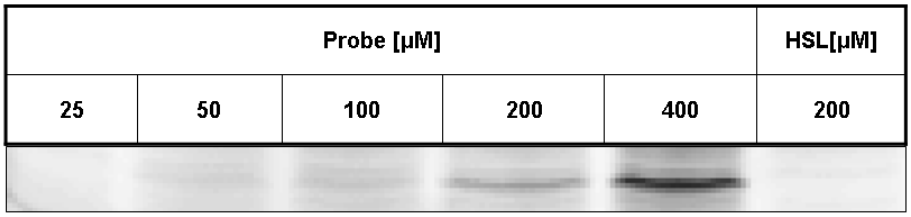

Fig. 4. Representation of the receptor in *C. albicans* SC5314 for $3OC_{12}$HSL bounded with increasing probe concentration (µM).

DISCUSSION

C. albicans and *P. aeruginosa* influence each other both through physical and chemical interactions. Physical interaction between *P. aeruginosa* and *C. albicans* result in adhesion to, biofilm formation on and killing of hyphae, but not yeast cells (1, 7). On the other hand, chemical interaction is based on detection of secreted small molecules that result in a wide spectrum of phenotypes. $3OC_{12}$HSL released by *P. aeruginosa* has an important effect on *C. albicans* biology by modulating its growth morphology. The exact mechanism of that inter-kingdom communication is not fully discovered, nevertheless we hypothesize that *C. albicans* has a dedicated

$3OC_{12}$HSL receptor that allows early detection of *P. aeruginosa* and a subsequent response to protect itself against an upcoming attack by this bacterium.

We showed the presence of a receptor for $3OC_{12}$HSL in *C. albicans* using the biomimetic probe designed previously (5). Exposure of *C. albicans* to the probe resulted in a detectable signal at 50 kDa. Separation of the total protein fraction showed that this prominent band remained in the membrane fraction illustrating that the $3OC_{12}$HSL receptor in *C. albicans* is a membrane or membrane associated protein.

Binding of the probe was competitively inhibited by addition of $3OC_{12}$HSL. In contrast, extensive background labeling was observed in the membrane fraction but not in the cytosolic fraction. A possible explanation for this could be that the probe, like $3OC_{12}$HSL, prefers to reside in the lipid bilayer, as illustrated by Davis *et al.* who showed that AHLs in micromolar concentrations interact with artificial membranes (3).

Properties of the binding to the receptor were determined. The binding was assessed to be specific based on a competition experiment where the probe competes for the same binding site with natural $3OC_{12}$HSL. $3OC_{12}$HSL concentration above 50 μM with a constant probe concentration of 200 μM out-competed the probe, indicating that there is an affinity difference between the probe and $3OC_{12}$HSL. Saturation of receptor binding sites by the ligand was tested based on the probe titration, where increasing concentration caused increased intensity of binding. Concentration of 100 μM probe was the lowest concentration with a detectable signal. In a previous study (Chapter 5) (5) it was shown that the probe has a lower affinity for LasR than $3OC_{12}$HSL, probably due to the small conformational differences in the acyl-chain. Also, the IC50 values for the inhibition of *C. albicans* germ tubes for the probe were higher than for $3OC_{12}$HSL. A full determination of affinity for the probe in comparison with $3OC_{12}$HSL remains to be performed in the future by applying a kinetic approach.

In conclusion, it is shown that *C. albicans* possesses a membrane-localized $3OC_{12}$HSL receptor. This conclusion provokes several questions, amongst others; what is the advantage for *C. albicans* of having a receptor for $3OC_{12}$HSL? Would *C. albicans* facilitate its survival? Based on the speculation that other species able to

sense $3OC_{12}$HSL, such as *Staphylococcus aureus* (10) and macrophages (12), could have $3OC_{12}$HSL receptors, we hypothesize that all these proteins might be homologous. Therefore, future research will focus on identification of the receptor in *C. albicans*, evaluation of its biological function(s) and identification of the $3OC_{12}$HSL receptor in unrelated species. For these purposes it is important to separate protein fractions on 2D gel electrophoresis, which will be difficult for membrane proteins, followed by MALDI-TOF/TOF. Another option might be receptor purification using e.g. magnetic Dynabeads with two-step hybridization including anti-TMR antibody or azide-beads.

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Chapter 8

The response of *Staphylococcus aureus* to
N-(3-oxododecanoyl) homoserine lactone is strain specific

ABSTRACT

Quorum sensing is the mechanism that bacteria use to communicate and coordinately regulate their gene expression. *Pseudomonas aeruginosa,* a Gram-negative bacterium uses acylated homoserine lactones such as *N*-(3-oxododecanoyl) homoserine lactone ($3OC_{12}HSL$) as signaling molecules. Recently it has been reported that virulence of the Gram-positive bacterium *Staphylococcus aureus* is antagonized by $3OC_{12}HSL$. The present study investigated the effect of $3OC_{12}HSL$ on several different *S. aureus* strains. While growth rates decreased by addition of $3OC_{12}HSL$ for all tested strains, the secretome was influenced by $3OC_{12}HSL$ in a strain specific manner. This response was not related to AIP-type. $3OC_{12}HSL$ oppositely regulated hemolysin and protein A production in *S. aureus* ATCC12600 and NCTC8325-4. Finally a synthetic analog of $3OC_{12}HSL$, with the potential to be used for identification of the membrane receptor for $3OC_{12}HSL$, was tested but did not show the same activity as the natural $3OC_{12}HSL$. This might be caused by the slight conformational changes in the acyl-chain of the probe, as the acyl-chain has been shown to be important for the *S. aureus* response to $3OC_{12}HSL$. Data obtained in the present study suggests that $3OC_{12}HSL$ affects *S. aureus* in a strain specific manner but this is not attributed to the AIP-type.

INTRODUCTION

Intercellular communication within a population of microorganisms is mediated by small diffusible signaling molecules, in a process termed quorum sensing (QS). QS is based on the production, external accumulation and subsequent detection of a small molecule resulting in coordination of gene expression in response to increasing cell density (33). The type of the secreted molecules and the mechanisms of detection differ per bacterial species. Gram-negative bacteria, for instance *Pseudomonas aeruginosa,* communicate using N-acyl homoserine lactones (AHLs) while Gram-positive bacteria, for instance *Staphylococcus aureus,* communicate by autoinducing peptides (AIPs) (15, 25).

In *P. aeruginosa*, QS is mediated by two complete acyl-HSL systems, LasR-LasI and RhlR-RhlI, and by an orphan receptor, QscR. LasI and RhlI are synthases that produce the signal molecules N-(3-oxododecanoyl)-HSL ($3OC_{12}HSL$) (25) and N-butyryl-HSL (C_4HSL) (26, 37), respectively. LasR and RhlR are their respective receptors that respond to their cognate signals and activate transcription of many virulence genes (28), (see Fig. 1 in Chapter 1).

In *S. aureus*, the accessory gene regulator (*agr*) locus contains two divergent transcripts: RNAII and RNAIII (13, 22). The RNAIII is an operon of four genes: *agrABCD* that encodes proteins required for synthesis of AIP and activates the regulatory cascade (Fig. 1) (22, 34). AgrA and C constitute a two-domain signaling module; AgrD is a precursor of autoinducing peptide (AIP), while AgrB is a membrane endopeptidase essential for AIP biosynthesis (21, 34). AIP binds to and activates AgrC followed by phosphorylation of AgrA, which up-regulates promoters P_2 and P_3 (22). This in turn autoactivates the *agr* system and up-regulates RNAIII transcription. RNAIII up-regulates expression of secreted proteins and down-regulates surface proteins (27, 31) many of which are virulence factors, for instance protein A (*spa*), fibronectin-binding protein but also exoproteins like hemolysins and toxic shock syndrome toxin 1 (TSST-1) (9, 35). The expression of the *agr* system is highly influenced by other regulatory systems, including *sarA*, which encodes numerous exotoxins and the fibronectin-binding proteins while repressing protein A production (4, 6).

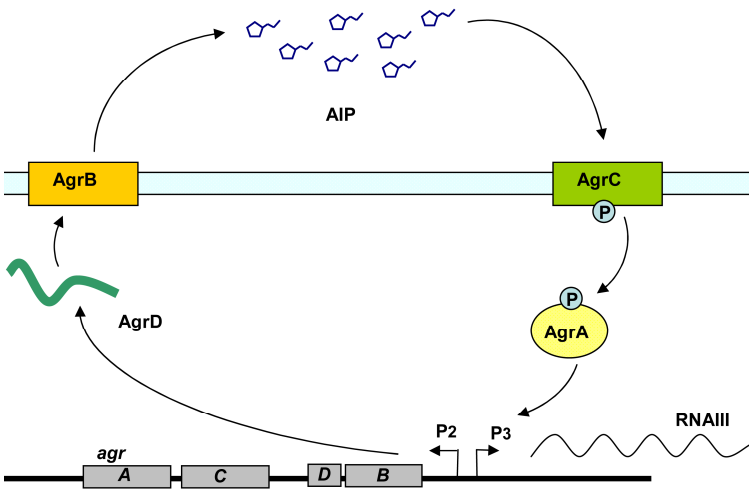

Fig. 1. Schematic representation of the *agr* system in *Staphylococci*. Peptide precursor AgrD is processed through AgrB at the cytoplasmic membrane and secreted in the form of AIP. At a threshold concentration AIP binds to AgrC inducing phosphorylation of the AgrA which activates transcription from two *agr* promoters. The P_2 promoter leads to circuit autoactivation, and P_3 drives to the production of RNAIII, which is the regulatory effector of the system. This figure is adapted from (21).

