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A multitask biosensor for micro-volumetric detection of *N*-3-oxo-dodecanoyl-homoserine lactone quorum sensing signal

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ABSTRACT

N-3-oxo-dodecanoyl-homoserine lactone ($3OC_{12}$-HSL) is the main quorum sensing (QS) signal produced by the human pathogen *Pseudomonas aeruginosa*, a major cause of hard-to-treat nosocomial infections and years-lasting chronic biofilm infections in the lungs of cystic fibrosis (CF) patients. $3OC_{12}$-HSL-dependent QS is considered a promising target for novel anti-pseudomonads drugs. However, the screening systems employed to date for the identification of QS inhibitors (QSI) were aimed at the identification of inhibitors of $3OC_{12}$-HSL signaling rather than of the synthesis or the export of this molecule. Moreover, the low concentration of $3OC_{12}$-HSL in CF sputum has hampered large scale studies aimed at addressing the role of this molecule in the CF lung infection. Here we describe the construction and characterization of PA14-R3, a new whole-cell biosensor for the quantitative detection of $3OC_{12}$-HSL. PA14-R3 provides fast and direct quantification of $3OC_{12}$-HSL over a wide range of concentrations (from pM to μM), and proved to be an easy-to-handle, cost-effective and reliable biosensor for high-throughput screening of $3OC_{12}$-HSL levels in samples of different origin, including CF sputum. Moreover, the specific features of PA14-R3 made it possible to develop and validate a novel high-throughput screening system for QSI based on the co-cultivation of PA14-R3 with the PA14 wild-type strain. With respect to previous screening systems for QSI, this approach has the advantage of being cost-effective and allowing the identification of compounds targeting, besides $3OC_{12}$-HSL signaling, any cellular process critical for QS response, including $3OC_{12}$-HSL synthesis and secretion.

1. Introduction

The Gram negative bacterium *Pseudomonas aeruginosa* is an important cause of infection, particularly in hospitals where it is responsible for about 10% of all nosocomial infections. Furthermore, *P. aeruginosa* chronic lung infection remains the major cause of morbidity and mortality among patients suffering from cystic fibrosis (CF), a genetic disease that affects about 1/2500 newborns in USA and Europe. *P. aeruginosa* infections are often recalcitrant to conventional antimicrobial chemotherapy since this bacterium is endowed with both innate and acquired resistance to many antibiotics, and adopts a biofilm mode of growth *in vivo* (Driscoll et al., 2007).

An appealing approach for identifying new drugs against *P. aeruginosa* is to search for inhibitors of virulence-related traits, as opposed to growth inhibitors *per se*. Selective targeting of the pathogenic potential of the bacterium by anti-virulence drugs would prevent or inhibit the establishment of the infection process, providing the host immune system with a better chance of clearing the infection (Cegelski et al., 2008; Rasko and Sperandio, 2010).

P. aeruginosa possesses a network of quorum sensing (QS) systems based on the production, secretion and perception of different signal molecules. The *N*-3-oxo-dodecanoyl-homoserine lactone ($3OC_{12}$-HSL) is required for optimal production of other QS signals, namely N-butanoyl-homoserine lactone (C_4-HSL) and 2-heptyl-3-hydroxy-4-quinolone (PQS) (Williams and Cámara, 2009).

The QS cascade functions in a way that when $3OC_{12}$-HSL signal molecule reaches a certain concentration in the *P. aeruginosa* growth environment, it binds and activates the LasR signal receptor protein, a transcriptional regulator that triggers the expression of hundreds of genes. These include, among others, the *lasI* gene encoding the $3OC_{12}$-HSL-producing synthase, the genes required for the synthesis of the other *P. aeruginosa* QS signals, and the genes for production of some virulence factors and biofilm formation (Williams and Cámara, 2009). A number of studies support the

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role of QS in *P. aeruginosa* infection and highlight its importance as a candidate for novel virulence-targeted antimicrobials (reviewed in Winstanley and Fothergill, 2009; Bjarnsholt and Givskov, 2007; Rasko and Sperandio, 2010). Therefore, search for QS inhibitors (QSI) to be used as lead compounds for the development of "non-antibiotic" drugs against *P. aeruginosa* has become an intensive field of industrial and academic research (Kjelleberg et al., 2008; Wang et al., 2008; Pan and Ren, 2009 and references therein).

