APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 2010, p. 6119–6127 Vol. 76, No. 18
0099-2240/10/$12.00 doi:10.1128/AEM.00677-10

Construction of Self-Transmissible Green Fluorescent Protein-Based Biosensor Plasmids and Their Use for Identification of *N*-Acyl Homoserine-Producing Bacteria in Lake Sediments▽

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Received 17 March 2010/Accepted 20 July 2010

Many bacteria utilize quorum sensing (QS) systems to communicate with each other by means of the production, release, and response to signal molecules. *N*-Acyl homoserine lactone (AHL)-based QS systems are particularly widespread among the *Proteobacteria*, in which they regulate various functions. It has become evident that AHLs can also serve as signals for interspecies communication. However, knowledge on the impact of AHLs for the ecology of bacteria in their natural habitat is scarce, due mainly to the lack of tools that allow the study of QS in bacterial communities *in situ*. Here, we describe the construction of self-mobilizable green fluorescent protein (GFP)-based AHL sensors that utilize the conjugation and replication properties of the broad-host-range plasmid RP4. We show that these novel AHL sensor plasmids can be easily transferred to different bacterial species by biparental mating and that they give rise to green fluorescent cells in case the recipient is an AHL producer. We also demonstrate that these sensor plasmids are capable of self-spreading within mixed biofilms and are a suitable tool for the identification of AHL-producing bacteria in lake sediment.

The term quorum sensing (QS) describes the phenomenon that many bacteria utilize cell-to-cell communication systems to coordinate their behavior according to their population size (20). QS systems rely on the production of signal molecules that are released into the environment. Bacteria will respond to these molecules only when their concentration has reached a certain threshold, upon which the expression of target genes is either activated or repressed. Among the various QS signal molecules identified to date, *N*-acyl-homoserine lactones (AHLs) have been investigated to the greatest extent (4, 49, 50). AHLs are usually synthesized by LuxI-type proteins and perceived by cognate receptor proteins belonging to the LuxR family of transcriptional regulators. A recent bioinformatic analysis identified complete QS circuits (i.e., at least one LuxI and LuxR homolog) in 68 (26%) of the 265 sequenced proteobacterial genomes investigated (8). QS systems have been experimentally analyzed in more than 50 different bacterial species and shown to control expression of a wide spectrum of functions, including bioluminescence, virulence, symbiosis, different forms of motility, biofilm formation, production of antibiotics and toxins, and conjugation (49).

Although the molecular basis of AHL-dependent signaling circuitries and their regulated functions have been investigated in reasonable detail in a few model bacteria, knowledge on the ecological role of QS is scarce (12). However, evidence has accumulated over the past years that many bacteria inhabiting diverse environments produce AHLs, and it has been suggested that these signal molecules may also serve for interspecies communication. For example, it has been demonstrated that 8% and 12% of the culturable bacteria from the wheat and tomato rhizospheres, respectively, produce AHLs (33, 42). More recently, Dulla and Lindow (16) showed that 7% of the culturable epiphytes of leaves synthesize AHLs. With the aid of different AHL biosensors, evidence for AHL-mediated cross talk among bacteria in the rhizosphere (21, 42) and on leaf surfaces (16) was obtained. More detailed ecological studies of QS are hampered by the following two problems: (i) only a minor proportion of the bacteria in the environment can be cultured on standard media and (ii) for *in situ* visualization of AHL-mediated communication suitable biosensors have to be introduced into the natural microbial consortium under investigation.

In this study, we report on the construction of novel green fluorescent protein (GFP)-based AHL sensor plasmids that are capable of self-spreading within microbial communities by means of conjugation. To this end, we have integrated three GFP-based sensor cassettes that respond to different spectra of AHL signal molecules into a derivative of the broad-host-range plasmid RP4. We show that the sensor plasmids will spread within mixed biofilms and are suitable to identify AHL-producing bacteria in lake sediment.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. All strains were grown aerobically in Luria-Bertani Lennox medium (Difco) at 30°C (*Pseudomonas putida*) or 37°C (*Escherichia*, *Burkholderia*, and *Serratia* species). Solid medium contained 15 g agar/liter (Conda, Madrid, Spain). For selection of *Burkholderia* and *Pseudomonas* strains, *Pseudomonas* isolation agar (Becton Dickinson, Sparks, MD) was used. Antibiotics were used at the following concentrations when required: ampicillin, 100 μg ml^{-1}; gentamicin, 20 μg ml^{-1}; kanamycin, 50 or 100 μg ml^{-1}; tetracycline, 20 μg ml^{-1}; rifampin, 50 μg ml^{-1}; nalidixic acid, 50 μg ml^{-1}; and streptomycin, 50 μg ml^{-1}.

Construction of RP4-based AHL sensor plasmids. We first cloned three AHL sensor cassettes, which respond to different spectra of AHL species, into the

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▽ Published ahead of print on 30 July 2010.