Based on the allelic variations in *agrBCD* and variation in amino acid sequence of their AIP, *S. aureus* strains can be subdivided into four different groups: AIP-type I - IV (8). Recognition of a specific AIP molecule by its cognate receptor is highly sequence-specific and a single amino acid substitution in AIP can change group specificity (14, 23).

Due to the extracellular nature of QS molecules, they are readily available to other species that can also respond to such signals (Chapter 1). Since *S. aureus* and *P. aeruginosa* are commonly co-isolated from infections of burn wounds (32) it would not be surprising that these two species are able to recognize each other. Recently it was shown that *S. aureus* responds to the presence of $3OC_{12}HSL$ secreted by *P. aeruginosa* (30). An important staphylococcal surface protein, protein A encoded by *spa*, involved in *S. aureus* defense against the host immune system was up-regulated in response to $3OC_{12}HSL$. In addition, $3OC_{12}HSL$ induced

down-regulation of expression of the global regulators *sarA* and *agr* and consequently, several other virulence factors such as hemolysin, exotoxin, fibronectin-binding protein and factors related to biofilm formation were down-regulated. This response of *S. aureus* to $3OC_{12}$HSL was specific, as no response was observed for $3OC_4$HSL or unsubstituted acyl-HSLs like C_{12}HSL.

Qazi *et al.* concluded that *S. aureus* has a specific membrane receptor for $3OC_{12}$HSL (30). The nature of this putative receptor remained unknown. We recently designed and synthesized a probe $3OC_{12}$HSL analog that can be used to detect $3OC_{12}$HSL binding receptors (Chapter 5, 6 and 7). This probe has only minimal structural deviations compared to natural $3OC_{12}$HSL and their biological activities are comparable. Through a small photoactive group (diazirine) the probe can be coupled to the receptor and after addition of a reporter/tag group the receptor-protein-probe complex can be identified.

The aim of the present study was firstly, to investigate the effect of $3OC_{12}$HSL on various *S. aureus* strains and attempt to relate the response to AIP-types, and secondly to detect the presence of a receptor for $3OC_{12}$HSL in *S. aureus* using the probe and determine its localization.

MATERIALS AND METHODS

Bacterial strains and growth conditions

All *S. aureus* strains were grown on tryptic soy broth (TSB) agar plates at 37°C from which 5 ml TSB pre-cultures were inoculated and incubated overnight at 37°C under shaking conditions at 150 rpm. *S. aureus* ATCC12600 was determined to be *agr* positive and belongs to *agr*/AIP type I according to the method published by Gilot *et al.* (12). *S. aureus* NCTC8325-4 is a derivative of the NCTC8325 strain but has been cured of prophages (24). This strain is *spa* positive (29), *agr* positive and like ATCC12600 affiliated with *agr*/AIP type I (20). *S. aureus* NCTC8325-4 is essentially the same as RN6390 (used by Qazi *et al.*) since RN6390 is also derived from NCTC8325 by curing it from prophages. *S. aureus* Newman has been isolated from a patient in 1952 (1), is *spa* positive (29) and *agr*/AIP type I. *S. aureus* Col is a methicilin resistant strain, isolated from an operating theatre in the early 60s (11)

and *agr*/AIP type I. *S. aureus* MN8 is an *agr*/AIP type III strain isolated form a case of menstrual toxic shock syndrome in the 80s (2, 19). *S. aureus* 090914D, *S. aureus* 072365T, *S. aureus* 7323 and *S. aureus* 5296 are clinical isolates that were isolated from infections associated with implants and typed by the department of Medical Microbiology, UMCG.

Fine chemicals

N-(3-oxododecanoyl)-L-homoserine lactone ($3OC_{12}HSL$) is naturally produced by *P. aeruginosa* as a QS molecule. The synthetic L-enantiomer was kindly provided by Dr. M. Meijler, (Department of Chemistry, Ben Gurion University of the Negev, Israel) and a stock solution of 25 mM in DMSO was stored at -20°C. The acyl-HSL diazirine probe was prepared as recently described by Dubinsky *et al.* (Chapter 5) dissolved in DMSO to received 10 mM solution and stored at -20°C.

Determination of growth rates

Growth rates of *S. aureus* were determined by measuring the optical density of the bacterial suspension over a 24-h period in a plate reader (FLUOstar Optima, BMG labtech) with a heated incubation chamber. A pre-culture of *S. aureus* was diluted 1:100 in 1.5 ml TSB in a 12-wells tissue polystyrene culture plate (Greiner Bio-One Cellstar) and a range of $3OC_{12}HSL$ or probe concentrations was added; TSB alone was used as a control. The plate was incubated at 37°C while the optical density at 575 nm (OD_{575}) was measured every 30 min, preceded by a short shaking cycle. The growth rate at a certain time point was calculated using the change in optical density divided by the time (h^{-1}).

Hemolysins assay

Hemolysin activity in spent medium of *S. aureus* ATCC12600 and NCTC8325-4 grown with a range of $3OC_{12}HSL$ concentrations was determined by placing 10 µl spent medium on a sheep blood agar plate, allowing it to dry and incubate overnight at 37°C. The color and the diameter of the halos, formed due to the lysis of red blood cells in the blood agar, were determined. The color of the halo was used to determine the type of hemolysin as followed: a darkish halo is indicative for

the presence of α-hemolysin and a transparent halo for the presence of β-hemolysin. The cross-section of the outer halo was used as a measure of total hemolysin present in the spent medium, as the size of the halo correlates with the amount of hemolysin secreted by *S. aureus* under specific conditions (38).

Western Blot analysis of protein A

Production of protein A was tested for *S. aureus* ATCC12600 and NCTC8325-4 grown with a range of $3OC_{12}HSL$ concentrations as follows: after overnight growth, equal numbers of cells were taken based on the OD_{600}. Cell wall proteins were extracted using lysostaphin in 30% raffinose medium as described previously (5). The supernatant samples containing the cell wall proteins were ran on a 12.5% polyacrylamide SDS gel together with a positive control containing increasing amounts of commercially available protein A (Sigma-Aldrich). The proteins in the gel were transferred onto a PVDF Immobilon-P transfer membrane (Millipore) following the protocol for semi dry blotting (18). Electro-blotting was performed for 35 min at a constant current of 0.8 mA/cm^2. The membrane was carefully removed and blocked with 1% bovine serum albumin solution (Sigma), either over night at 4°C, or for 1 h at room temperature, with mild shaking. The membrane was subsequently exposed to biotinylated goat anti-rabbit protein A IgG antibody (Abcam) prepared in 1:10,000 dilution for 1.5 h at room temperature with mild shaking. Finally, streptavidin IRDye (LI-COR) was added in 1:10,000 dilution for 1 h at room temperature with mild shaking. The membrane was washed and subjected to detection using an Odyssey Infrared Imaging system (LI-COR).

Isolation and separation of secreted proteins

Secreted proteins of all staphylococcal strains were precipitated from the spent media of 24 h cultures grown with a range of $3OC_{12}HSL$ or probe concentrations. Briefly, the cells were removed by centrifugation (4,000 x *g* for 10 min) and the supernatant was filter-sterilized using a 0.22 μm filter (Millipore). Proteins were precipitated by addition of 1 volume trichloroacetic acid to 4 volumes of protein sample, followed by 10 min incubation at 4°C. Proteins were pelleted by centrifugation in a tabletop microcentrifuge (Eppendorf) for 5 min at 16,000 x *g*

and the pellet was washed three times with 200 µl ice-cold acetone. The remaining pellet was dried in a heat block at 95°C for 5 - 10 min.

SDS-PAGE gel electrophoresis

Dried protein pellets were resuspended in 1x sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue, 5% 2-mercaptoethanol or 350 mM DTT, demineralized water), and warmed for 10 min at 80°C. Protein concentrations were determined using Quant-iT™ Protein Assay Kit (Invitrogen) and measured on Qubit® fluorometer (Invitrogen). Equal amounts of proteins were loaded on a 10% polyacrylamide SDS gel in a Bio-Rad Mini Protean® Tetra System. Each gel contained a lane with pre-stained marker (Kaleidoscope Broad Range Molecular Weight Ladder, Fermentas) for reference. The gel was run for approximately 1 h at a constant potential of 150 V. Subsequently, the gel was rinsed once with demineralized water, stained with Coomassie brilliant blue (10% acetic acid, 25% isopropanol, 0.5 g/l Coomassie) and de-stained using de-staining solution (10% acetic acid, 10% isopropanol).

RESULTS

3OC_{12}HSL inhibits the growth rate of *S. aureus* ATCC12600 and NCTC8325-4 strains

Growth of *S. aureus* ATCC12600 in the presence of a range of 3OC_{12}HSL concentrations resulted in inhibition of the growth rate in a concentration dependent manner (Fig. 2A). Growth rates in the exponential phase were inhibited for all concentrations of 3OC_{12}HSL. At concentrations of 5 and 15 µM 3OC_{12}HSL the growth rate reached the same plateau height as a control without 3OC_{12}HSL, but it was delayed in time. The plateau phase decreased considerably at concentrations of 30 and 60 µM 3OC_{12}HSL (data not shown).