Several reporter strains have been constructed, endowed with different specificity and sensitivity for QS signal molecules produced by different bacterial species. These biosensors consist of a reporter gene expressed under the control of a QS-dependent promoter, introduced into a strain unable to produce QS signal molecules (*e.g.*, *Escherichia coli* or a *P. aeruginosa* QS mutant) but expressing the signal receptor specific for that promoter (*e.g.*, LasR or its homologs). The presence of the specific QS signal molecule is recognized by the cognate signal receptor which triggers the expression of the reporter gene. Biosensors of this kind have been constructed primarily for the non-quantitative detection of different QS signal molecules produced by bacteria, but some of them have occasionally been employed for the quantification of QS signals in laboratory cultures and/or clinical samples (reviewed in Steindler and Venturi, 2007), or for the screening of QSI. A QSI screening procedure is generally carried out by detecting a reduction of the biosensor response to exogenously added synthetic signal molecule in the presence of a putative inhibitory compound. These QSI screening procedures have proven to be useful for the identification of inhibitors of signal receptor activity. However, since the signal molecule is exogenously provided, this approach is not suited to identify inhibitors of the synthesis and export of the signal molecule or of other key processes related to QS (reviewed in Wang et al., 2008; Kjelleberg et al., 2008).

Few biosensors based on the *P. aeruginosa* LasR/LasI system have been generated so far, all showing low sensitivity (*i.e.*, high detection limit) and thus requiring solvent extraction to concentrate the signal molecule before testing (Pearson et al., 1994; Riedel et al., 2001; Lee et al., 2006). One exception is a biosensor generated in *E. coli* able to detect $3OC_{12}$-HSL levels in the pM range (Winson et al., 1998), which however cannot be employed to identify inhibitors of specific *P. aeruginosa* QS-related processes other than signal-mediated activation of LasR.

On this basis, we have undertaken a study aimed at the construction of a simple multi-task biosensor suitable for the direct detection of $3OC_{12}$-HSL levels in both laboratory and clinical samples, and for the high-throughput screening of molecules targeting the *P. aeruginosa* $3OC_{12}$-HSL-dependent QS. To our knowledge, the screening method based on this newly developed biosensor represents the first QSI screening system suitable for the identification of inhibitors of any process related to $3OC_{12}$-HSL-dependent QS, including LasR activity as well as $3OC_{12}$-HSL synthesis and export/import across the cell envelope.

2. Materials and methods

2.1. Bacterial strains, growth media and reagents

Bacterial strains and plasmids used in this study are listed in Table S1. *E. coli* and *P. aeruginosa* cultures were grown in Luria–Bertani broth (LB; Sambrook et al., 1989), LB supplemented with 1.5% agar (LA) or Pseudomonas Isolation Agar (PIA). Where required, antibiotics were used at the following concentrations: ampicillin (Ap, 100 μg/ml), kanamycin (Km, 50 μg/ml), tetracycline (Tc, 12.5 μg/ml), nalidixic acid (Nal, 20 μg/ml), gentamycin (Gm, 10 μg/ml) for *E. coli*; Gm (200 μg/ml), carbenicillin (Cb, 250 μg/ml), trimethoprim (Tm, 100 μg/ml) for *P. aeruginosa*.

Synthetic acyl-HSLs were purchased from the University of Nottingham, UK. The stock solutions (25 mM) were prepared in ethyl acetate acidified with 0.1 % acetic acid. Furanone C-30 (FC30) was a gift from Dr. Naresh Kumar (University of New South Wales, Australia). The stock solution (100 mM) was prepared in dimethyl sulfoxide.

2.2. Generation of plasmids and reporter strains

Standard cloning techniques (Sambrook et al., 1989) were used to construct the plasmids listed in Table S1. In *P. aeruginosa*, the divergently oriented *rsaL* and *lasI* genes are transcribed by a bi-directional promoter. For the construction of plasmid pMS402 P*rsaL*::*lux*, a BamHI DNA fragment corresponding to the entire *rsaL-lasI* bidirectional promoter was excided from pDK201 (Duan and Surette, 2007) and ligated to the same plasmid in the opposite orientation, so that the transcription of the reporter gene is under the control of the *rsaL* promoter.

Plasmid pUCP18 P*rsaL*::*lux* was constructed in two-steps. First, a NotI/XhoI DNA fragment containing the *rsaL* promoter fused to the entire *luxCDABE* luminescence operon from *Photorhabdus luminescens* was excided from pMS402 P*rsaL*::*lux* and ligated to compatible sites of plasmid pBluescript2 KS+ (Stratagene), generating the plasmid pBS P*rsaL*::*lux*. Subsequently, a SacI–KpnI DNA fragment containing the P*rsaL*::*luxCDABE* transcriptional fusion was excided from pBS P*rsaL*::*lux* and ligated into the same sites of pUCP18 plasmid.

For the construction of mini-CTX P*rsaL*::*lux*, a BamHI DNA fragment encompassing the *rsaL* promoter was excided from pMS402 P*rsaL*::*lux* and ligated into BamHI-digested mini-CTX-*lux* plasmid. The orientation of the insert was checked by PCR.

