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Fluorescent Labeling Agents for Quorum-Sensing Receptors (FLAQS) in Live Cells

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Live-cell fluorescent labeling of bacterial cell-to-cell communication pathways has emerged as a challenging problem. The use of labeled ligands with a fluorescent tag represents the simplest method of choice. However, such approaches are often accompanied by insufficient binding affinity towards the receptor and competition problems with the natural agonists. Herein, the first example of a fluorescently labeled bacterial quorum-sensing signaling compound is presented. The synthetic labeling agent mimics the activity of the natural agonist throughout a large concentration range, and excellent labeling of bacterial quorum-sensing receptors in live cells was achieved. This operationally simple, fast, and inexpensive method was successfully applied to the selective labeling of the *Burkholderia cenocepacia* quorum-sensing receptor CepR. Furthermore, selective labeling was achieved in mixed bacterial cultures, demonstrating the potential of this approach as a very powerful tool to visualize quorum sensing in bacteria in their natural habitat.

B. cenocepacia, a member of a bacterial group collectively referred to as the *B. cepacia* complex (Bcc), has emerged as an important pathogen for patients suffering from cystic fibrosis (CF) or immunocompromised persons.[1,2]These pathogens are known to form mixed biofilms with *Pseudomonas aeruginosa* in the lungs of CF patients[3]and are often associated with reduced survival and the risk of developing a fatal pneumonia known as cepacia syndrome.[4–6] *B. cenocepacia* is considered to be the most problematic species, particularly because some strains have the ability to spread epidemically between CF individuals.[7] Many pathogens have developed mechanisms to overcome the host defenses and remain invisible until a critical population density is reached. Typically, these bacteria use a cell-to-cell signaling system to coordinate expression of virulence factors in a cell-density-dependent manner, which is referred to as quorum sensing (QS).[8–10] Bacteria utilize QS to synchronize their behavior to regulate functions that benefit the entire population, such as biofilm development, synthesis of virulence factors, and the production of antibiotics and extracellular hydrolytic enzymes.[11]

QS in Gram-negative bacteria is mediated by the exchange of diffusible small-molecule signals termed autoinducers (AI).[12] The most commonly used signaling molecules are *N*-acyl-L-homoserine lactones (AHLs).[13] Evidence has emerged that AHLs are also recognized by eukaryotes and induce specific responses often affecting the immune system of the organism.[14,15] The QS system of *B. cenocepacia* is comprised of the LuxR-family AHL receptor CepR and the LuxI-type CepI synthase, which directs the synthesis of *N*-octanoyl homoserine lactone (C_8-AHL) and minor amounts of *N*-hexanoyl homoserine lactone (C_6-AHL) signaling molecules.[16,17] The CepIR system regulates multiple functions, including virulence, biofilm formation, swarming motility, and the production of proteases, siderophores, and antifungal compounds.[18]

Several *Burkholderia* identification methods have been developed including fluorescence in situ hybridization (FISH)[19] and rRNA gene-based PCR assays.[20] However, in spite of the importance of QS for virulence and biofilm development, knowledge of the temporal and spatial production of AHL signaling molecules within biofilms or within the infection host is scarce. This is largely due to the lack of techniques to visualize AHL-mediated communication at the single-cell level. Although GFP-based biosensors have been used for this purpose,[21] their use is restricted to genetically engineered experimental model settings. An alternative approach was recently presented by Meijler and co-workers, who reported on an aniline-catalyzed two-step labeling strategy for the visualization of the *Pseudomonas aeruginosa* LasR QS receptor in live cells by using a selective bio-orthogonal ligation.[22] This method is based on the selective covalent binding of the isothiocyanate functionalized AHL to LasR and good labeling quality was achieved by using the double-mutant strain *P. aeruginosa* JP2 (*lasI*/*rhlI* deleted). However, this approach is restricted to the absence of native AHLs and cannot be applied for the investigation of bacteria–host communication pathways. A more general approach by using a labeled ligand with a fluores-