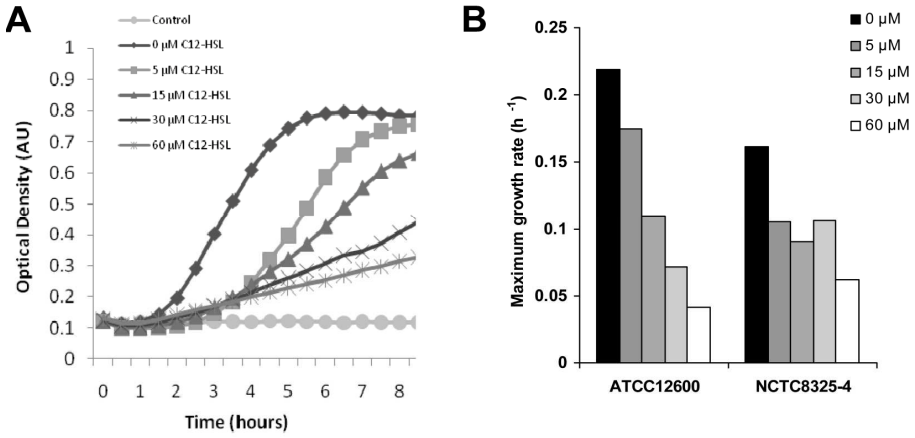

Fig. 2. (A) Typical growth curves of *S. aureus* ATCC12600 grown in the presence of a range of 3OC12HSL concentrations. Growth rates were determined by measuring OD575. Control indicates medium without inoculum. (B) Maximum growth rate of *S. aureus* ATCC12600 and NCTC8325-4 strain under influence of increasing concentration of 3OC12HSL. In A and B data of a representative experiment is shown.

Similar growth inhibition by $3OC_{12}HSL$ was detected for *S. aureus* NCTC8325-4, for which the maximum growth rate was slightly lower than the growth rate of ATCC12600 (Fig. 2B).

$3OC_{12}HSL$ influence on secreted hemolysins is strain dependent

Qazi *et al.* previously reported that $3OC_{12}HSL$ inhibited production of hemolysins α and δ in *S. aureus* (30). We therefore tested the effect of $3OC_{12}HSL$ on hemolysin production in *S. aureus* ATCC12600 and NCTC8325-4. Spent medium placed on sheep blood agar plates resulted in halo formation after incubation (Fig. 3A). S. aureus ATCC12600 revealed increase of a single darkish halo upon exposure to 3OC12HSL up to 15 µM. Addition of 30 µM resulted in a slight decrease of the halo size (Fig. 3B). This dark halo is indicative of α-hemolysin, while the bright halo, indicative of β-hemolysin activity was not observed for ATCC12600. In contrast, S. aureus NCTC8325-4 showed decrease of the outer darkish halo size with rising 3OC12HSL concentration, indicative of inhibition of α-hemolysin. The inhibition was also detectable for the inner transparent halo (β-hemolysin), which disappeared with increasing 3OC12HSL concentration, as reported previously by

Qazi et al. (30). Remarkably, quantification of the diameter of the outer darkish halo revealed that for the ATCC12600 strain the halo size increased, whilst for the NCTC8325-4 the halo size decreased, with the increasing concentration of 3OC12HSL (Fig. 3B).

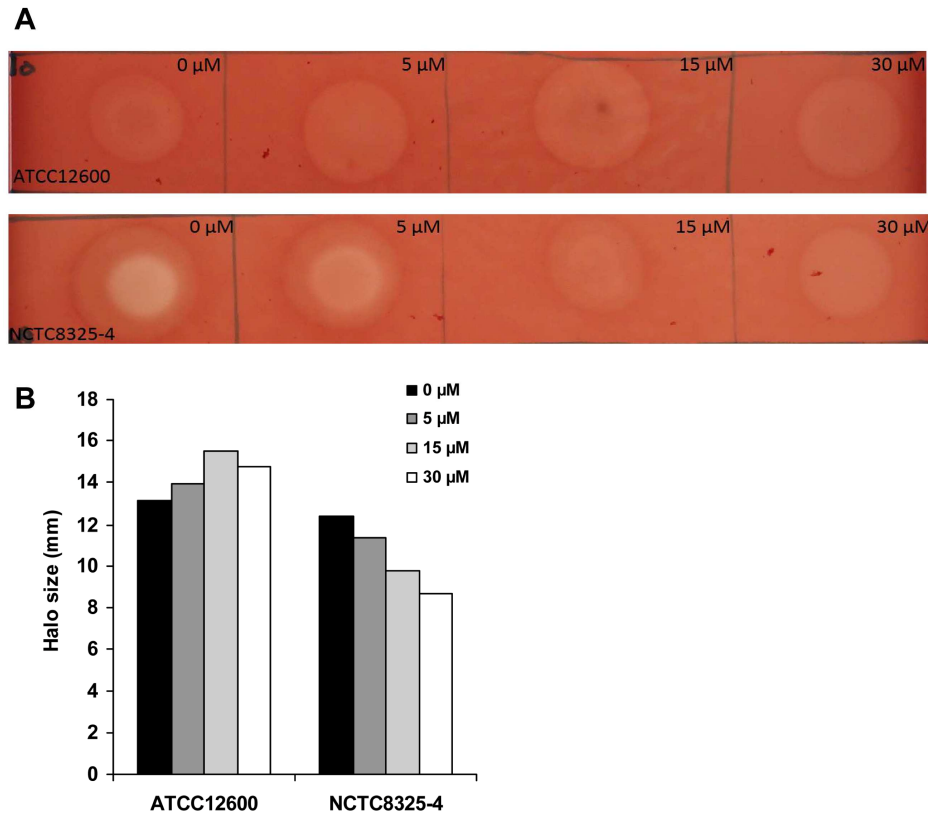

Fig. 3. (A) Hemolysin assay performed on sheep blood agar plates for *S. aureus* ATCC12600 (upper panel) and NCTC8325-4 (lower panel). Droplets of filter sterilized spent medium obtained from the cultures grown with the indicated $3OC_{12}HSL$ concentrations were placed on the plates. A transparent halo represents β-hemolysin while a darkish halo α-hemolysin. (B) Measurement of the cross-section of the outer halos showed an opposite trend in halo size development upon addition of $3OC_{12}HSL$ to *S. aureus* ATCC12600 and NCTC8325-4 culture.

3OC12HSL inhibits protein A expression in ATCC12600 but enhances expression in NCTC8325-4

Cell wall proteins isolated from S. aureus ATCC12600 and NCTC8325-4 were analyzed for protein A presence using Western blotting. For S. aureus ATCC12600, protein A is down-regulated by 15 µM 3OC12HSL (Fig. 4) while, in contrast, for S. aureus NCTC8325-4 an up-regulation of protein A production was observed at the same 3OC12HSL concentration.

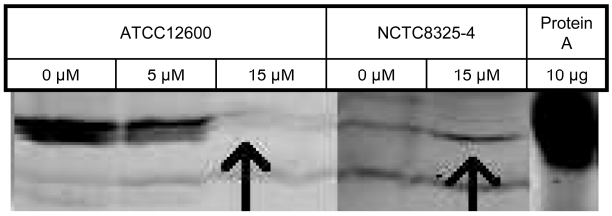

Fig. 4. Western blot analysis of cell wall proteins of *S. aureus* ATCC12600 and NCTC8325-4 in response to $3OC_{12}HSL$. Cell wall proteins were isolated from cultures exposed to different concentrations of $3OC_{12}HSL$ and protein A was specifically detected using a protein A antibody conjugated with infrared dye, scanned on Odyssey infrared scanner. Up- and down-regulated proteins A (around 50 kDa) are indicated with an arrow. A positive control of 10 µg of purified protein A was included.

$3OC_{12}HSL$ influences the secretome of *S. aureus* ATCC12600 and NCTC8325-4 in a strain dependent manner

Secretome analysis of *S. aureus* ATCC12600 exposed to $3OC_{12}HSL$ revealed that expression of some proteins, especially in the region between 20 and 40 kDa was increased with increasing concentrations of $3OC_{12}HSL$ (Fig. 5). Expression of other proteins, especially those with a molecular weight between 50 and 75 kDa were down-regulated at concentrations of 15 and 30 µM $3OC_{12}HSL$. The secretome of *S. aureus* NCTC8325-4 showed an opposite response to $3OC_{12}HSL$ compared to ATCC12600. Expression of proteins of sizes 25 and 30 kDa was inhibited upon addition of $3OC_{12}HSL$, while expression of a 75 kDa protein was activated with increasing concentration of $3OC_{12}HSL$ (Fig. 5).

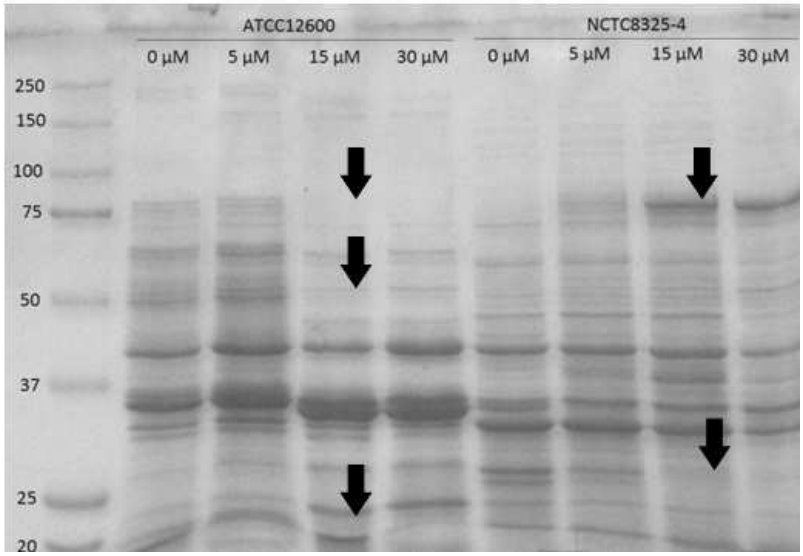

Fig. 5. Secretome of *S. aureus* ATCC12600 and NCTC8325-4. An opposite response to $3OC_{12}HSL$ was observed especially for the proteins of 30 and 75 kDa. Proteins that are markedly up- or down-regulated due to $3OC_{12}HSL$ are indicated with an arrow.