Plasmids were introduced into *P. aeruginosa* by transformation or, in the case of miniCTX *PrsaL*::*lux*, by conjugal transfer. Excision of the miniCTX plasmid from the *attB* neutral site was achieved by Flp-mediated recombination as described (Hoang et al., 2000).

2.3. Micro-volumetric detection of $3OC_{12}$-HSL

Reporter strains PA14-R1, PA14-R2 and PA14-R3 were grown overnight at 37 °C on LA plates supplemented with the required antibiotics. Bacterial colonies were suspended in LB and adjusted to an absorbance at 600 nm (A_{600}) of 0.045. Two hundred μl of these suspensions was dispensed in black, clear-bottom 96-wells microtiter plates (Perkin Elmer) in the presence of increasing concentrations (152 pM to 3 μM) of exogenously added $3OC_{12}$-HSL. Plates were incubated at 37 °C with shaking, and cell density (A_{600}) and bioluminescence (light counts per second, LCPS) were simultaneously measured at each time point in a Wallac 1420 Victor^{3}V multilabel plate reader (Perkin Elmer). Relevant parameters for bioluminescence measurement were: emission aperture, large; counting time, 1 s. Relevant parameters for absorbance measurement were: filter 595/60; excitation aperture, normal; reading time, 0.1 s.

Luminescence values were normalized per cell density. The dose–response relationship between the concentration of $3OC_{12}$-HSL and luminescence emission was inferred for each strain at each time point, and both linear and logarithmic regression lines were drawn from the normalized luminescence values.

To assess the specificity of the PA14-R3 reporter, this strain was cultured in microtiter plates as described above in the presence of 10-fold dilutions of different acyl-HSL molecules, the final concentrations ranging from 100 μM to 1 pM (see Table 1 for details). Cell density and bioluminescence were measured after 4 h of incuba-

Table 1
Sensitivity of PA14-R3 to *P. aeruginosa* and Bcc acyl-HSLs.

	P. aeruginosa		Bcc		
Acyl-HSL	$3OC_{12}$-HSL	C_4-HSL	C_{10}-HSL	C_8-HSL	C_6-HSL
Detection limit[a]	10 pM	10 μM	100 μM	>100 μM	>100 μM

[a] The detection limit has been defined as the lowest concentration of acyl-HSL able to increase of ≥20% the luminescence produced by the biosensor in the absence of any added molecule.

tion at 37 °C. The detection limit has been defined as the lowest concentration of acyl-HSL able to increase of ≥20% the luminescence produced by the biosensor in the absence of any added molecule.

For the micro-volumetric determination of $3OC_{12}$-HSL levels in culture supernatants, PAO1, PAO1 *rsaL* and PAO1 *lasR* were grown overnight at 37 °C in LB. Cultures were diluted 1:100 in 100 ml LB and grown at 37 °C. At each time point the A_{600} was measured, and 2 ml of culture were centrifuged and the supernatant was filtered and stored at −20 °C until used. Twenty μl of appropriate dilutions of culture supernatants was added to 180 μl of LB inoculated with PA14-R3 (final A_{600} = 0.045). Microtiter plates were incubated at 37 °C with shaking, and A_{600} and LCPS were measured after 4 h of incubation at 37 °C. A calibration curve was generated with $3OC_{12}$-HSL at known concentrations, and used to calculate the concentration of $3OC_{12}$-HSL in each sample.

For the micro-volumetric determination of $3OC_{12}$-HSL levels in CF sputum, different sputum samples were collected from CF patients cared at the Cystic Fibrosis Center, University "Sapienza", Rome, Italy. Sputa were fluidified by addition of an equal volume of Sputasol solution (dithiothreitol 1.0 g/l, potassium chloride 0.2 g/l, sodium chloride 7.8 g/l, di-sodium hydrogen phosphate 1.1 g/l, potassium hydrogen phosphate 0.2 g/l; Oxoid) and incubation at 4 °C with gentle shaking for 1 h. To determine the *P. aeruginosa* load in CF sputa, 10-fold serial dilutions of the fluidified sputum were plated both on PIA and LA plates and the number of CFU/g of sputum were counted on PIA after overnight incubation at 37 °C. The rest of the sample was centrifuged at 30,000 × *g* for 20 min at 4 °C, and the supernatant was sterilized by filtration and stored at −20 °C for subsequent use. One hundred μl of fluidified sputum samples was added to 100 μl of a PA14-R3 culture in LB (final A_{600} = 0.045) in microtiter plates. Dedicated calibration curves were obtained by adding 100 μl of LB inoculated with PA14-R3 and containing known concentrations of $3OC_{12}$-HSL to 100 μl of Sputasol solution (final A_{600} of PA14-R3 = 0.045; final $3OC_{12}$-HSL concentrations ranging from 152 pM to 3 μM). Microtiter plates were incubated at 37 °C with shaking, and A_{600} and LCPS were measured after 4 h of incubation. The equations derived from the calibration curves were used to calculate the concentration of $3OC_{12}$-HSL present in each sputum sample.