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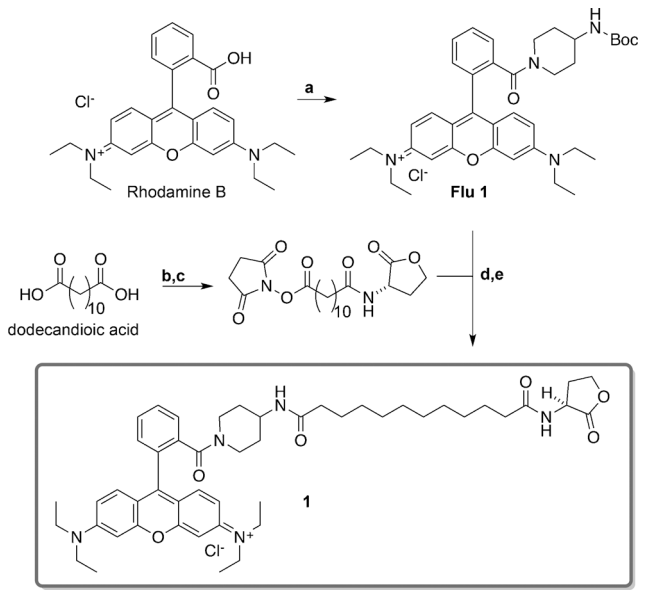
cent tag represents an operationally simpler labeling method, but this often results in decreased affinity of the modified ligand for its target protein. Furthermore, competition with native AHLs constitutes a challenging problem.

The design of our target molecules was guided by recent observations that the termini of certain AHL compounds can be modified while retaining their agonistic activity. In fact, terminal modification[23] of AHL fragments with catecholate groups allowed their immobilization on surfaces and concomitant induction of QS in the GFP-based biosensor *P. putida* F117 (pAS-C8),[21] in which *cepR* is recombinantly overexpressed. We hypothesized if we could take advantage of this molecular plasticity and introduce fluorescent labels at the terminal position of AHL derivatives with the goal of selectively labeling quorum sensing in bacterial strains. Herein, we report the development of a fluorescent-labeling agent for QS receptors (FLAQS) and demonstrate selective tagging of CepR in *B. cenocepacia*. This highly selective and operationally simple method for live-cell labeling of QS receptors by using a fluorescent AHL-analogue represents a new in situ imaging method for AHL-mediated communication pathways.

A first challenge consisted in finding a suitable fluorophore that could be used for visualization with the GFP-based *P. putida* F117 (pAS-C8) biosensor to monitor the correlation between activity and labeling. The advantage of this reporter strain, in which the AHL synthase gene *ppuI* is knocked out, is the absence of AHL production, preventing competition with added synthetic AHLs. In addition, a better labeling compared with the wild type (WT) can be expected in this sensor, because *CepR* is overexpressed. We synthesized various FLAQS containing different rhodamine B dyes. Rhodamine B was selected due to its high luminescence quantum yield and red fluorescence, which can be clearly separated from the green fluorescence of the biosensor. Most fluorescent hybrids proved to be difficult to purify, and decomposition was observed. The only compound that could be purified by preparative reverse-phase HPLC without degradation was hybrid **1**, which could be synthesized in five steps from commercially available rhodamine B and dodecandioic acid as shown in Scheme 1. The desired fluorescently labeled AHL analogue was obtained with an overall yield of 71% from the known fluorescent dye **Flu 1**.[24] The rhodamine moiety of this hybrid cannot build the nonfluorescent spirolactone form due to the presence of the non-nucleophilic secondary amide making it pH independent and water soluble. The fluorescence properties of FLAQS **1** were evaluated in aqueous medium by recording the fluorescence spectrum (Figure 1). Both bands have almost no overlap leading to improved fluorescence properties of hybrid **1** relative to rhodamine B. The absorption maximum is located at $\lambda = 560$ nm, and only little absorption occurs at 510 nm, which is the maximum GFP emission wavelength. This important attribute should preclude fluorescence quenching, which otherwise could negatively influence the activity assays. The maximum emission of FLAQS **1** is located in the red spectral area at $\lambda = 600$ nm.



Scheme 1. Reaction conditions: a) Ref. [24]; b) *N,N'*-disuccinimidyl carbonate, pyridine, MeCN, 6 h, RT; c) L-homoserine lactone (HSL), NEt_3, MeCN, 7 h, RT; d) CH_2Cl_2, trifluoroacetic acid (TFA), 5 h, RT; e) MeCN, 15 h, RT, 71% (over four steps).

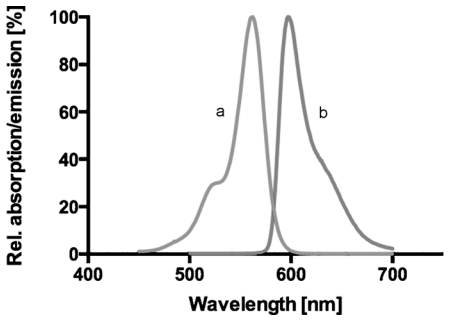


Figure 1. Fluorescence spectrum of FLAQS **1**; a = absorption; b = emission.