Response of other *S. aureus* strains to $3OC_{12}HSL$ is strains specific

In order to determine if the opposite response to $3OC_{12}HSL$ of *S. aureus* ATCC12600 and NCTC8325-4, both belonging to the AIP-type I, was an exception, we analyzed the response of several other strains to 15 µM $3OC_{12}HSL$. All strains showed significant inhibition of growth by $3OC_{12}HSL$ as indicated by a decrease of the maximum growth rates (Fig. 6). Inhibition of growth by $3OC_{12}HSL$ is not strain dependent.

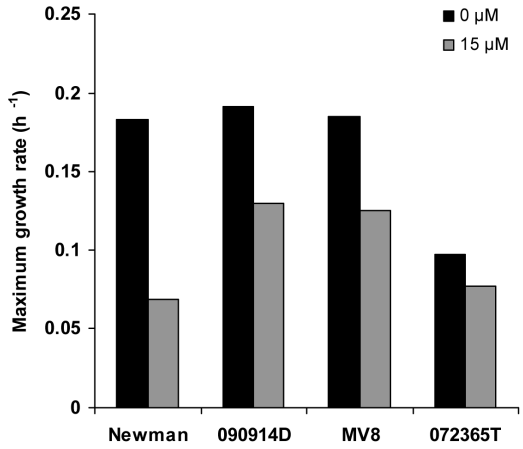

Fig. 6. Maximum growth rates calculated for various *S. aureus* strains exposed to 15 µM $3OC_{12}HSL$. Data of a representative experiment is shown.

In contrast to the effect of $3OC_{12}HSL$ on growth, ATCC12600 and NCTC8325-4 showed a different secretome pattern in response to $3OC_{12}HSL$. We therefore analyzed the secretome response of other *S. aureus* strains to 15 µM $3OC_{12}HSL$. Each strain displayed their own specific secretome pattern in response to $3OC_{12}HSL$ (Fig. 7). *S. aureus* Newman and Col showed increased expression of a protein of 30 kDa, similar to the response of ATCC12600 strain to $3OC_{12}HSL$. *S. aureus* Col showed down-regulation of a 75 kDa protein, also similar to ATCC12600, while in the Newman strain that protein is up-regulated, reflecting a similar response of NCTC8325-4 to $3OC_{12}HSL$.

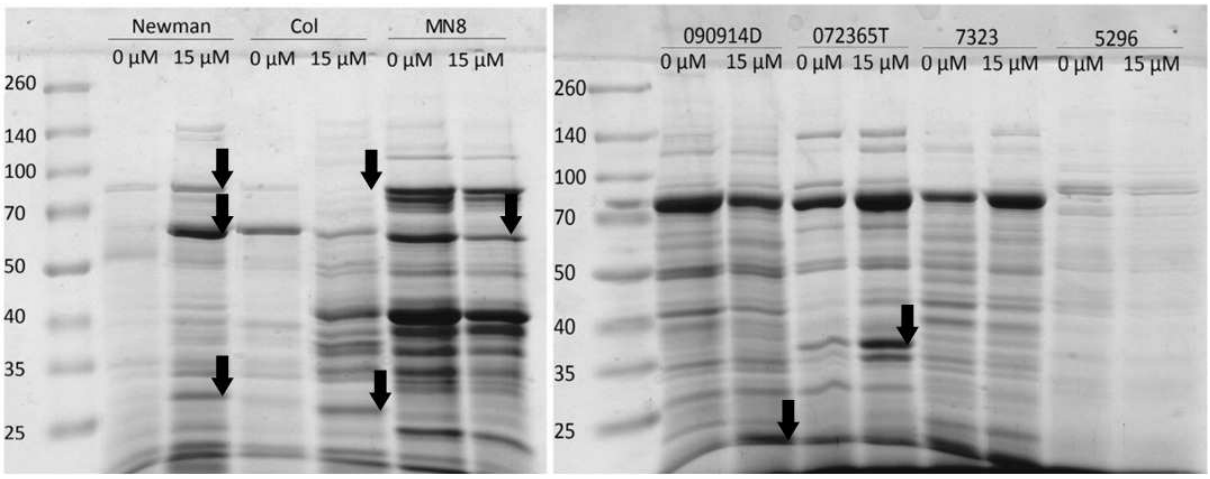

Fig. 7. Secreted proteins of commonly used lab strains and clinical isolates of *S. aureus*. A selection of proteins, that are notably up- or down-regulated in response to 15 µM $3OC_{12}HSL$, are marked with arrows.

Interestingly, expression of a 65 kDa protein in the Newman strain increased upon addition of 15 µM $3OC_{12}HSL$, while for the Col and MN8 strains expression of this protein was reduced. An irregular pattern was shown in the response of the proteins between 30 and 40 kDa. These seem to be up-regulated in NCTC8325-4, Newman and Col, while down-regulated in MN8. For the strains 090914D, 072365T and 7323 the protein of 75 kDa was highly expressed upon addition of $3OC_{12}HSL$. The other secreted proteins of 090914D strain were not changed drastically under influence of $3OC_{12}HSL$. There was however a slight decrease visible for 40 kDa protein and an increase for 20 kDa protein. *S. aureus* 072365T secreted more proteins of 37 and 75 kDa after the addition of 15 µM $3OC_{12}HSL$.The strains 7323 and 5296 showed only slight response on $3OC_{12}HSL$. In conclusion, all secretome patterns were different and there was no clear classification pattern for the secretome response to $3OC_{12}HSL$ among the strains tested.

The effect of acyl-HSL diazirine probe on *S. aureus*

In order to validate the acyl-HSL diazirine probe, the effect of addition of this probe up to 30 µM on the growth-rate of *S. aureus* was determined. The results of the experiments performed with the acyl-HSL diazirine probe on *S. aureus*

ATCC12600 and NCTC8325-4 revealed that there was no growth-rate inhibiting activity (data not shown). We also analyzed the effect of addition of the acyl-HSL diazirine probe on the secretome. None of the tested strains showed a different protein pattern on SDS-PAGE in presence of the acyl-HSL diazirine probe compared to the control (data not shown). We could therefore not validate activity of the acyl-HSL diazirine probe.

DISCUSSION

Interspecies communication has received increasing attention in the past years. The present study describes the effect of the *P. aeruginosa* QS molecule, $3OC_{12}HSL$, on several different *S. aureus* strains. $3OC_{12}HSL$ inhibited growth of all tested *S. aureus* strains, confirming the results described by Qazi *et al.* (30) for a single strain. However, $3OC_{12}HSL$ regulated gene expression in a strain specific manner, most notably production of the cell wall localized protein A, secreted hemolysins and other proteins of the secretome.

Growth-rate inhibition would provide *P. aeruginosa* with a competitive advantage over *S. aureus* allowing it to out-competing that bacterium from a shared ecological niche. At high concentrations of 30 µM and 60 µM $3OC_{12}HSL$ the growth-rate was severely inhibited. Possible cytotoxic effects of $3OC_{12}HSL$ have been reported earlier by Kaufmann *et al.* (16). They showed that growth inhibition of various bacterial species occurred through the breakdown product of $3OC_{12}HSL$, tetramic acid, which is formed upon opening of the homoserine lactone ring (lactonolysis). This reaction is favored by alkaline pH, creating 3-oxo-dodecanoyl-homoserine (3, 39). The precise mechanism of growth inhibition by tetramic acids has previously been suggested to involve destabilization of the cytoplasmic membrane (17). This means that the observed growth inhibition for *S. aureus* is a non-specific response to $3OC_{12}HSL$, i.e. it would not require a receptor protein in the membrane. Alternatively, the response to $3OC_{12}HSL$ on genes expression is specific, as un-substituted acyl-HSLs like $C_{12}HSL$ does not have any effect (30). In our study, the probe that also contains changes in the acyl chain showed no effect.

Further studies are required to confirm a specific or non-specific growth-rate inhibition by $3OC_{12}HSL$.

The secretome response of *S. aureus* to $3OC_{12}HSL$ was strain dependent as illustrated by variable effects on expression of protein A and hemolysins as well as different secretome patterns. The *agr* system in *S. aureus* down-regulates expression of exponential-phase proteins such as protein A, while expression of post-exponential phase proteins such as enterotoxins B, C and D, toxic shock syndrome toxin-1 and hemolysins (5) are up-regulated. Qazi *et al.* (29) showed that RNAIII levels (effectors of *agr* system) were reduced in *S. aureus* RN6390 (essentially the same as NCTC8325-4 strain) exposed to $3OC_{12}HSL$, leading to up-regulation of protein A and down-regulation of hemolysins (30). Our data showed an opposite response for the ATCC12600 strain: down-regulation of protein A and up-regulation of hemolysins. This suggests activation instead of inhibition of RNAIII and thus possibly *agr* or *sarA*. Since *S. aureus* strains can be subdivided in four types that display slight differences in regions *agrB-D-C* (23), therewith the strain specific response on $3OC_{12}HSL$ can be explained by differences in AgrC. This could indicate that in NCTC8325-4 strain $3OC_{12}HSL$ disturbs the binding of AIP to AgrC inhibiting the *agr* system, while ATCC12600 strain could have slightly different AgrC receptors for which $3OC_{12}HSL$ is not able to disturb the binding of AIP, meaning activation of the *agr* system. Another way of disturbing the *agr* system is by interfering with AgrB, the processor of the AIP molecule, which is possibly the case in NCTC8325-4.