2.4. Co-cultivation assays

Wild-type *P. aeruginosa* PA14 and the reporter strain PA14-R3 were grown overnight at 37 °C on LA plates. Bacteria were scraped from plate surfaces, suspended in LB and diluted in LB at a 3/1 reporter/wild type ratio (A_{600} = 0.045 and 0.015 for PA14-R3 and PA14, respectively). As negative control, the same procedure was followed to prepare a co-culture with the signal-defective PA14 *lasI* mutant instead of PA14. Two hundred μl of co-cultures was dispensed in microtiter plates, incubated for 8 h at 37 °C with shaking, and A_{600} and LCPS were measured every hour. Where indicated, $3OC_{12}$-HSL and/or FC30 were added to the samples at final concentrations of 3 and 10 μM, respectively.

A patent concerning biosensor PA14-R3 and its applications is pending (No. ITRM2010A000541O).

3. Results and discussion

3.1. Construction of reporter strains for the detection of $3OC_{12}$-HSL

A transcriptional fusion between the LasR-dependent *rsaL* promoter (P*rsaL*) and the *luxCDABE* operon was constructed and evaluated as reporter system for $3OC_{12}$-HSL quantification. P*rsaL* was selected because this promoter is directly activated by LasR and several hundred-fold inducible in the presence of $3OC_{12}$-HSL (Rampioni et al., 2007; Whiteley and Greenberg, 2001; Schuster et al., 2003), whereas the *luxCDABE* operon is a convenient reporter system that has no background in *P. aeruginosa* and provides direct signal detection without addition of exogenous substrate(s) (Meighen and Szittner, 1992). The *P. aeruginosa* PA14 *lasI* mutant was chosen as the host strain for the P*rsaL*::*luxCDABE* fusion because it expresses LasR signal receptor protein but is impaired in the LasI synthase activity and hence does not produce $3OC_{12}$-HSL. If a P*rsaL*::*luxCDABE* fusion is introduced into PA14 *lasI*, this strain will produce luminescence only upon addition of the $3OC_{12}$-HSL signal molecule to the growth medium.

To generate reporter strains with different ranges of sensitivity to $3OC_{12}$-HSL, the P*rsaL*::*luxCDABE* fusion was introduced into PA14 *lasI* either as high-copy number construct in the pUCP18 plasmid (Schweizer, 1991) or as low-copy number construct in the pMS402 plasmid (Duan et al., 2003) or chromosomally integrated as single copy at the *attB* neutral site (Hoang et al., 2000). The resulting strains were named PA14-R1, PA14-R2 and PA14-R3, respectively. In order to test the ability of the reporter strains to respond to $3OC_{12}$-HSL, the three strains were grown for 8 h in 200 μl of LB medium in microtiter plates at 37 °C in the presence of increasing $3OC_{12}$-HSL concentrations, and luminescence was measured every hour (Fig. S1). The results were used to generate both linear and logarithmic functions describing the dose–response relationships for each reporter strain at each hour of measurement (Fig. S2).

PA14-R1 and PA14-R2 gave optimum response at 5 h of growth, with luminescence emission directly proportional to $3OC_{12}$-HSL concentration in the range of 152 pM to 37 nM, and to the log of $3OC_{12}$-HSL concentration in the range of 12 nM to 1 μM (Fig. S2A and B). On the other hand, the PA14-R3 biosensor showed the best results at 4 h of growth, with luminescence directly proportional to $3OC_{12}$-HSL concentration in the range of 152 pM to 12 nM, and to the log of $3OC_{12}$-HSL concentration in the range of 1.4 nM to 3 μM (Fig. 1 and Fig. S2C). Thus, PA14-R3 was able to measure $3OC_{12}$-HSL levels over a wider range of concentrations compared with PA14-R1 and PA14-R2. The higher reproducibility of response data in the different experiments and the stability of the reporter system integrated into the chromosome are additional advantages of PA14-R3 with respect to the other two reporter strains. For these reasons, PA14-R3 was chosen for further studies.