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant genotype and/or characteristics[a]	Source or reference
Strains		
E. coli		
MT102	*araD139* (*ara-leu*)7697 Δ*lac thi hsdR*; Nal^r	Laboratory collection
CC118(λpir)	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1* λ*pir* lysogen	26
HB101	*recA thi pro leu hsdM*$^+$; Sm^r	5
MTRP40	MT102 carrying RP4; Tc^r	This study
MTRP41	MT102 carrying RPL4lux; Nal^r Km^r	This study
MTRP42	MT102 carrying RPL4las; Nal^r Km^r	This study
MTRP43	MT102 carrying RPL4cep; Nal^r Km^r	This study
P. putida		
IsoF	Wild-type isolate from tomato roots; AHL positive	41
IsoF *dsRed*	IsoF tagged with pUT-Gm-*dsRed*; Gm^r	This study
KT2442	Wild-type, AHL-negative strain; Rif^r	18
EL101	IsoF derivative; carrying RPL4lux; Km^r	This study
EL102	IsoF derivative; carrying RPL4las; Km^r	This study
EL103	IsoF derivative; carrying RPL4cep; Km^r	This study
EL104	KT2442 derivative; carrying RPL4lux; Rif^r Km^r	This study
EL105	KT2442 derivative; carrying RPL4las; Rif^r Km^r	This study
EL106	KT2442 derivative; carrying RPL4cep; Rif^r Km^r	This study
P. aureofaciens		
ATCC 13985	River isolate	ATCC
EL107	ATCC 13985 derivative; carrying RPL4lux; Km^r	This study
EL108	ATCC 13985 derivative; carrying RPL4las; Km^r	This study
EL109	ATCC 13985 derivative; carrying RPL4cep; Km^r	This study
B. cenocepacia		
H111	Wild type	
H111 *dsRed*	H111 tagged with pUT-Km-*dsRed*; Km^r	This study
Plasmids		
RP4 modified	IncPα plasmid sensitive to kanamycin; Ap^r Tc^r	22
pUT/mini-Tn*5*	Tn*5*-based delivery plasmid; Ap^r Km^r	14
pJBA132	Cloning vector containing *luxR*-P_{luxR}-P_{luxI}-RBSII-*gfp*(ASV)-T_0-T_1; Tc^r	3
pKR-C12	AHL sensor plasmid; pBBR::*lasR*-P_{lac}-P_{lasB}-*gfp*(ASV); Gm^R	36
pAS-C8	AHL sensor plasmid; pBBR::P_{lac}-*cepR*-*gfp*(ASV)-P_{cepI}; Gm^r	36
pPLlux	pUT/mini with Km^r::*luxR*-P_{luxR}-P_{luxI}-RBSII-*gfp*(ASV)-T_0-T_1 in the NotI site	This study
pPLlas	pUT/mini with Km^r::*lasR*-P_{lac}-P_{lasB}-*gfp*(ASV)-T_0-T_1 in the NotI site	This study
pPLcep	pUT/mini with Km^r::P_{lac}-*cepR*-*gfp*(ASV)-P_{cepI}-T_0-T_1 in the NotI site	This study
RPL4lux	RP4 with Km^r::*luxR*-P_{luxR}-P_{luxI}-RBSII-*gfp*(ASV)	This study
RPL4las	RP4 with Km^r::*lasR*-P_{lac}-P_{lasB}-*gfp*(ASV)	This study
RPL4cep	RP4 with Km^r::P_{lac}-*cepR*-*gfp*(ASV)-P_{cepI}	This study
pUT-Km-*dsRed*	Tn*5*-based delivery plasmid, carrying P_{lac}-*dsRed*-T_0-T_1; Km^r	
pUT-Gm-*dsRed*	Tn*5*-based delivery plasmid, carrying P_{lac}-*dsRed*-T_0-T_1; Gm^r	
pRK600	*ori* ColE1 RK2-*mob*$^+$ RK2-*tra*$^+$; Cm^r	13

[a] Tc^r, tetracycline resistance; Rif^r, rifampin resistance; Km^r, kanamycin resistance; Gm^r, gentamicin resistance; Str^r, streptomycin resistance; Nal^r, nalidixic acid resistance; T_0, transcriptional terminator derived from phage λ; T_1, transcriptional terminator derived from the *rrnB* operon of *E. coli*.

transposon delivery vector pUT/mini-Tn*5*Km. Plasmid pPLlux was generated by cloning a 2.85-kb EcoRI-MluI fragment of pJBA132 blunt ended with Klenow fragment into the filled-in NotI site of pUTKm. Using the primers *las-gfpF* (5′-GCGCGCGTAATACGACTCAC-3′) and *las-gfpR* (5′-TGACCATGATTACGCCAAGC-3′), a 3.5-kb fragment of plasmid pKR-C12 containing a *las*-based AHL sensor cassette was amplified with the aid of the Long Expand polymerase (Roche). The DNA fragment was cloned into pUT/mini-Tn*5*Km cut with NotI and blunt ended with Klenow fragment, resulting in plasmid pPLlas. A 2.2-kb fragment containing the *cep*-based AHL sensor cassette of plasmid pAS-C8 was obtained by PCR analysis using the primers *cep-gfpF* (5′-AGCGGCCGCTCACGACGTTGTAAAACGAC-3′) and *cep-gfpR* (5′-AGCGGCCGCATGACCATGATTACGCCAAG-3′). The PCR product was cloned into pCR2.1 (Invitrogen) and then subcloned as a NotI fragment (restriction sites underlined in the primer sequences) into the corresponding sites of pUT/mini-Tn*5*Km to yield plasmid pPLcep.

Insertion of the three sensor cassettes into the broad-host-range RP4 derivative pRK24 was performed as follows. First, the delivery plasmids pPLlux, pPLlas, and pPLcep were transferred from the donor strain, CC118λpir, through triparental mating with the helper plasmid pRK600 into the recipient strain, *Escherichia coli* MT102Rif, harboring pRK24, a Km^s derivative of the broad-host-range plasmid RP4. Selection on LB plates containing 50 μg/ml kanamycin, 10 μg/ml tetracycline, and 50 μg/ml rifampin resulted in MT102Rif derivatives carrying the sensor cassettes inserted either in the chromosome or in RP4. To discriminate between chromosomal and plasmid insertions, approximately 5,000 transconjugants were pooled and mated with the nalidixic acid-resistant strain MT102Nal. Selection on plates containing 50 μg kanamycin/ml and 50 μg nalidixic/ml resulted in derivatives carrying plasmid pRK24 with the AHL sensor cassettes inserted at random positions. For each sensor cassette, one transconjugant was chosen on the basis that (i) GFP fluorescence was induced in the presence of appropriate AHLs and (ii) the conjugation frequencies on plates were similar to that of the parent plasmid pRK24. Employing an arbitrary PCR procedure, we determined that the sensor cassettes have inserted into *fiwA*, *traC2*, and a gene of unknown function on RP4lux, RP4las, and RP4cep, respectively.