S. aureus ATCC12600, NCTC8325-4, Newman and Col all belong to *agr* or AIP type I, while MN8 is an AIP type III strain (19). When comparing the differences in the secreted proteins of type I and type III strains no pattern is observed that completely coincides with the AIP groups.

It is important from the clinical point of view that a strain specific response of *S. aureus* to $3OC_{12}HSL$ can be detected. An elaborate mapping of the response of various strains on $3OC_{12}HSL$ could provide a certain advantage in the steps towards using $3OC_{12}HSL$ as an antibacterial/therapeutic agent (23, 36). Inhibiting virulence is potentially achieved by influencing protein A expression which *S. aureus* uses to protect itself from being phagocytised (10). Protein A deficient mutants have shown

to be phagocytosed more efficiently and display less virulence in animal infection models. $3OC_{12}HSL$ could potentially be used to disturb the anti-opsonic nature of protein A and decrease virulence in the host.

In our attempt to identify the receptor for $3OC_{12}HSL$ using acyl-HSL diazirine probe we tested its activity in comparison to natural $3OC_{12}HSL$. These experiments did not indicate any signs of activity for *S. aureus* ATCC12600 nor NCTC8325-4. The growth-rate was not inhibited and there was no change in the pattern of secreted proteins. This was somewhat unexpected as the probe was previously been shown to have very similar activity compared to the natural $3OC_{12}HSL$ in inhibition of germ tube formation in *Candida albicans* (7), (Chaper 5). The fact that the probe is not active in *S. aureus* suggests that the binding of $3OC_{12}HSL$ and thus the probe is specific among species. The small deviations, such as the diazirine in the alkyl chain of the probe could disturb the binding orientation and therefore its activity for one (*S. aureus*), but not for another (*C. albicans*) species. This suggestion coincides with the report of Kaufmann *et al.* (16) and Qazi *et al.* (30) that the homoserine lactone ring is not necessary for a growth inhibiting effect. The homoserine lactone ring of the probe did not undergo any changes, in contrast to the tail. This generates the idea that the tail chain of the $3OC_{12}HSL$ molecule is necessary for a growth inhibiting response, disturbing the cytoplasmic membrane. Small structural deviations could make the probe potentially more susceptible to hydrolysis resulting in a non-toxic acyl alcohol product (16, 39). Normally created tetramic acid which was shown to have antibacterial properties, formed from the probe degradation might lack its activity or is not formed at all.

In conclusion, the response of *S. aureus* to $3OC_{12}HSL$ is strain specific, but not linked to AIP-type. The acyl chain of $3OC_{12}HSL$ appears to be very important for recognition of $3OC_{12}HSL$ by *S. aureus*. Further understanding of the systems governing $3OC_{12}HSL$ recognition by *S. aureus* could facilitate novel drug development.

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Chapter 9

General discussion

INTRODUCTION

Understanding how quorum sensing (QS) systems function in communication between bacteria and eukaryotes is a big challenge due to the complexity of the system. Deciphering these complex interactions; identification and comparison of QS receptors from different species, especially from different kingdoms, would improve our understanding as it can provide insight into the evolutionary development of interspecies communication.

The studies described in this tahesis focuse on unraveling of QS mediated interspecies and interkingdom communication, which occurs between bacteria, but also between bacteria and eukaryotic cells, in this thesis represented by *Candida albicans*. The human body provides suitable growth conditions for many microorganisms, commonly organized in multispecies biofilms, where *C. albicans* constitutes a serious threat. This fungus is an opportunistic pathogen and a growing problem for immunocompromised individuals. *C. albicans* often exists in multispecies biofilms where QS molecules are crucial. Not surprising, the role of QS molecules in interspecies communication has received increasing attention over the past years.

Interkingdom communication using quorum sensing molecules

The human body constitutes several places of complex interspecies communication. For instance in the oral cavity Gram-positive commensals frequently encounter Gram-negative pathogens. Furthermore, fungi and the host represent the cross-kingdom counterpart in such interactions. Competence stimulating peptide (CSP) produced by the Gram-positive bacterium *Streptococcus mutans* (**Chapter 3**), and autoinducer-2 (AI-2) produced by the Gram-negative bacterium *Aggregatibacter actinomycetemcomitans* (**Chapter 4**) exert an inhibiting effect on *C. albicans* germ tubes and biofilm formation. Interestingly, AI-2 of *Streptococcus gordonii* had an opposite effect on *C. albicans*. This could be due to the different molecular conformations that AI-2 can have, and would indicate that the receptor in *C. albicans* is able to discriminate at least two different conformations of AI-2 and respond oppositely. Alternatively, *C. albicans* can have two receptors that are

specific for either AI-2 conformation. In order to answer this question, identification of the receptor(s) is necessary.

Another example of a complex niche is represented by burn-wound infections, in which *Staphylococcus aureus* and *C. albicans* regularly meet *Pseudomonas aeruginosa*. The Gram-negative opportunistic pathogen *P. aeruginosa* secretes N-(3-oxododecanoyl) homoserine lactone ($3OC_{12}HSL$), a QS molecule that represses *C. albicans* germ tubes formation (2). *P. aeruginosa* is also able to inhibit growth and modify gene expression of *S. aureus* but the latter response is strain specific (**Chapter 8**). The question remains whether the growth response of *S. aureus* is a non-specific response, in which the 3OC12HSL receptor is not involved and if the modulation of gene expression, as exemplified by the differences in secretome response involves specific receptors.

Presence of a receptor is supported by previous work of Qazi *et al.*, who have illustrated saturatable binding of 3OC12HSL to *S. aureus* membranes (7). Again, identification of a receptor would help answer these questions.

Identification of the receptors for quorum sensing molecules using a biomimetic probe

Identification of QS receptors would elucidate the mechanisms and pathways involved in interspecies interactions, and possibly clarify some of the unexpected results mentioned below. We therefore aimed at finding the receptors for $3OC_{12}HSL$ using a diazirine probe, designed to mimic the natural $3OC_{12}HSL$ molecule (**Chapter 5**). As a proof of concept the probe was optimized on the model strain *P. aeruginosa ΔlasI* (**Chapter 6**) which allowed us to find the receptor in *C. albicans* model for $3OC_{12}HSL$ (**Chapter 7**). The probe was also tested on *S. aureus* strains but did not have any influence on the growth rate or on the secretome (**Chapter 8**), in contrast to previous data showing that the probe had a very similar activity compared to the natural $3OC_{12}HSL$ in *P. aeruginosa* and *C. albicans* (**Chaper 5**). This could suggest that the binding of $3OC_{12}HSL$ and thus the probe is specific among species. The small deviations, such as the diazirine in the alkyl chain of the probe could disturb the binding orientation and therefore its activity for one (*S. aureus*), but not for another (*C. albicans*, *P. aeruginosa*) species.

These differences in activity of the probe in different species could mean that such receptors are not homologous and only distantly related. The structure of the probe and its modification appear to be important factors and needs to be carefully tested for each species.

Difficulties encountered while studying interspecies communication and methods of improvement

As described in this thesis, interspecies communication is ubiquitous and its clinical importance is dependent on the niche and site of infection. Investigation of clinical importance of interspecies communication brings many difficulties and requires a good model for testing. Like many models, they should mimic natural conditions as close as possible and include naturally existing factors. Implementation of those circumstances, combined with multiple species of microbes in presence and absence of QS molecules is complicated, often hard to achieve and analyze.

In our research we limited interspecies communication to two organisms mixed *in vitro* in one culture, or mixed with synthetic QS molecules. We did not perform experiments *in vivo*, although that would be the best model. Next studies could involve *Caenorhabditis elegans* as a host for *C. albicans*, *P. aeruginosa* and *S. aureus* infections, describing specific defense systems and multifactorial transcriptional responses (1, 4, 6, 8). Even more complex but highly relevant would be the use of an *in vitro* burn-wound infection model e.g. skin discarded during abdominoplasty would be burned and infected by *C. albicans*, *P. aeruginosa* and *S. aureus*. This model would amend the use of QS mutants and exogenous addition of synthetic QS molecules in such a way that the host immune response would be partly intact.

The second part of that thesis studying the probe also brought some difficulties within the designed methodology (**Chapter 6**). While we were able to label and tag several proteins, identification was unsuccessful, probably due to a low abundance of the protein (illustrating the high sensitivity of our approach). To allow receptor identification, the described method needs further optimization by enriching for labeled and tagged proteins. This could be achieved for instance by

partial purification of labeled and tagged proteins using affinity chromatography, or direct purification of alkyne-probe-labeled proteins to engineered clickable azide beads. These examples present feasible solutions to the problem of low protein abundance and need to prove their value in future experiments.