3.2. Micro-volumetric determination of $3OC_{12}$-HSL levels in laboratory cultures and in clinical samples

The wide range of response and the high sensitivity of the PA14-R3 biosensor suggested that this strain could be used for the direct micro-volumetric determination of $3OC_{12}$-HSL levels in *P. aeruginosa* laboratory cultures as well as in sputa of CF patients colonized by *P. aeruginosa*. To our knowledge, among the microorganisms colonizing the CF lung, only *P. aeruginosa* and members of the *Burkholderia cepacia* complex (Bcc) produce acyl-HSL signal molecules (Foweraker, 2009; Lyczak et al., 2002), and both species can be isolated from the same CF sputum (Govan et al., 2007). Given that *P. aeruginosa* produces also C_4-HSL and Bcc members produce N-hexanoyl-HSL (C_6-HSL), N-octanoyl-HSL (C_8-HSL), and N-decanoyl-HSL (C_{10}-HSL) (Venturi et al., 2004; Table 1), it was

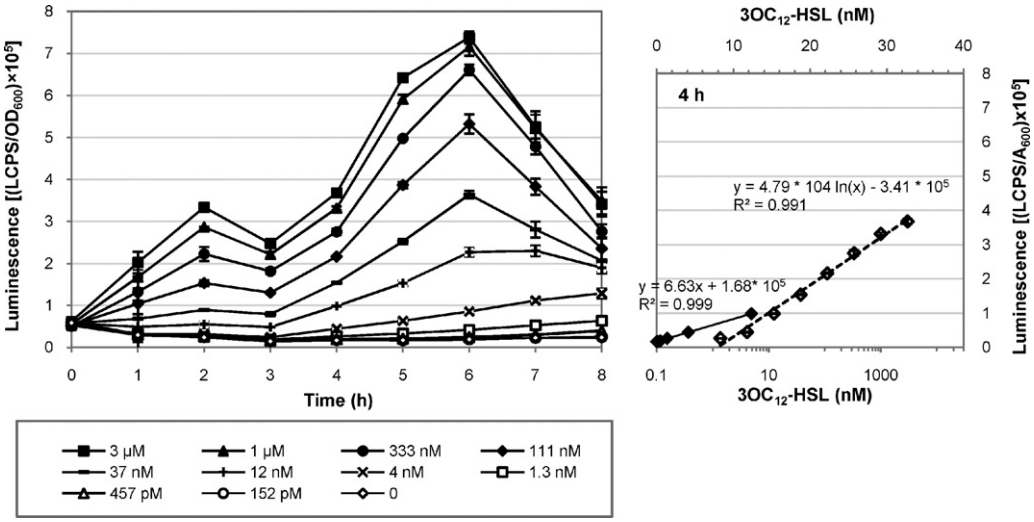

Fig. 1. Detection of $3OC_{12}$-HSL by the PA14-R3 reporter strain. The left panel shows the luminescence produced by the reporter strain grown in LB in microtiter plates at 37 °C in the presence of increasing concentrations of exogenously-added $3OC_{12}$-HSL. $3OC_{12}$-HSL concentrations are given in the legend below the figure. Luminescence was measured every hour. The right panel shows the functions describing the dose–response relationships inferred from the data shown in the left panel, at 4 h of growth. Regression lines were drawn on both logarithmic (dashed, lower axis) and linear scale (solid, upper axis). The equation and the R^2 value for each regression line are shown. Data are the mean ± SD of three independent experiments.

necessary to rule out the possibility that these acyl-HSLs could influence PA14-R3 response to $3OC_{12}$-HSL. Therefore, we compared the sensitivity of PA14-R3 for all acyl-HSLs produced by *P. aeruginosa* and Bcc.

PA14-R3 did not respond to C_6-HSL and C_8-HSL up to 100 μM concentration, while it responded to C_4-HSL and C_{10}-HSL, although at concentrations 6 and 7 orders of magnitude higher than the detection limit for $3OC_{12}$-HSL, respectively (Table 1). According to previous studies, the highest concentration of acyl-HSLs in laboratory cultures and in CF sputa are ≤28 μM and ≤21 nM, respectively (Chugani et al., 2001; Erickson et al., 2002; Chambers et al., 2005). Considering that in our assays biological samples are 4- to 10-fold diluted (see Section 2), the above results rule out any interference of C_4-, C_6-, C_8- and C_{10}-HSLs with the response of PA14-R3 to $3OC_{12}$-HSL and suggest that the PA14-R3 biosensor could be suitable to determine the levels of $3OC_{12}$-HSL in both laboratory cultures and CF sputa.