Having achieved the desired fluorescence properties with FLAQS **1**, we proceeded to compare the activities of the synthetic and the natural agonists with respect to binding to the receptor proteins. The GFP fluorescence of the *P. putida* F117 (pAS-C8) biosensor induced by both compounds over a broad range of concentrations (58 pM to 488 μM) were compared. The results with the natural agonist C_8-AHL are in good agreement with literature (blue in Figure 2) with a maximum activity at a concentration of 15 μM.[21] A proportional decrease of activity and concentration was observed at lower concentrations. In contrast, higher concentrations resulted in inhibition of GFP synthesis. FLAQS **1** shows a very similar profile compared to C_8-AHL (red in Figure 2) with an activity maximum at a concentration of 4 μM. As a negative control, the *P. putida* F117 (pAS-C8) sensor was also incubated with only the fluorophore **FLU 1** under identical conditions, and no activity could be observed at any

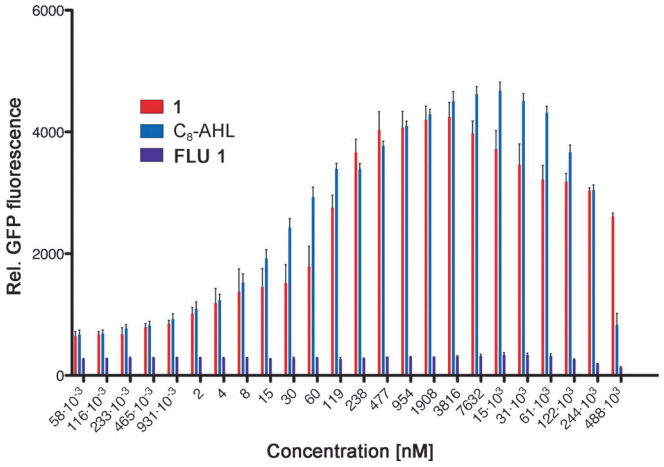


Figure 2. Activity tests with the *P. putida* F117 (pAS-C8) biosensor comparing FLAQS **1**, **Flu 1**, and the natural C_8-AHL (data is reported as mean ± SEM, $N=3$).

concentration (purple in Figure 2). These results demonstrate that FLAQS **1** shares the activity of C_8-AHL over the desired concentration range and possesses the desired fluorescent properties for our bioassays.

As a next challenge, we addressed the labeling of the wild-type strain *B. cenocepacia* H111. This strain was isolated from a CF patient, and its *cepIR* QS system was shown to control biofilm formation and swarming motility.[25] The use of this strain is especially advantageous due to its lack of the CciR/I QS system that is present in some *B. cenocepacia* strains.[26] The labeling of the wild type is more problematic than the reporter strain, because the receptor is not overexpressed, and competition between the natural agonist and the synthetic FLAQS can create challenges for fluorescent detection. To label *B. cenocepacia* H111 cultures were incubated in the presence of 244 μM FLAQS **1** for 3 h, subsequently washed, and examined by confocal microscopy. We were pleased to find that the entire bacterial population could be labeled with the red FLAQS **1** (Figure 3a). The red fluorescence was found to be uniformly distributed in the cell, suggesting that the CepR receptor is a soluble cytoplasmic protein. *B. cenocepacia* H111 cells were incubated with different concentration of FLAQS **1** to determine the required minimal concentration to achieve good labeling. A good yield of red-fluorescence labeling was achieved with a concentration of 31 μM (Figure 3b), whereas only minor red fluorescence was seen at 8 μM, and almost no fluorescence was detected at a concentration of 2 μM (see the Supporting Information).