Future perspectives

Discovery of QS receptor in eukaryotic organism is of high importance because of its possible role in e.g. inhibition of virulence. That mechanism may be represented by the inhibiting effect exerted by *P. aeruginosa* on *C. albicans* where virulence of that fungus is reduced. In addition, other eukaryotic cells are able to detect the *P. aeruginosa* QS molecule (**Chapter 1**), most notably cells from the human immune system (3). Understanding the complicated interkingdom communication is therefore of clear fundamental and medical significance. As reported before, eukaryotic and bacterial signaling systems revealed many common features within protein-receptor complexes, transducers, effectors and autoinducing molecules (5). Considering that, our methodology might assist in studying homology of proteins and unraveling the co-evolution of eukaryotic and bacterial cells.

The study performed and methodology developed in this thesis may contribute in the future to clinical research where attenuation of human pathogens would be feasible by blocking and influencing QS receptors, modifying QS molecules and therefore decreasing virulence. It could be a new step towards the use of QS molecules as antibacterial agents.

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Summary

Interspecies cross-talk is an attractive subject for many researchers due to its fundamental nature but also the clinical relevance. In this thesis we studied some aspects of interspecies communication between eukarya and prokarya. We focus on *Candida albicans* as a representative of eukarya, communicating with various Gram-positive and Gram-negative bacteria, each using a range of structurally distinct signaling molecules. This thesis contains two parts; in the first chapters we describe development of an assay to study the effect of bacterial small molecules on *C. albicans* hypha formation (Chapter 2) and identification of two additional small bacterial quorum sensing (QS) molecules that affect hypha formation (Chapter 3&4). In the second part (Chapters 5-8) we describe development and validation in different species (*Pseudomonas aeruginosa*, *C. albicans* and *Staphylococcus aureus*) of a newly designed methodology using a biomimetic molecule that can be used to identify the receptors for QS molecules.

Chapter 1 reviews microbial capabilities to obtain, encrypt and share information based on 3-oxo-C12-homoserine lactone ($3OC_{12}HSL$), a small molecule secreted by *P. aeruginosa*. This molecule is sensed by a number of other microbes (for instance *S. aureus* and *C. albicans*) as well as by the host immune system (most notably macrophages). Here we discussed the role of sharing encrypted information among microbes at different levels in relation to clinical infections. Further, in light of specialized systems within interspecies communication we stated the two-fold aim of this thesis: 1) to identify non-HSL bacterial QS molecules that affect *C. albicans* biology and 2) to develop a new approach based on a biomimetic molecule, to identify specific receptors for bacterial QS molecules.

Compounds that affect the yeast to hypha transition have been subject of many studies. Traditional microscopy-based analysis of compounds is elaborated and testing of large numbers of compounds that affect the yeast to hypha transition would benefit from a fast and efficient method for activity screening. Therefore, in **Chapter 2** we propose a rapid screening method using a real time thermocycler in combination with *C. albicans* GFP reporter strains. Using P_{ACT1}-GFP and P_{HWP1}-GFP as reporters for growth and hypha formation, respectively, we showed that for a wide range of known compounds the effect of growth and hypha formation could be assessed in a quantitative fashion within 3 h after inoculation. Importantly, not

only repression but also enhancement of hypha formation could be assessed as demonstrated with the QS molecule tyrosol.

C. albicans is commonly found in the human oral cavity where it interacts with S*treptococcus mutans*, a Gram-positive bacterium. The aim of **Chapter 3** was to investigate inter-kingdom communication between these two microorganisms, based on production of secreted molecules. *S. mutans* UA159 inhibited *C. albicans* germ-tube (GT) formation in co-cultures, even when physically separated from *C. albicans*. Only spent media of *S. mutans* that was collected in the early exponential phase (4 h-old cultures) inhibited GT formation of *C. albicans*. During this phase, *S. mutans* UA159 produces a QS molecule, competence stimulating peptide (CSP). The role of CSP in inhibiting GT formation was confirmed using synthetic CSP and a *comC* deletion strain of *S. mutans* UA159, which lacks the ability to produce CSP. Other *S. mutans* strains and other *Streptococcus spp.* also inhibited GT formation but to different extents, possibly reflecting differences in CSP amino acids sequences among *Streptococci spp.* or differences in the level of CSP accumulation in the media. This study demonstrated that CSP, a *S. mutans* quorum sensing molecule secreted during the early stages of growth, inhibited the *C. albicans* morphological switch.

Another example of interaction within the oral microflora, occurring between *C. albicans* and the Gram-negative bacterium *Aggregatibacter actinomycetemcomitans*, was described in **Chapter 4**. A QS molecule produced by *A. actinomycetemcomitans* named AI-2, synthesized by LuxS, inhibited *C. albicans* hypha- and biofilm formation. *C. albicans* biofilm formation increased significantly when co-cultured with *A. actinomycetemcomitans luxS*, lacking AI-2 production. Addition of wild-type-derived spent medium or adding synthetic AI-2 to spent medium of the *luxS* strain, restored inhibition of *C. albicans* biofilm formation to wild-type levels. Addition of synthetic AI-2 significantly inhibited hypha formation of *C. albicans* possibly explaining the inhibition of biofilm formation.

In **Chapter 5** we describe a new approach to identify specific receptors for N-acyl homoserine lactones (AHLs) using a biomimetic probe was described. An AHL analog was designed with minimal structural changes in the alkyl chain to increase chances of recognition. In this approach the probe covalently linked to its

cognate receptor through irradiation of a photoactive moiety. This receptor-probe complex is subsequently labeled with a cycloaddition partner, which itself carries a fluorescent tag such as biotin or rhodamine. This enabled isolation of the probe-bound protein, followed by identification by mass spectrometry. The probe was tested for its ability to mimic natural $3OC_{12}HSL$ in an *Escherichia coli* reporter strain, *P. aeruginosa*, macrophages and *C. albicans* what revealed a very similar inhibiting effect as with the natural molecule. Furthermore, using the probe heterologous expressed LasR could be covalently and specifically labeled. This study highlights the potential of the biomimetic probe to isolate and identify putative receptors that binds $3OC_{12}HSL$ in eukaryotic cells.

The methodology developed in Chapter 5 was optimized in **Chapter 6** using a *P. aeruginosa lasI* mutant unable to produce $3OC_{12}HSL$. The receptor for $3OC_{12}HSL$ in *P. aeruginosa* is LasR and therefore, as proof-of-principle of our methodology, we aimed to detect that protein and possibly other $3OC_{12}HSL$-binding proteins. Optimal conditions for probe photoactivation, labeling and tagging were determined. We separated cytosolic proteins bound with the probe and tagged with tetramethylrhodamine (TMR) on 2D gel electrophoresis, which resulted in imaging of several unique proteins. Furthermore we tried to identify proteins with MS/MS but without success, probably due to low abundance.

In **Chapter 7** the optimized protocol for detecting probe-labeled proteins using TMR was applied to *C. albicans* SC5314 to identify the receptor for $3OC_{12}HSL$. The results showed the existence of the unique protein localized in the membrane of *C. albicans*, which is the putative receptor for $3OC_{12}HSL$. Specificity and affinity of probe binding was provisionally studied, however further optimization steps are needed to complete this research.

The effect of $3OC_{12}HSL$ on *S. aureus* is described in **Chapter 8**. It was shown that growth of *S. aureus* is inhibited by $3OC_{12}HSL$ in all tested strains in a concentration dependent manner. The secreted protein profiles of *S. aureus* were influenced by addition $3OC_{12}HSL$ in a strain specific manner. When *S. aureus* ATCC12600 was compared with *S. aureus* NCTC8325-4 an opposite response to $3OC_{12}HSL$ in the hemolysis assay and expression of protein A was revealed. This research suggests that $3OC_{12}HSL$ influences the *agr* system of *S. aureus* in a strain

specific manner directly or indirectly. Finally, the biomimetic analog of $3OC_{12}HSL$, with the potential to be used for identification of the receptor for $3OC_{12}HSL$, was tested but did not show comparable activity as the natural $3OC_{12}HSL$.

In **Chapter 9**, the general discussion, aspects and methods of studying interspecies communications using small signaling molecules are disserted.

Samenvatting

Cross-talk tussen soorten is een aantrekkelijk onderwerp voor veel onderzoekers door de fundamentele maar ook de klinische relevantie van het onderwerp. In dit proefschrift hebben we een aantal aspecten van interspecies communicatie tussen eukarya en prokarya bestudeerd. We hebben ons gericht op *Candida albicans* als vertegenwoordiger van eukarya, die communiceert met verschillende Gram-positieve en Gram-negatieve bacteriën, die elk een verscheidenheid aan structureel verschillende signaal moleculen gebruiken. Dit proefschrift bestaat uit twee delen; in de eerste hoofdstukken beschrijven we de ontwikkeling van een methode om het effect van bacteriële moleculen op hyfe vorming van *C. albicans* te bestuderen (Hoofdstuk 2) en identificatie van twee nieuwe bacteriële quorum sensing (QS) moleculen die hyfe vorming beïnvloeden (Hoofdstukken 3&4). In het tweede deel (Hoofdstukken 5-8) beschrijven we de ontwikkeling en validatie, in verschillende soorten (*Pseudomonas aeruginosa*, *C. albicans* en *Staphylococcus aureus*), van een nieuw ontworpen methodologie om receptoren voor QS moleculen te identificeren, welke gebruik maakt van een biomimetisch molecuul.