In order to check this hypothesis, the PA14-R3 biosensor was used to directly measure the levels of $3OC_{12}$-HSL in micro-volumes of culture supernatants of *P. aeruginosa* strains that differ in $3OC_{12}$-HSL production (Fig. 2). Considering the well-known role of the QS regulators LasR and RsaL as activator and repressor of *lasI* expression and $3OC_{12}$-HSL production, respectively, we used cultures of the wild-type strain PAO1 and its isogenic mutants PAO1 *rsaL* and PAOI *lasR* as source of the $3OC_{12}$-HSL inducer (Rampioni et al., 2007; Schuster and Greenberg, 2006). Twenty μl of culture supernatants (or appropriate dilutions) was diluted in 200 μl (final volume) of a PA14-R3 culture, and luminescence was measured in microtiter plates after 4 h of growth (see Section 2 for details). With respect to the wild-type strain, a dramatic reduction and a strong increase of $3OC_{12}$-HSL levels were observed in the *lasR* mutant and in the *rsaL* mutant, respectively (Fig. 2), in good agreement with the opposite effects of these genes on *lasI* expression and $3OC_{12}$-HSL production (Rampioni et al., 2007; Schuster and Greenberg, 2006). These results confirmed that PA14-R3 can be used as a convenient and easy-to-handle tool to quantify $3OC_{12}$-HSL in *P. aeruginosa* culture supernatants. For this reason, this biosensor could be useful for large-scale studies aimed at investigating mutations and/or cultural conditions affecting $3OC_{12}$-HSL production in *P. aeruginosa*.

A pilot assay was also performed to test the possibility of using PA14-R3 for the quantification of the levels of $3OC_{12}$-HSL in micro-volumes of sputa from CF patients. As preliminary test, we verified whether the CF sputum composition may influence the response of the PA14-R3 biosensor. To this aim, fluidified sputum samples (100 μl) of five CF patients not colonized by *P. aeruginosa* were added to an equal volume of the PA14-R3 culture in the absence or presence of known concentrations of $3OC_{12}$-HSL. No CF sputum activated the biosensor unless $3OC_{12}$-HSL was exogenously added, and the response of PA14-R3 to $3OC_{12}$-HSL in the presence of Sputasol alone or of the fluidified *Pseudomonas*-negative sputum samples was comparable (Fig. S3), indicating that CF sputum composition does not affect $3OC_{12}$-HSL detection. Then, sputum samples were collected from 20 CF patients with known history of colonization by *P. aeruginosa*. Selective plate count confirmed the presence of *P. aeruginosa* cells in the sputum of 15 out of the 20 patients analyzed, even if the bacterial load was highly variable among samples (Table S2). The PA14-R3 biosensor detected measurable amounts of $3OC_{12}$-HSL (from 7 to 237 nM) in eight out of the 15 *P. aeruginosa*

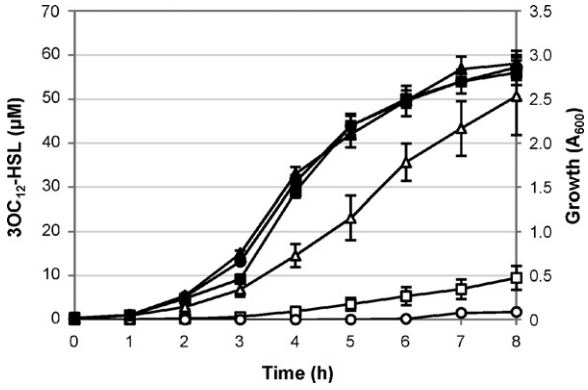

Fig. 2. Micro-volumetric determination of $3OC_{12}$-HSL levels in laboratory cultures using the PA14-R3 biosensor. Cell density (A_{600}; filled symbols) and $3OC_{12}$-HSL level in culture supernatants (empty symbols) were measured along the growth curve in *P. aeruginosa* PAO1 (squares), PAO1 *rsaL* (triangles) and PAO1 *lasR* (circles). $3OC_{12}$-HSL concentrations were inferred using dedicated calibration curves. Data are the mean ± SD of two independent experiments.