Characterization of AHL monitor strains. To determine the specificity and sensitivity of the different GFP-based AHL monitor strains, respective overnight cultures were diluted four times into fresh LB medium, incubated for 1 h at 30°C, and then distributed in 200-μl aliquots into wells of a microtiter plate. C_4-, C_6-, C_8-, C_{10}-, C_{12}-, C_{14}-, C_6-oxo-, C_8-oxo-, C_{10}-oxo-, and C_{12}-oxo-homoserine lactone (HSL) were added at final concentrations ranging from 9.76 nM to 10 μM (in 2-fold serial dilutions). Following 6 h of incubation at 30°C, green fluorescence was measured using the microtiter plate reader Synergy *HT* (MWG Biotech, Germany) with an excitation wavelength of 474 nm and emission detection at 515 nm. Data were processed with KC4 software (BioTek Instruments). The fluorescence measurements were corrected for autofluorescence and plotted as a function of AHL concentration.

Filter mating procedure. Cultures of donor and recipient strains were grown overnight at 37°C or 30°C in LB medium supplemented with the appropriate antibiotics. Following dilution to an optical density at 600 nm (OD_{600}) of 0.1, the cultures were incubated with shaking for 4 h. Thereafter, 2 ml of the cultures were harvested, washed with fresh LB, and resuspended in 500 μl of LB. Donor and recipient cells (100 μl each) were mixed 1:1 and then applied as 50-μl drops on the surface of a sterile disk (0.22 μm pore size; Millipore), which were placed on LB plates. After overnight incubation at 37°C, cells were scraped off, washed, and resuspended in 0.9% NaCl. Serial dilutions were plated on selective media containing appropriate antibiotics.

Microcosm experiments. Sediment from Lake Zürich was collected at 47°15′45.69″N, 8°39′40.47″E at a depth of 3 m in July 2009. Samples were stored at 4°C for further analysis. For the isolation of AHL-producing bacteria, overnight cultures of the donor strain *Pseudomonas putida* KT2442 (RP4lux) were washed, resuspended in 0.9% NaCl, and then mixed with 1 ml of lake sediment. The mixtures were incubated in 2.5-ml tubes without shaking over a period of 10 days at room temperature. On days 6, 8, and 10, samples of 10 μl were withdrawn for microscopic inspection. Additionally, 50-μl samples were plated on LB agar plates containing 50 μg ml^{-1} kanamycin and incubated at 30°C for 10 days. Green fluorescent colonies, i.e., AHL-producing transconjugants, were identified using a stereo microscope (M1665FC; Leica). Transconjugant colonies were toothpicked and further purified on selective agar plates prior to the extraction of chromosomal DNAs. PCR amplification of 16S rRNA genes was performed by the aid of the primer pair (5′-AGAGTTTGATYMTGGCTC-3′) and (5′-CA KAAAGGAGGTGATCC-3′).

Cultivation of biofilms, CLSM, and image analysis. Biofilms were grown in artificial flow cells supplied with modified FAB medium (27) containing 0.3 mM glucose. The flow system was assembled and prepared as described previously (11). The substratum consisted of a microscope glass coverslip (Knittel Gläser, Braunschweig, Germany). Overnight cultures in LB medium were subcultured to an OD_{600} of 0.7 before dilution in 0.9% NaCl to an OD_{600} of 0.1. Aliquots (250 μl) of these dilutions were used to inoculate the flow channels. Medium flow was kept at a constant rate of 0.2 mm s^{-1} by a Watson-Marlow 205S peristaltic pump. The incubation temperature was 30°C. Microscopic inspection and image acquisition were performed on a confocal laser scanning microscope (CLSM) (DM5500Q; Leica) equipped with a ×40/1.3 oil objective and a ×63/1.4 oil objective. Data were analyzed with Leica Application Suite (Mannheim, Germany) and the Imaris software package (Bitplane, Switzerland). Images were prepared for publication using Photoshop CS (Adobe) and PowerPoint (Microsoft) software.

Partial 16S rRNA gene sequence accession numbers. The partial 16S rRNA gene sequences of representatives of the three genera identified were deposited in the NCBI database under the accession numbers HM627635, HM627636, and HM627637.

RESULTS

Construction and characterization of self-transferable GFP-based AHL sensor plasmids. A mini-derivative of the broad-host-range plasmid RP4 was chosen as a backbone for the construction of GFP-based AHL sensor plasmids that are capable of self-transferring into and within natural bacterial communities. The construction of these plasmids is described in Materials and Methods and is outlined in Fig. 1. The three resulting RP4 derivatives carry AHL sensor cassettes that are expected to respond to different spectra of AHL molecules, depending on the components used for their construction (see also reference 42): RP4lux is based on the *lux* QS system of