Various control experiments were performed to exclude random binding of FLAQS **1** to the bacterial population. The double knockout mutant *B. cenocepacia* H111Δ*cepRI*, in which both *cepR and cepI* have been inactivated, showed no fluorescence labeling after incubation under the same conditions used for the WT (Figure 3c). The presence of the receptor CepR appears to be necessary for labeling. We were also pleased that in the absence of the QS receptor, no unspecific binding was detected. In contrast, enhanced red-fluorescence labeling could be observed with a strain overexpressing *cepR* as compared to the WT (see the Supporting Information). Labeling of *E. coli* XL1-Blue, which does not encode an AHL-dependent QS system, also showed no fluorescence under the same incubation conditions (Figure 3d). As an additional control experiment, *B. cenocepacia* H111 was incubated with the fluorophore **Flu 1** alone to confirm that the bioactive moiety is indeed responsible for the selective binding towards CepR. As was expected, no labeling of the cells could be detected (Figure 3e). This clearly indicates

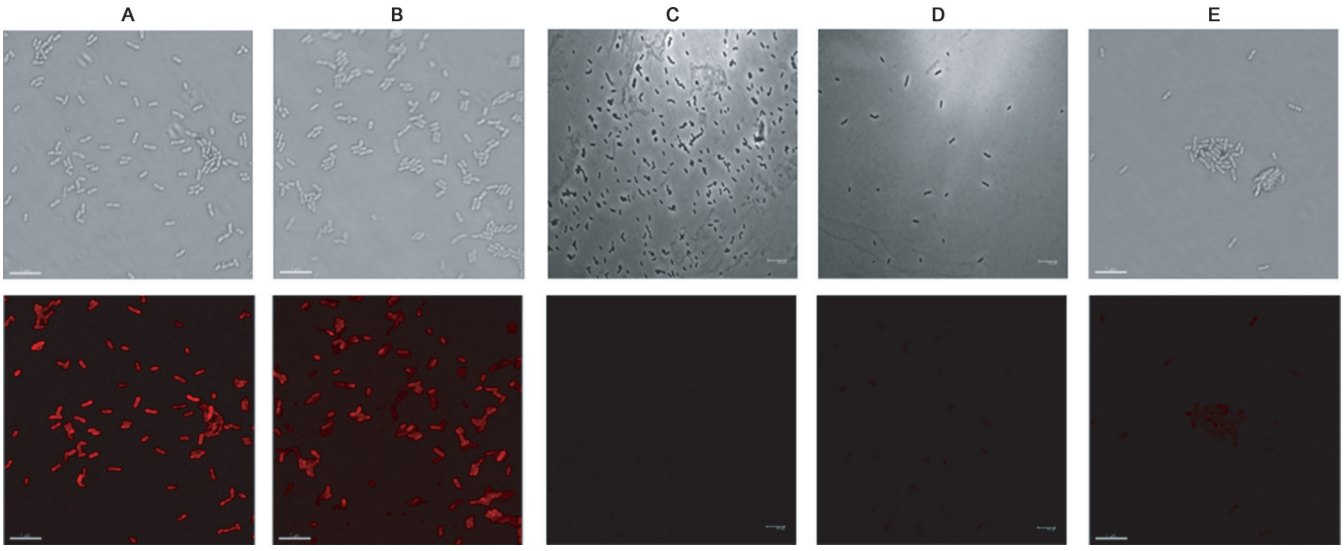


Figure 3. Imaging of different strains incubated with fluorescent probes. *B. cenocepacia* H111 incubated in the presence of A) 244 μM or B) 31 μM FLAQS **1**. *B. cenocepacia* H111Δ*cepRI* incubated in the presence of C) 244 μM FLAQS **1**. *E. coli* XL1-Blue incubated in the presence of D) 244 μM FLAQS **1**. *B. cenocepacia* H111 incubated in the presence of E) 244 μM **Flu 1**. Phase-contrast images are shown at the top, red-fluorescence images at the bottom. Scale bars are 7 μm.

that the rhodamine derivative by itself is not binding to the bacterial cells.

We next wanted to investigate if FLAQS **1** actually binds to the same target site as the natural AHL molecule. To evaluate this hypothesis, competition assays between the synthetic and the natural ligands were performed. *B. cenocepacia* H111 was incubated with the same concentration of FLAQS (244 μM) and varying concentrations of pure C_8-AHL (1 and 100 μM). Microscopic inspection of the cultures revealed a significant decrease in labeling when the concentration of C_8-AHL was increased (see the Supporting Information). These results are in agreement with the hypothesis that both compounds compete for the same binding pocket of the CepR receptor in *B. cenocepacia* H111.