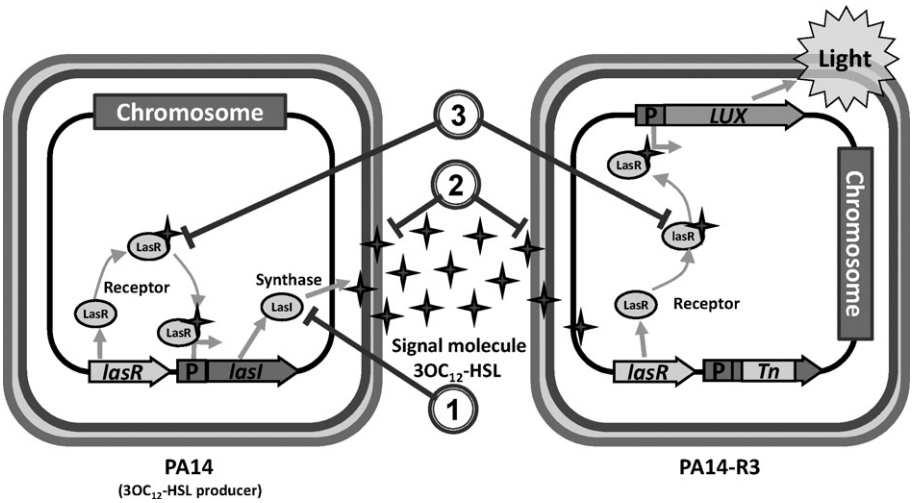

Fig. 3. Schematic representation of the PA14/PA14-R3 co-cultivation screening system. The wild-type PA14 produces the $3OC_{12}$-HSL signal that in turn activates PA14-R3 bioluminescence emission. Molecules interfering with (1) synthesis of the signal molecule, (2) transport of the signal molecule across the cell envelope, and (3) activity of the signal molecule receptor will cause a reduction of luminescence with respect to the untreated control. *Tn*, transposon MrT7 (Liberati et al., 2006).

positive sputum samples, but no apparent correlation between the bacterial load and $3OC_{12}$-HSL levels was observed (Table S2). This result is in line with some previous studies in the field (Erickson et al., 2002; Middleton et al., 2002; Chambers et al., 2005) and can be explained by the coexistence of QS-defective and -proficient clonal variants in the lung of CF patients (Winstanley and Fothergill, 2009). The ratio between these populations is variable and seems to increase during years, suggesting that a reduction of $3OC_{12}$-HSL production could occur during late stages of the chronic infection (Hoffman et al., 2009; Winstanley and Fothergill, 2009). It must be noted, however, that in previous studies only a limited number of CF sputum samples was analyzed, likely due to the technical limits imposed by previous $3OC_{12}$-HSL measurement methods. Thus, to draw any conclusion about the relationship between $3OC_{12}$-HSL levels, *P. aeruginosa* colonization and the severity of the chronic infection, a larger number of samples should be analyzed and the results correlated to the clinical history of the patient. Although out of the scope of the present work, the above results highlight that PA14-R3 represents a powerful tool to deal with this still unexplored issue.

Finally, it must be pointed out that sputum samples collected from CF patients exposed to antibiotic treatment inhibited the growth of the biosensor strain (data not shown), indicating that the PA14-R3 biosensor can be used only for CF patients who had stopped antibiotic treatment at least three days before sampling, as in the case of the patients described above.

3.3. Development and validation of a co-cultivation method for the micro-volumetric, high-throughput screening of QSI

The last aim of this study was the development of a novel QSI screening system based on the co-cultivation of PA14-R3 with wild-type *P. aeruginosa* PA14. In this system, the wild-type strain provides the biosensor with the $3OC_{12}$-HSL required for luminescence emission. The addition of a molecule with inhibitory activity towards any process related to QS would reduce the emission of luminescence by the biosensor with respect to the control co-culture without any inhibitor added (Fig. 3). As expected, the co-culture of PA14-R3 with PA14 emitted significant luminescence levels, while the control co-culture of PA14-R3 with the PA14 *lasI* mutant, which is impaired in $3OC_{12}$-HSL production, did not (Fig. 4). The addition of exogenous $3OC_{12}$-HSL (3 μM) to the PA14-R3/PA14 co-culture increased the luminescence levels during the first 4 h of growth, after which the luminescence levels were comparable to those measured in the co-culture without addition of synthetic $3OC_{12}$-HSL (Fig. 4). The anticipated response of the biosensor in the presence of exogenously added $3OC_{12}$-HSL is a feature of QS-regulated systems and is due to the presence of high concentrations of signal molecule at low cell densities. This experiment shows that, under our experimental conditions, the PA14-R3/PA14 co-culture did not reach a saturation response to $3OC_{12}$-HSL for the first 5 h of growth, suggesting that during this period any decrease in $3OC_{12}$-HSL synthesis and/or sensing can be detected by the system. Since at 4 h of growth the system disclosed a good response and was far below the saturation level, we chose 4 h as the optimal time point for the high-throughput screening of potential QSI.