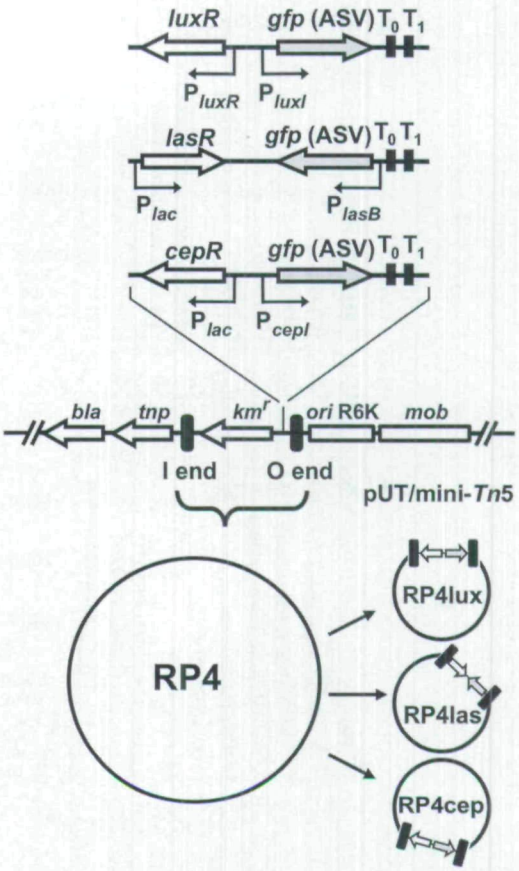

FIG. 1. Schematic outline of the construction of self-transferable GFP-based AHL biosensors. The three AHL sensor cassettes were first assembled on pUT/mini-Tn*5*Km. These constructs were then used to deliver the sensor cassettes into the broad-host-range RP4 derivative pRK24.

Vibrio fischeri, which is most sensitive for 3-oxo-C_6-HSL; RP4las is engineered from components of the *las* QS system of *Pseudomonas aeruginosa*, which exhibits the highest sensitivity for 3-oxo-C_{12}-HSL but also responds to other long-chain AHLs; and RP4cep has been constructed from components of the *cep* QS system of *Burkholderia cenocepacia*, which is most sensitive for C_8-HSL.

In the first step we wished to characterize the new AHL sensor plasmids for their responsiveness to different AHL molecules. We used two background strains in these experiments, *Pseudomonas putida* KT2442 and *Escherichia coli* MT102, both of which do not produce AHLs. As depicted in Fig. 2, the two strains harboring the three RP4-based AHL sensor plasmids responded to different spectra of AHL molecules in a way that was predicted from the genetic components used for their construction. However, in agreement with a previous study (42), we observed that the sensitivity as well as the specificity of the sensor plasmids were strongly influenced by the bacterial host. For example, *P. putida* KT2442 harboring plasmid RP4lux exclusively responded to 3-oxo-C_6-HSL and 3-oxo-C_8-HSL, while the same plasmid responded to a wide range of different AHL molecules in the *E. coli* MT102 background (Fig. 2A and D). On the other hand, the sensor plasmids RP4las and RP4cep were highly sensitive for AHLs, with acyl

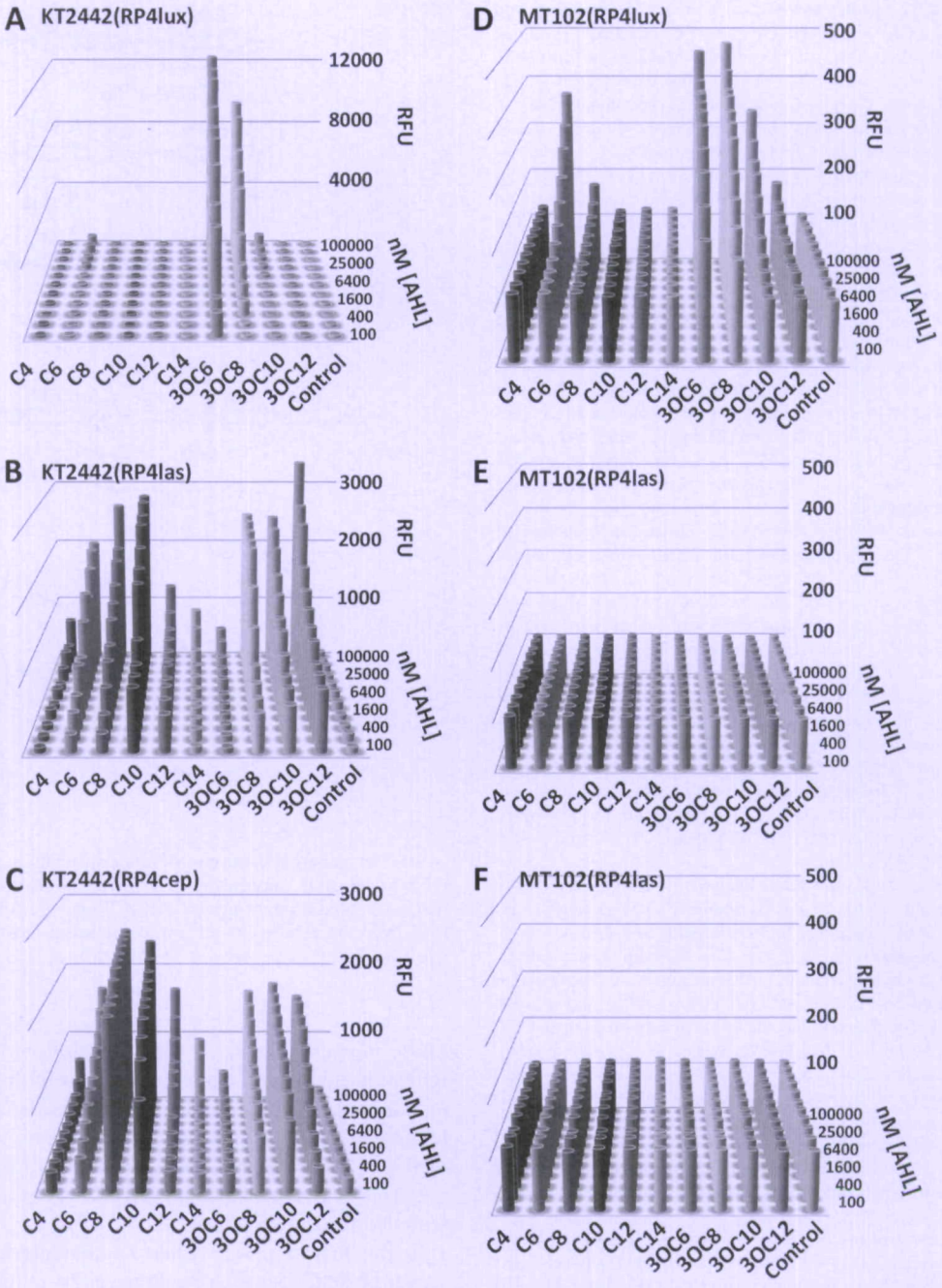

FIG. 2. Response of AHL to different sensor plasmids in *P. putida* and *E. coli*. Sensors were grown in the presence of various AHL compounds in a concentration range from 9.76 nM to 10 μM. Controls were cells grown without the addition of AHLs in the medium. Green fluorescence was recorded with a microtiter plate reader at 515 nm. AHL compounds assayed are shown at the bottom of each panel. RFU, relative fluorescence units.