Hoofdstuk 1 geeft een overzicht van de microbiële mogelijkheden om informatie te verkrijgen, versleutelen en delen. Dit wordt gedaan op basis van 3-oxo-C12-homoserine lacton ($3OC_{12}HSL$), een QS molecuul van *P. aeruginosa*. Dit molecuul wordt door een aantal andere micro-organismen waar genomen (bijvoorbeeld *S. aureus* en *C. albicans*) maar ook door het immuunsysteem van de gastheer (bijvoorbeeld macrofagen). We bediscussiëren de rol van uitwisseling van versleutelde informatie tussen micro-organismen op verschillende niveaus in relatie tot het infectie proces. Vervolgens wordt met het oog op de gespecialiseerde systemen voor interspecies communicatie een tweevoudig doel van dit proefschrift geformuleerd: 1) identificatie van niet-HSL bacteriële QS moleculen die de biologie van *C. albicans* beïnvloeden en 2) ontwikkeling van een nieuwe manier, gebruikmakend van biomimetische moleculen, om specifieke receptoren voor bacteriële QS moleculen te identificeren.

Stoffen die de gist naar hyfe transitie beïnvloeden zijn veel bestudeerd. Analyse van dit proces op basis van traditionele microscopie is arbeidsintensief. Het testen van de activiteit van een grote hoeveelheid verbindingen die de gist naar hyfe transitie beïnvloeden zou baat hebben bij de ontwikkeling van een snelle en

efficiënte methode. Daarom wordt in **Hoofdstuk 2** een snelle screenings methode voorgesteld die gebruik maakt van een real time thermocycler in combinatie met GFP reporter stammen van *C. albicans*. Door gebruik te maken van P_{ACT1}-GFP en P_{HWP1}-GFP als reporters voor respectievelijk groei en hyfe vorming, is aangetoond dat voor een serie bekende stoffen het effect op groei en hyfe vorming bepaald kan worden op kwantitatief niveau binnen 3 uur na inoculatie. Voor het bekende QS molecuul tyrosol is aangetoond dat zowel remming als versnelling van hyfe vorming kan worden gemeten.

C. albicans wordt vaak aangetroffen in de mondholte van mensen waar het contact heeft met *Streptococcus mutans*, een Gram-positieve bacterie. Het doel van **Hoofdstuk 3** was om de communicatie op basis van QS moleculen tussen deze twee micro-organismen te onderzoeken. *S. mutans* UA159 remde hyfe-vorming van *C. albicans* in co-cultures, zelfs wanneer de organismen fysiek gescheiden waren door een membraan. Alleen gebruikt medium van vroeg-exponentieel groeiende *S. mutans* cultures (4 uur oude cultures) remde hyfe-vorming van *C. albicans*. Tijdens deze fase van groei produceert *S. mutans* UA159 een QS molecuul genaamd "competence stimulating peptide" (CSP). De rol van dit molecuul in de remming van hyfe vorming van *C. albicans* werd bevestigd door gebruik te maken van synthetisch CSP en een *comC* deletie mutant van *S. mutans* UA159, welke geen CSP meer kan maken. Andere *S. mutans* stammen en andere *Streptococcus* soorten remde hyfe-vorming van *C. albicans* ook, maar tot verschillende niveaus. Dit zou kunnen komen door de verschillen tussen *Streptococcus* soorten in aminozuur volgorde van CSP of verschil in hoeveelheid CSP dat in het groeimedium terecht komt. Deze studie bewijst dat CSP, een QS molecuul van *S. mutans* dat vroeg in de groei geproduceerd wordt in staat is om hyfe-vorming van *C. albicans* te remmen.

De interactie tussen *C. albicans* en de Gram-negatieve bacterie *Aggregatibacter actinomycetemcomitans*, eveneens micro-organismen uit de mondholte is beschreven in **Hoofdstuk 4**. AI-2, een QS molecuul dat door *A. actinomycetemcomitans* geproduceerd wordt door middel van LuxS, gaf remming van hyfe- en biofilm vorming van *C. albicans*. *C. albicans* biofilm vorming nam significant toe in co-cultures met *A. actinomycetemcomitans luxS*, (zonder AI-2 productie) in vergelijking met het wild-type. Toevoeging van gebruikt medium van

het wild-type, of toevoeging van synthetisch AI-2 aan het gebruikte medium van de *luxS* stam, herstelde de remming van *C. albicans* biofilm vorming tot vergelijkbare niveaus als gevonden voor co-cultures met het wild-type. Toevoeging van synthetisch AI-2 gaf een significante remming van hyfe-vorming van *C. albicans* en is mogelijk de verklaring voor de gevonden remming van biofilm vorming.

Een nieuwe methode om specifieke receptoren voor N-acyl homoserine lactonen (AHLs) te identificeren middels synthese en validatie van een biomimetische probe staat beschreven in **Hoofdstuk 5**. We hebben een AHL analoog ontworpen met minimale structurele afwijking in de alkyl keten om de kans op herkenning door de receptoren zo groot mogelijk te maken. In deze aanpak wordt de probe eerst gebonden aan zijn receptor door activatie van een lichtgevoelige groep. Dit probe-receptor complex ondergaat een reactie met een cyclo-additie partner, die zelf weer gebonden is aan een "tag" zoals bijvoorbeeld een biotine of rhodamine groep. Hierdoor kon probe-gebonden eiwit geïsoleerd worden en vervolgens geïdentificeerd worden met behulp van massa spectrometrie. De probe is getest op de mogelijkheid om natuurlijk $3OC_{12}HSL$ te vervangen in *Escherichia coli* pSB1075 als reporter stam, *P. aeruginosa*, macrofagen en *C. albicans* wat een zeer vergelijkbaar remmend effect liet zien vergeleken met het natuurlijke molecuul. Kunstmatig in *E. coli* tot expressie gebrachte LasR kon ogenschijnlijk covalent en specifiek worden gelabeld met de probe. Deze studie toont het potentieel van de biomimetische probe om $3OC_{12}HSL$ receptoren van eukaryoten te isoleren en identificeren.

In **Hoofdstuk 6** is de in Hoofdstuk 5 ontwikkelde methodologie geoptimaliseerd in een *lasI* mutant van *P. aeruginosa* met een defect in productie van $3OC_{12}HSL$. LasR is eerder al beschreven als de receptor voor $3OC_{12}HSL$ in *P. aeruginosa*. De probe is gebruikt om LasR en andere $3OC_{12}HSL$-bindende eiwitten te identificeren. De beste condities voor licht activatie van de probe, labeling en "tagging" van het probe-receptor complex zijn bepaald. Vervolgens zijn cytosolische eiwitten gebonden met probe en getagged met tetramethylrhodamine (TMR) gescheiden met behulp van 2D gel elektroforese wat resulteerde in de visualisatie van unieke eiwitten. Verder is geprobeerd om de eiwitten te identificeren met

behulp van MS/MS maar zonder succes, waarschijnlijk door een zeer lage concentratie van de eiwitten.

Het geoptimaliseerde protocol voor weergave van eiwitten die met de probe gelabeld en vervolgens getagged konden worden hebben we toegepast op *C. albicans* SC5314 om daarin de receptor voor $3OC_{12}HSL$ te vinden (beschreven in **Hoofdstuk 7**). De resultaten toonden de aanwezigheid aan van een in de membraan gelokaliseerd eiwit in *C. albicans* wat waarschijnlijk de receptor voor $3OC_{12}HSL$ is. Specificiteit en affiniteit van de binding zijn kort bestudeerd echter verdere optimalisatie is nodig om dit onderzoek af te ronden.

Het effect van $3OC_{12}HSL$ op *S. aureus* wordt beschreven in **Hoofdstuk 8**. Daar wordt aangetoond dat de groei van *S. aureus* geremd wordt door $3OC_{12}HSL$ op een concentratie afhankelijke manier, in alle stammen die getest werden. Uitscheiden van eiwitten door *S. aureus* wordt ook door $3OC_{12}HSL$ beïnvloed en de manier waarop dat gebeurt is stam afhankelijk. Een vergelijking van *S. aureus* ATCC12600 met *S. aureus* NCTC8325-4 liet een tegengestelde reactie op $3OC_{12}HSL$ zien in de hemolyse assay en in expressie van een oppervlakte eiwit: protein A. Dit onderzoek suggereert dat $3OC_{12}HSL$ het *agr* systeem van *S. aureus* beïnvloed in een stam-afhankelijke manier, ofwel rechtstreeks, of indirect. Tenslotte, is de biomimetische probe voor $3OC_{12}HSL$, gebruikt om de receptor voor $3OC_{12}HSL$ te identificeren, maar de probe bleek niet dezelfde activiteit te hebben als het natuurlijke $3OC_{12}HSL$.

In **Hoofdstuk 9**, de algemene discussie, worden enkele aspecten en methodes om interspecies communicatie middels QS moleculen te bestuderen besproken.

Streszczenie

Wyjaśnienie molekularnej komunikacji między mikroorganizmami

Opisana w tej książce praca doktorska z dziedziny mikrobiologii molekularnej dotyczy zjawiska quorum sensing które zostało zbadane na poziomie molekularnym. W celu zrozumienia tej pracy chciałabym najpierw wyjaśnić podstawowe pojęcia:

Co to jest mikrobiologia?

Mikrobiologia jest działem biologii, który dotyczy mikroorganizmów takich jak bakterie, grzyby i niektóre pierwotniaki. W mojej pracy badałam różne gatunki bakterii oraz jeden gatunek grzyba. Te dwa mikroorganizmy znacznie się od siebie różnią, gdyż bakterie są jednokomórkowymi organizmami bez jądra komórkowego, które należą w systemie klasyfikacji do królestwa *Prokaryota* (z greki *pros* - przed i *karyon* - jądro). Grzyby są natomiast wielokomórkowymi organizmami i należą do *Eukaryota* (z greki *eu* - prawdziwy i *karyon* - jądro), są też bardziej zaawansowane ewolucyjnie od bakterii gdyż zawierają jądro komórkowe.