To validate the efficacy of using the PA14-R3/PA14 co-culture as screening system for the identification of QSI compounds, we tested the response of the system to FC30, a well-known QSI (Hentzer et al., 2003). The mechanism of action of FC30 remains poorly understood, although preliminary studies suggest that this syn-

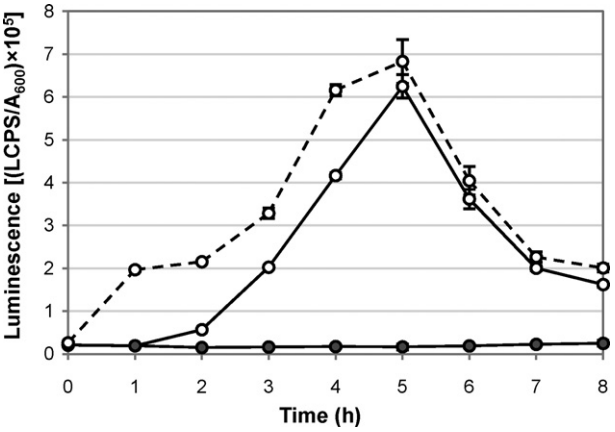

Fig. 4. Development of the PA14-R3/PA14 co-cultivation as QSI screening system. Luminescence emitted by PA14-R3 co-cultured with *P. aeruginosa* PA14 (empty circles) in the absence (solid line) or in the presence of 3 μM exogenously added $3OC_{12}$-HSL (dashed line). As control, PA14-R3 was co-cultivated with *P. aeruginosa* PA14 *lasI* (filled circles). The co-cultures were grown in microtiter plates at 37 °C, and cell density and bioluminescence were measured every hour. Data are the mean ± SD of three independent experiments.

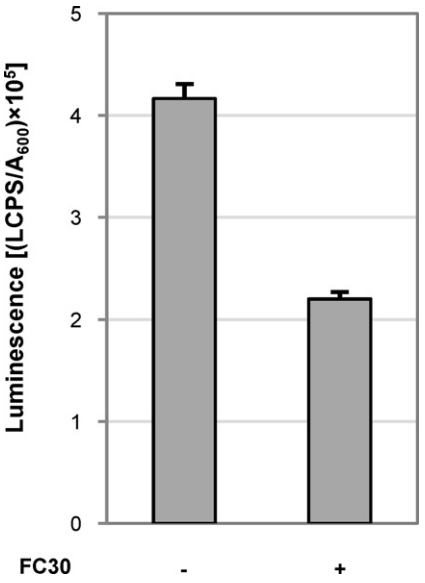

Fig. 5. Validation of the PA14-R3/PA14 co-cultivation as QSI screening system. Luminescence emitted by the PA14-R3/PA14 co-culture at 4 h of growth in the absence or in the presence of 10 μM FC30. Data are the mean ± SD of three independent experiments.

thetic derivative of natural furanones interferes with LasR signaling in *P. aeruginosa* (Kjelleberg et al., 2008). Accordingly, transcriptomic studies showed that FC30 reduces the transcription of many $3OC_{12}$-HSL-regulated genes, affects biofilm structure, and promotes the clearance of *P. aeruginosa* in a mouse model of pulmonary infection (Hentzer et al., 2003). However, to our knowledge, any direct effect of FC30 on $3OC_{12}$-HSL production by *P. aeruginosa* has not yet been demonstrated.

The level of $3OC_{12}$-HSL produced by *P. aeruginosa* PAO1 was decreased by 50% in the presence of 10 μM FC30, demonstrating that FC30 negatively affects $3OC_{12}$-HSL production (Fig. S4). Coherently, the addition of 10 μM FC30 to the PA14-R3/PA14 co-culture determined a comparable reduction (ca. 50%) of the luminescence levels with respect to the control co-culture without any added compound (Fig. 5), indicating that the inhibitory effect of FC30 on $3OC_{12}$-HSL production by the wild-type strain is faithfully recorded by the PA14-R3 biosensor. Overall, these results indicated that the PA14-R3/PA14 co-cultivation system is suitable for the screening of QSI.

4. Concluding remarks

The PA14-R3 biosensor provides direct quantification of $3OC_{12}$-HSL over a wide range of concentration (from pM to μM) in micro-volumes of laboratory or clinical samples. Since PA14-R3 can be used in microtiter plates and results are made available in approximately 4 h, it looks well suited for high-throughput studies. For instance, we showed that it can be successfully used for studying mutants with altered $3OC_{12}$-HSL production or for determining $3OC_{12}$-HSL concentration in sputum samples from CF patients. Of note, besides not requiring substrate addition for signal detection, this biosensor is very stable, since it carries the P*rsaL*::*luxCDABE* fusion permanently integrated in the chromosome.

The specific features of PA14-R3 made it possible to develop and validate a novel high-throughput QSI screening system based on the co-cultivation of PA14-R3 with the PA14 wild-type strain. The most innovative feature of this screening system is that $3OC_{12}$-HSL is not exogenously added to the biosensor, but provided by a *P. aeruginosa* producer strain during co-cultivation with the biosensor. Besides being handy and cost-effective, this approach allows the identification of compounds targeting any cellular process critical for QS response, in addition to LasR activity (as schematized in Fig. 3), and in the future it could represent a valuable tool to search for novel QSI-based drugs active against *P. aeruginosa* infection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.01.022.

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