side chains ranging from C_8 to C_{12} in *P. putida* KT2442, whereas the same sensor plasmids did not function in the *E. coli* MT102 background (Fig. 2B to F).

We next tested the functionality of the sensor plasmids after transfer to AHL-producing strains. To this end, the three sensor plasmids were conjugated into *P. putida* IsoF and *Pseudomonas aureofaciens* by biparental agar surface matings. The resulting recombinant strains were grown in liquid LB medium, and expression of GFP was measured along the growth curves. In agreement with the fact that *P. putida* IsoF harbors the PpuIR QS system, which is highly homologous to the LasIR QS system of *P. aeruginosa* and utilizes mainly 3-oxo-C_{10}-HSL and 3-oxo-C_{12}-HSL as signal molecules (41), we observed that GFP fluorescence was strongly induced in cells harboring RP4las at the transition from the exponential to the stationary growth phase (Fig. 3A). Similar induction kinetics and levels were also seen with *P. putida* IsoF harboring RP4cep, likely because 3-oxo-C_{10}-HSL is well recognized by the *cep*-based sensor cassette (Fig. 3A). However, when the sensor RP4lux was transferred to *P. putida* IsoF, no GFP ex-

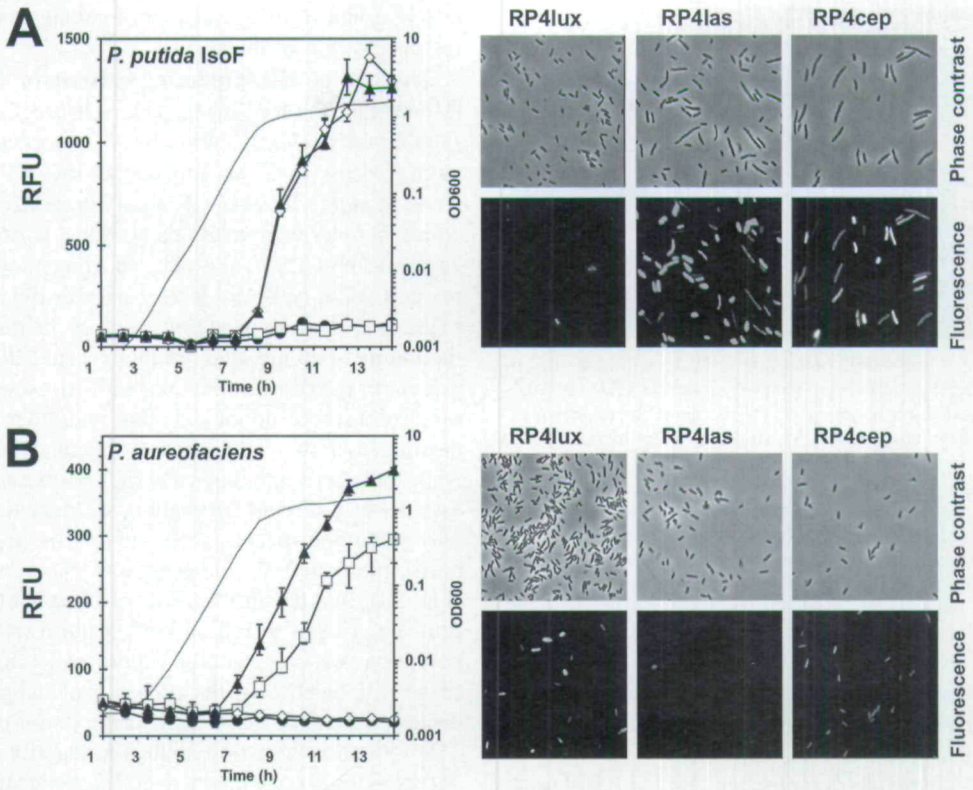

FIG. 3. Induction of fluorescence of two AHL-producing bacteria, *P. putida* IsoF (A) and *P. aureofaciens* (B), harboring the sensor plasmids RP4lux (□), RP4las (◇), and RP4cep (▲) and control cells without the plasmid (●). The recombinant strains were grown in LB medium, and expression of GFP was measured along the growth curves. Images representing the same field of view of the cells harboring the sensor plasmids were visualized by phase contrast and epifluorescence microscopy. Data represent mean values ± standard deviations of three independent experiments. RFU, relative fluorescence units.

pression was observed, despite the fact that the sensor plasmid is very sensitive for 3-oxo-C_8-HSL when present in *P. putida* KT2442 (Fig. 2A). In agreement with these measurements, we observed that only 20% of the cells expressed GFP in samples of a stationary phase culture of *P. putida* IsoF (RP4lux), whereas most cells were fluorescent in cultures of *P. putida* IsoF harboring RP4las (98%) or RP4cep (86%) (Fig. 3A).

When the sensor plasmids were tested in another strain background, namely, *P. aureofaciens*, both RP4lux and RP4cep were found to be activated while RP4las was not (Fig. 3B). These results are in good agreement with the fact that *P. aureofaciens* produces mainly C_6-HSL (52), which activates the *lux*- and *cep*-based sensor plasmids at low concentrations while the *las*-based sensor plasmid is very insensitive for short-chain AHLs. Microscopic inspection confirmed the measurements and showed that only about 10% of the cells harboring RP4las were activated, whereas 40% and 87% showed GFP fluorescence in the case of RP4lux and RP4cep, respectively (Fig. 3B).