Co to jest mikrobiologia molekularna?

Mikrobiologia molekularna jest to dział mikrobiologii który zajmuje się badaniami na poziomie molekuł czyli cząsteczek oraz oddziaływań między nimi. Przykładem takich cząsteczek są białka, które stanowią podstawowy budulec każdej komórki. Budowa białek jest bardzo złożona gdyż są one tak zwanymi biopolimerami zbudowanymi z aminokwasów połączonych wiązaniami peptydowymi. Białka mają różne wielkości, tzw. masa molekularna białka; pełnią one też wiele różnych funkcji, niektóre z nich są receptorami, które odbierają sygnały z otoczenia. W mikrobiologii molekularnej często stosuje się techniki biochemiczne do odkrywania i identyfikacji nowych, nieznanych białek.

Co to jest quorum sensing?

Słowo quorum (z łaciny *quorum - praesentia sufficit*) oznacza minimalną liczbę członków określonego zgromadzenia, niezbędną żeby rozpocząć działanie lub obrady. Quorum sensing jest to zjawisko odkryte w latach 70 XX wieku u bakterii *Vibrio fischeri*, która żyje na hawajskiej kałamarnicy *Euprymna scolopes* i umożliwia jej świecenie (bioluminescencje). Quorum sensing określa sposób porozumiewania się ze sobą mikroorganizmów za pomocą wysyłanych cząsteczek sygnalnych. Żeby ta komunikacja była możliwa w środowisku musi być określona liczba komórek, inaczej mówiąc musi być odpowiednie zagęszczenie populacji. Wtedy sąsiadujące komórki zaczynają wysyłać sygnały w postaci różnych cząsteczek sygnalnych. Cząsteczki te gromadzą się w otoczeniu żeby kolejno zostać rozpoznane i odczytane przez inne komórki. Na poziomie molekularnym odbywa się to w ten sposób, że receptory znajdujące się w każdej komórce łączą się z pasującymi do nich cząsteczkami, co działa tak jak mechanizm klucza i zamka. Po włożeniu klucza do zamka rozpoczyna się pewien proces: otwierają się drzwi. W komórce dzieje się podobnie. Receptor z przyłączonym do niego związkiem sygnalnym rozpoczyna cały cykl reakcji prowadzący do ekspresji określonych genów, co dalej powoduje powstawanie nowych białek.

Zjawisko quorum sensing jest bardzo ważne w świecie mikroorganizmów, gdyż odpowiada za produkcje antybiotyków, toksyn, produktów metabolicznych, bioluminescencje (wytwarzanie światła) oraz formowanie biofilmu (zbita kolonia różnych gatunków mikroorganizmów ściśle przyklejona do podłoża, ułatwiająca im przeżycie, np. płytka nazębna).

Jeszcze do niedawna sądzono że quorum sensing zachodzi jedynie między mikroorganizmami z tego samego gatunku, lecz to stwierdzenie jest mylne, gdyż bakterie różnych gatunków występujące w tym samym miejscu, np. w jamie ustnej mogą się ze sobą komunikować. Odkryto nawet że komunikacja ta łamie granice królestwa *Prokaryota* i odbywa się pomiędzy bakteriami i grzybami należącymi do *Eukaryota*.

Badanie zjawiska quorum sensing jest bardzo atrakcyjnym tematem nie tylko ze względu na jego fundamentalny charakter, lecz także możliwość zastosowania w medycynie i w odkryciu nowych leków lub hamowaniu chorobotwórczości.

Wszystkie mikroorganizmy opisane w niniejszej pracy żyją w organizmie człowieka i mogą u niego wywołać chorobę lecz nie muszą, są to tak zwane patogeny oportunistyczne. Podczas mojej pracy skupiłam się na odkrywaniu w jaki sposób komunikują się te bakterie pomiędzy sobą oraz jak przebiega komunikacja pomiędzy tymi bakteriami a grzybem *Candida albicans*.

C. albicans należy do drożdżaków, które wywołują oportunistyczne zakażenia u ludzi z obniżoną odpornością (np. z HIV/AIDS, po radioterapii lub antybiotykoterapii). U większości ludzi grzyb ten należy do normalnej flory fizjologicznej np. pochwy u kobiet i jest wówczas niegroźny lecz gdy nadarzą się sprzyjające dla niego warunki, np. pH pochwy zmieni się z kwaśnego na zasadowy ze względu na przebytą chorobę i osłabienie lub ciążę, może dojść do stanu zapalnego i infekcji. *C. albicans* jest grzybem polimorficznym (z greki *poly* -wiele, *morfe* - kształt) i może występować w wielu postaciach, takich jak drożdże (okrągłe komórki) i strzępki (wydłużone, rozgałęzione komórki). Przemiana pomiędzy stadium drożdży a strzępek odpowiedzialna jest za chorobotwórczość i zachodzi w określonych warunkach: podwyższona temperatura i pH. Uważa się też że postać drożdży jest formą ochronną grzyba, pod którą może on przetrwać niekorzystne warunki bądź atak innego organizmu. Z medycznego punktu widzenia ważne jest zahamowanie tworzenia się strzępek i tym samym ograniczenie chorobotwórczości tego grzyba. *C. albicans* również wykorzystuje zjawisko quorum sensing do komunikacji. Cząsteczką sygnalną produkowaną przez tego grzyba jest m.in. farnesol.

Podczas moich badań pracowałam nad wieloma gatunkami bakterii Gram-dodatnich oraz Gram-ujemnych, które są zdolne do produkcji różnorodnych strukturalnie cząsteczek sygnalnych. Bardzo ważnym aspektem w mojej pracy jest quorum sensing bakterii *Pseudomonas aeruginosa* (pałeczka ropy błękitnej) która produkuje $3OC_{12}HSL$ lakton. Cząsteczka ta wywiera hamujący wpływ na formowanie strzępek grzyba *C. albicans*, który pod jej wpływem występuje w formie drożdży, co jest formą obronną. Grzyb ten prowadzi rodzaj swoistej wojny z bakterią *P. aeruginosa* i również może ją atakować wysyłając farnesol co z kolei powoduje zahamowanie toksyczności tej bakterii.

Negatywny wpływ $3OC_{12}HSL$ laktonu jest wywierany nie tylko na *C. albicans* ale też na różne bakterie które występują w sąsiedztwie *P. aeruginosa*, takie jak *Staphylococcus aureus* (gronkowiec złocisty) oraz na układ immunologiczny (**rozdział 1**, Fig. 2). Przykładem takiej sytuacji może być infekcja rany oparzeniowej, którą niestety jest bardzo trudno leczyć.

W **rozdziale 2** opisujemy szybką i wydajną metodę testowania związków, które wywierają hamujący bądź indukujący wpływ na formowanie strzępek u *C. albicans*. Metoda ta z użyciem *C. albicans* GFP (zielone fluorescencyjne białko połączone z genem odpowiadającym za powstawanie strzępek) oraz sprzętu PCR do odczytywania sygnału fluorescencyjnego jest metodą niezależną od obserwatora w przeciwieństwie do tradycyjnej metody mikroskopowej.

Kolejne rozdziały tej pracy opisują badania nad efektami bakteryjnych cząsteczek sygnalnych na formowanie strzępek u *C. albicans* takimi jak CSP produkowane prze *Streptococcus mutans* (paciorkowiec przyczyniający się do próchnicy zębów) oraz AI-2 produkowane przez inne bakterie wchodzące w skład flory jamy ustnej. Oba te związki hamują powstawanie strzępek *C. albicans* co zostało przedstawione w **rozdziale 3** i **4**.

Dalsza część tej pracy przedstawia syntezę sztucznej cząsteczki $3OC_{12}HSL$ (**rozdział 5**). Cząsteczka ta funkcjonuje tak samo jak naturalna produkowana przez *P. aeruginosa*, lecz ma małe różnice w strukturze (Fig. 1). Została ona tak zaprojektowana żeby umożliwić jej lokalizację po przyłączeniu się do białka-receptora i tym samym identyfikacje tego receptora (abstrakt). **Rozdział 6** opisuje użycie tej sztucznej cząsteczki u *P. aeruginosa* niezdolnej do produkcji naturalnego $3OC_{12}HSL$ laktonu. Doświadczenia zostały zaprojektowane w ten sposób żeby po dodaniu do kultury bakteryjnej sztucznego $3OC_{12}HSL$, udowodnić, że cząsteczka ta, pomimo swoich modyfikacji łączy się z przeznaczonym dla niej białkiem-receptorem, nazywanym LasR.

W **rozdziale 7** stosujemy powyżej opisaną technikę u *C. albicans* w celu identyfikacji nieznanego receptora dla cząsteczki $3OC_{12}HSL$ (Fig. 1 i 2).
Ostatni doświadczalny **rozdział 8** opisuje efekt wywierany przez $3OC_{12}HSL$ lakton na *S. aureus*. Cząsteczka ta wpływa na syntezę wielu białek tej bakterii, hamująco lub pobudzająco, co zależy od szczepu bakteryjnego.

W **rozdziale 9** przedstawiona jest ogólna dyskusja dotycząca metod i aspektów poszczególnych rozdziałów dotyczących studiowania komunikacji między mikroorganizmami.

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