Finally, we wanted to test if the *cepR* overexpression strain H111 (*cepR*$^+$) and the GFP-tagged *cepR* knockout mutant H111Δ*cepR*:GFP can be differentiated by our CepR labeling approach. The mixed strains were incubated with FLAQS **1** for 3 h and visualized by fluorescence microscopy. The results clearly demonstrate that the GFP-tagged knockout mutant could not be labeled with FLAQS **1**, whereas the strain-overexpressing CepR was clearly labeled (Figure 4). Based on these results, we expect that FLAQS

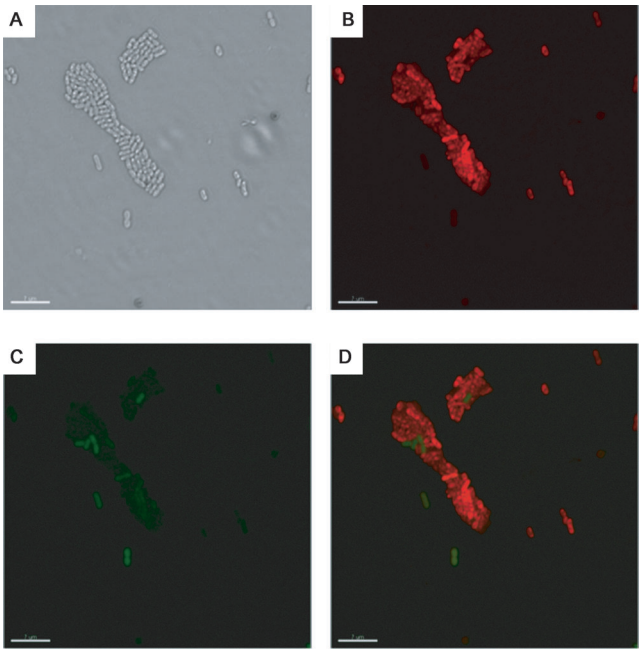


Figure 4. Imaging of mixed bacterial populations with FLAQS **1**: A) Phase contrast, B) red fluorescence, C) GFP fluorescence, and D) overlay of B) and C) of a bacterial mixture of the CepR-overexpressing strain *B. cenocepacia* H111 (*cepR*$^+$) and the GFP-tagged QS mutant *B. cenocepacia* H111Δ*cepR*:GFP with FLAQS **1** (244 μM). Scale bars are 7 μm.

will be able to label bacteria based on their unique AHL signaling system. Therefore, FLAQS might also be applied for the study of bacterial interspecies communication pathways based on a given AHL chemotype. Although our work focused on agonists of C_8-AHL, we are confident that other QS receptors using different AHL molecules may be selectively labeled by using FLAQS with varying alkyl-chain lengths. We are currently investigating *P. aeruginosa* selective FLAQS, which, in combination with FLAQS **1**, could lead to a very fast, simple, and inexpensive tool to analyze sputa of CF patients. Furthermore, as FLAQS possess similar properties as the natural agonists, they could be used as a new and potentially useful method for investigations of pathogenic and symbiotic interactions between bacteria and their eukaryotic hosts.

Our results demonstrate that easily synthesized FLAQS can be successfully used for the operationally simple and selective labeling of QS receptors in *B. cenocepacia* H111, a pathogen isolated from a CF patient. This represents a new, operationally simple, fast, and inexpensive tool for the imaging of QS receptors by using fluorescently labeled AHL analogues. Furthermore, application of FLAQS **1** suggested the presence of CepR as a soluble cytoplasmic protein. The selectivity of FLAQS **1** has been applied to specific QS labeling in a mixed bacterial culture. We think that FLAQS could be utilized for the fast analysis of QS in various environmental and clinical samples, such as, for example, sputa from CF patients and samples of plant roots to deliver a better understanding of bacteria–host communication pathways.

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Keywords: *Burkholderia cenocepacia* · cell recognition · fluorescent probes · lactones · quorum sensing

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Cell Recognition

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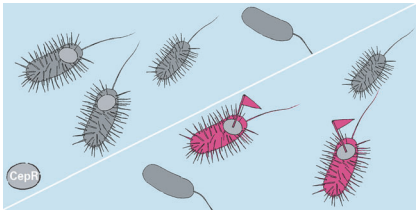
Fluorescent Labeling Agents for Quorum-Sensing Receptors (FLAQS) in Live Cells



Lighten up: A selective fluorescent-labeling agent for quorum sensing (FLAQS) can be used for the visualization of the communication pathway of bacteria in live cells (see figure). This represents a new, operationally simple, fast, and inexpensive tool for the imaging of quorum-sensing receptors by using fluorescently labeled signaling-molecule analogues.