In conclusion, these experiments demonstrate that the three GFP-based AHL biosensors can be easily transferred to different bacterial species and that they are induced in a way that correlates well with the AHL species produced by the strain.

***In situ* monitoring of AHL production in mixed biofilms.** It is now generally assumed that in nature most bacteria exist as mixed biofilms and that QS plays a particular important role in these consortia, not only because the bacteria are in close vicinity to each other and thus have high population densities but also due to the fact that some bacteria utilize AHL-regulated functions to form biofilms (for reviews, see references 1, 13, and 28). We therefore wished to investigate whether the three RP4-based AHL sensor plasmids are capable of spreading within a mixed biofilm and thus would allow the identification of AHL-producing bacteria. For a proof of principle, we cultured mixed biofilms consisting of *P. putida* KT2442 harboring the sensor plasmid RP4cep (the donor) and the AHL-producing strain *B. cenocepacia* H111 (the recipient) in flow chambers. To be able to distinguish between donor and recipient cells we used a *B. cenocepacia* H111 strain that was marked with the red fluorescent protein (RFP) as a recipient. When the mixed biofilms were analyzed by CSLM after 4 days of cultivation we observed a large number of green fluorescent cells, indicating that the sensor plasmid was induced by the AHLs produced by the recipient strain. Most importantly, a fraction of the induced cells (10%) had both red and green fluorescence (Fig. 4), indicating that the AHL sensor plasmid had been successfully transferred to the recipient H111. We were also able to extract plasmid RP4cep from the transconjugants obtained in this experiment (data not shown). Cells with only green fluorescence, i.e., donor cells that were stimulated by the AHLs released by the recipient strain to the medium, were also observed. Similar results were obtained when RP4lux and RP4las were tested in mixed biofilms with an RFP-tagged *P. putida* IsoF strain as recipient, albeit the intensity of the fluorescent signal, as well as the number of double

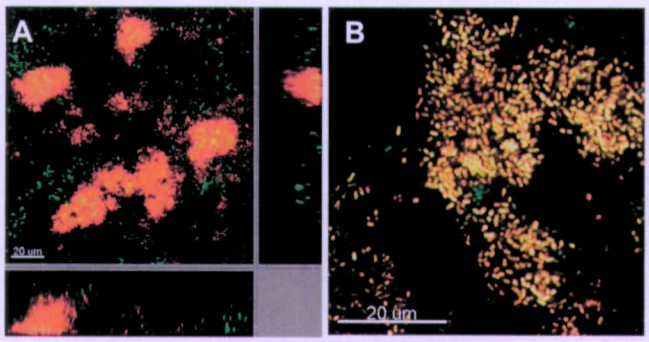

FIG. 4. Transfer of the AHL biosensor RP4cep within a mixed biofilm. *P. putida* KT2442(RP4cep) (donor) and the RFP-marked, AHL-producing strain *B. cenocepacia* H111 (recipient) were grown as mixed biofilms in flow chambers. (A) Biofilms were inspected by CSLM 5 days after inoculation. Green fluorescent cells indicate that the donor strain was induced by the AHLs produced by the recipient strain. Yellow cells, i.e., cells exhibiting red and green fluorescence, depict H111 cells that have acquired the AHL sensor plasmid through horizontal gene transfer. (B) Higher magnification of cells exhibiting green (activated donor), red (recipient), or green and red (yellow, activated recipient) fluorescence. Bars, 20 μm.

fluorescent cells, were found to be reduced relative to the situation using the sensor plasmid RP4cep and *B. cenocepacia* H111 as the recipient (data not shown).

In conclusion, these results demonstrate that the RP4-based sensor plasmids can spread in surface-associated biofilms and will give rise to green fluorescent cells when they are transferred into cells producing appropriate AHLs. Activation of donor cells due to exogenous AHLs may also occur but can be distinguished from activated transconjugants by the red fluorescence of the recipient.

Detection of AHL-producing bacteria in lake sediment. To test whether the RP4-based AHL sensor strains would also be suitable for *in situ* detection of AHL-producing bacteria in a natural ecosystem, we inoculated the RFP-marked donor strain *P. putida* KT2442 (RP4lux) into a lake sediment microcosm. Samples were taken daily over a period of 10 days and inspected by CLSM. We observed that the number of RFP-marked donor cells was decreasing over time, presumably due to grazing, as we noticed that many of the protozoa present in the samples had ingested red fluorescent cells, which were not present in the control (data not shown). However, after 5 days we also observed donor cells that had green fluorescence, indicating that the indigenous sediment community produces AHL molecules in biologically significant concentrations (Fig. 5A). After 8 days of incubation, we detected cells that were only green fluorescent, indicating that the sensor plasmid had been transferred to indigenous AHL-producing organisms (Fig. 5B). In a few instances we also detected activated donor cells, i.e., cells with red and green fluorescence and thus appearing yellow in the double-exposure picture (Fig. 5C). These cells are indicative of the presence of indigenous AHL-producing bacteria in the vicinity of the donor cells.

To obtain information about the identity of the AHL producers in the lake sediment samples, we incubated subsamples on LB agar plates containing kanamycin. After 2 days of incubation, green fluorescent colonies were observed and these were purified. Sequencing of the 16S rRNA genes of 42 strains revealed that they were members of the genera *Aeromonas*

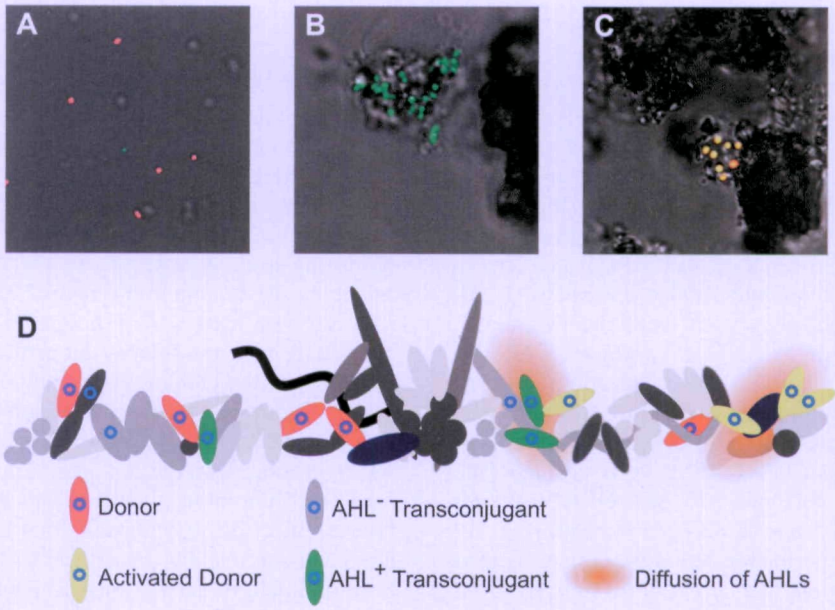

FIG. 5. Analysis of AHL-mediated communication in lake sediment by the aid of a conjugative AHL sensor plasmid. (A to C) Combined epifluorescence and phase contrast micrographs. (D) Schematic representation of the possible outcomes of the transfer of the self-transferable AHL sensor plasmids. Lake sediment microcosms were inoculated with the RFP-marked donor strain *P. putida* KT2442 (RP4lux). Samples were inspected by CLSM over a period of 10 days. Indigenous AHL-producing bacteria which have taken up the AHL sensor plasmid were identified and are evident by their green fluorescence after 5 days (A) and 8 days (B). (C) Activated donor cells, i.e., cells with red and green fluorescence and thus appearing yellow in the double-exposure picture, were detected after 10 days and are indicative of the presence of indigenous AHL-producing bacteria in the vicinity of the sensor cells. In some cases the green fluorescent cells were surrounded by activated donor cells, suggesting that the identified bacteria release signal molecules.

(97% similarity to *Aeromonas hydrophila*), *Providencia* (98% similarity to *Providencia alcalifaciens*), and *Pseudochrobactrum* (98% similarity to *Pseudochrobactrum asaccharolyticum*).

A thin-layer chromatography (TLC) analysis of dichloromethane extracts of spent culture supernatants of the three transconjugants identified spots that activated the LuxIR-based biosensor *E. coli* MT102(pSB403). In the case of *Aeromonas* species, the R_f values of the spots observed were similar to the ones of C_4-HSL and C_6-HSL (data not shown). This is in good agreement with previous work (6, 45) and is supported by the fact that the molecules also activated the AHL biosensor *Chromobacterium violaceum* CV026, which is most sensitive for C_6-HSL and to a lesser degree for C_4-HSL (data not shown). The spots observed with the two other strains could not activate *C. violaceum* nor could their mobilities unambiguously be correlated with any AHL molecule included as references. Additional work will be required to identify the structure of these AHL agonists.

DISCUSSION

In 1993, Swift et al. (46) introduced a novel strategy for the identification of AHL synthase genes, which employed a *lux* plasmid-based bioluminescent sensor that responded to the presence of AHLs. This approach was quickly adapted and advanced by many research groups who used various *luxR* family genes in combination with transcriptional or translational fusions of cognate target promoters to different reporter genes for the construction of AHL biosensors. Depending on the genetic components used for their construction, these sensors vary greatly with respect to sensitivities as well as specificities (for a recent review, see reference 43). The use of these bacterial biosensors has greatly facilitated screening of bacteria for the production of AHLs, has accelerated the identification of *luxI* homologues, and has made high-throughput screens for AHL mimics (agonists and antagonists) possible. For *in situ* studies of AHL-dependent signaling in complex habitats, biosensors utilizing fluorescent proteins (GFP and RFP) as reporter genes have been developed. These biosensors were employed to demonstrate AHL-mediated communication in swarm colonies of *Serratia liquefaciens* (3), to detect AHL production in *P. aeruginosa*-infected lung tissue (53), to visualize interspecies communication between *P. aeruginosa* and *B. cenocepacia* (36), to detect AHLs produced by bacteria colonizing the rhizosphere of tomato plants (42) and to estimate the calling distance (i.e., the distance over which bacteria can communicate by the aid of signal molecules) in this habitat (21), and to measure the quorum size of *Pseudomonas syringae* on the leaves of bean plants (17).

Although fluorescent protein-based biosensors have proven to be extremely valuable for *in situ* studies of AHL-mediated signaling at the single-cell level, their disadvantage is that they have to be, at least temporarily, introduced into the investigated ecosystem. For this purpose the AHL sensor cassettes need to be transferred to suitable strains isolated from the habitat under investigation. Given that the vast majority of microorganisms cannot be cultured (2), this is often a difficult task. Furthermore, although introduced biosensors have been used to demonstrate AHL production in natural habitats, they do not allow conclusions to be drawn on the identity of the producing organisms.

A few studies have employed a metagenomic approach to increase our knowledge of QS in natural communities and particularly to identify QS systems in uncultured microorganisms. To this end, metagenomic cosmid clones were transferred into different biosensor strains and screened for AHL production. Williamson et al. (51) reported the isolation of *luxI* and *luxR* homologues from a metagenomic library with DNA from soil on the floodplain of the Tanana River in Alaska. More recently, Hao and colleagues (25) identified and characterized three novel *luxI* and *luxR* gene pairs from libraries constructed from DNA from activated sludge and soil. The same approach led also to the isolation of a monooxygenase gene from gypsy moth gut microbiota that is involved in the synthesis of AHL signal mimics (23). Furthermore, in two studies metagenome-derived clones that encode quorum-quenching lactonases, which interfere with bacterial QS, were identified (35, 37). Phylogenetic analyses of the identified genes (as well as of other genes that were present on the same DNA fragments) were used to tentatively trace back the organisms from which the cloned genes originated. However, as *luxIR* homologues can be part of mobile elements that are horizontally transferred (34, 47), such investigations have to be interpreted with caution. Another disadvantage of the metagenomic approach is that it will not allow the identification of QS-controlled functions, except for cases where the regulated gene(s) is located next to the identified *luxIR* genes.

In this study, we have constructed novel tools that allow the identification of AHL-producing bacteria in their natural habitat. To this end we have integrated three GFP-based AHL sensor cassettes with different specificities into a self-transmissible mini-RP4 derivative and we show that transfer of these constructs to an AHL-producing organism gives rise to green fluorescent cells. In agreement with an earlier report (42), we observed that the functionality of the sensor plasmids was highly dependent on the background strain (Fig. 2 and 3). There are several possible explanations for this finding: (i) bacteria are thought to be freely permeable for short-chain AHLs but evidence has accumulated that long-chain AHLs are actively transported (7, 9, 32), and it is therefore tempting to speculate that differences in the presence and/or specificity of long-chain AHL transporters in different strains may account for the observed differences in sensitivity; (ii) previous work has shown that LuxR family transcriptional activators need to make contact with the C-terminal domain of the RNA polymerase (RNAP) α-subunit to stimulate transcription of target genes (19, 29, 48); variations in the respective RNAP domains may be responsible for the observed malfunction of the biosensor plasmid in certain strain backgrounds; and (iii) given that many bacteria are capable of degrading AHLs (for a review, see reference 15) it is also possible that some strains effectively inactivated some of the AHLs added in our bioassays. Additional work will be required to resolve this issue.

We have demonstrated that the sensor plasmids are capable of spreading within a defined model biofilm community as well as in lake sediment. A clear limitation of our approach is that not all bacteria of a natural community are capable of receiving and maintaining the plasmid. However, RP4 has been shown to exhibit a very broad host range and has been demonstrated to

efficiently transfer in activated sludge (22, 40), chicken manure (24), and the rhizospheres of various plants (38, 39). More recently, it has been demonstrated that RP4 can transfer to bacteria in soil suspensions with a frequency of up to 1 transfer per 10^4 cells (31). Interestingly, the affiliation of the transconjugants by the analysis of their 16S rRNA gene sequences revealed that they all belonged to the *alpha*, *beta*, and *gamma* subclasses of the *Proteobacteria*. As the large majority of species, for which AHL production has been demonstrated so far, belong to the same phylogenetic groups (8), we believe that the use of plasmid RP4 as a backbone for our conjugative biosensors is particularly effective for the identification of AHL producers.

We have used RFP-marked donor strains in this study to (i) follow the fate of the donor after introduction into the lake sediment microcosms and (ii) to differentiate between donor cells that were induced by external AHLs and green fluorescent cells that represent indigenous AHL-producing bacteria carrying the sensor plasmid (Fig. 5). Although it is also possible that the sensor plasmid is transferred to a cell in the community that is not producing AHLs itself but is activated by external signal molecules produced by another bacterium in the vicinity, this will only give rise to transient fluorescence and will be lost after isolation or prolonged incubation, when sensor cells and AHL producers become separated. It is noteworthy that none of the transconjugants isolated in this study lost its fluorescence during the isolation procedure, indicating that the strains were not activated by an external AHL source.

McLean and colleagues (30) demonstrated, with the aid of an *Agrobacterium tumefaciens* AHL-responsive reporter strain, the presence of naturally occurring AHL production in aquatic biofilms growing on submerged stones. Using a conjugative AHL biosensor we show here that AHLs are also produced in the microbial consortium inhabiting lake sediment. Furthermore, three activated transconjugants, i.e., strains potentially synthesizing AHLs, were isolated and through sequence analyses of their 16S rRNA genes tentatively affiliated to the genera *Aeromonas*, *Providencia*, and *Pseudochrobactrum*. A TLC analysis of a dichloromethane extract of *Aeromonas* sp. spent culture supernatants suggested the production of C_4-HSL, which is in good agreement with previous work showing that *Aeromonas hydrophila* and *Aeromonas salmonicida* produce mainly this AHL species (45). Spots activating the LuxIR-based biosensor *E. coli* MT102(pSB403) were also observed with the two other isolates, but their mobilities could not be clearly correlated with any AHL molecule included. Additional work will be required to investigate whether the compounds activating the biosensor are AHLs or AHL mimics. Intriguingly, the production of a QS molecule has previously been suggested for *Providencia stuartii* (44).

Although we have used a cultivation-based approach to identify the producing organisms in this study, we would like to emphasize that it should also be possible to affiliate induced transconjugants by fluorescent *in situ* hybridization using rRNA-targeting probes. A similar methodology was previously used for the identification of transconjugants in biofilms or activated sludge that had taken up a GFP-marked plasmid (10, 22). Work currently under progress aims at collecting activated cells by fluorescence-activated cell sorting followed by the amplification and phylogenetic analysis of their 16S rRNA genes. This approach may allow affiliation of a larger number of AHL-producing bacteria in a cultivation-independent manner, circumventing the tedious search for green fluorescent transconjugants by epifluorescence microscopy and allowing a comprehensive survey of AHL-producing bacteria in natural microbial communities.

ACKNOWLEDGMENTS

We thank Stefanie Heller for technical assistance.

Financial support for work on QS and biofilm formation from the ESF project FP065-COMIX and the Swiss National Fund (project 3100A0-114215) is gratefully acknowledged.

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