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A Novel Plasmid For Detection of *N*-acyl Homoserine Lactones

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Abstract

Many bacteria utilize acyl-homoserine lactones as cell to cell signals that can regulate the expression of numerous genes. Structural differences in acyl-homoserine lactones produced by different bacteria, such as acyl-side chain length and the presence or absence of an oxy group, make many of the commonly used detection bioassays impractical for broad range detection. Here we present a simple, broad range acyl-homoserine lactone detection bioassay that can be used to detect a wide range of these chemical signals. A plasmid (pEAL01) was constructed and transformed into *Pseudomonas aeruginosa* strain QSC105 to allow for detection of a broad range of acyl-homoserine lactones through induction of a *lasB'-lacZ* transcriptional fusion. Monitoring β -galactosidase activity from this bioassay showed that *P. aeruginosa* strain QSC105 (pEAL01) could detect the presence of eight acyl-homoserine lactones tested at physiological concentrations. This novel strain could also detect acyl-homoserine lactones from the extracts of four different bacteria that produce different acyl-homoserine lactones signals. These data indicate that strain QSC105 (pEAL01) can be used to detect a wide variety of acyl-homoserine lactones by a simple β -galactosidase assay and this bioassay could be a useful and inexpensive tool to quickly identify the presence of these signal molecules.

Keywords

quorum sensing; acyl-homoserine lactone; bioassay

1. Introduction

Greater than 50 species of gram-negative bacteria have been found to communicate intercellularly via acyl-homoserine lactone signals (AHLs) (Fuqua, 2001). These signals function as “quorum sensing” molecules in that their concentration is an indicator of the relative bacterial population density. When the signal concentration reaches a threshold level, it interacts with a transcriptional activator protein which will then induce specific genes. These cell to cell signals control a wide variety of bacterial functions such as symbiosis, virulence, competence, conjugation, antibiotic production, motility, sporulation, and biofilm formation (Miller and Bassler, 2001). The AHL signal systems are comprised of two genes, the first of which encodes a signal synthase protein that is a member of the LuxI-type family of proteins. The second gene encodes a member of the LuxR-type transcriptional activator protein family (see Fuqua et al., 1994 for a review). The activity of these transcriptional activators is dependent

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on their cognate AHL signal, which binds to the transcriptional activator protein and thereby leads to its activation.

AHL signals share a common base structure of a homoserine lactone ring but they are highly variable with regard to the attached acyl side chain. When comparing signals from different systems, the acyl side chain varies in length, oxidation state, and saturation (Fuqua et al., 1996). Such signal differences are what provides specificity for each LuxR-type transcriptional activator. The specificity of LuxR-type proteins is the limiting factor for detecting signals with the use of bioassays.

The ability to detect AHL signals has enabled quorum sensing systems to be identified in many gram-negative bacteria. Several bioassays have been designed for detecting AHLs (reviewed in Steindler and Venturi, 2007). However, most are limited in their detection range due the incorporation of only one quorum sensing system. Improved bioassays have been developed for detection of a wide range of AHLs (Farrand et al., 2002; Zhu et al., 2003). While these can detect a variety of AHLs, they are limited in their ability to detect combinations of several important AHLs (Shaw et al., 1997). One broad specificity assay utilizes thin layer chromatography in conjunction with a bioassay strain (Zhu et al., 2003). This is an excellent assay, but it requires the use of multiple organic solvents, something that some laboratories may find prohibitive. Other AHL detection bioassays have been developed that required multiple plasmids for functionality (Farrand et al., 2002), which may be prohibitive if the system needed to be transferred to another organism for a given application. We wanted to develop a simple and inexpensive broad-specificity bioassay for the detection of acyl-homoserine lactone signals that is based on measuring enzymatic activity from a *lacZ* reporter fusion. The bioassay only requires extracts and a spectrophotometer to measure light absorbance at 660 nm and 420 nm. This single plasmid bioassay is based on the two *Pseudomonas aeruginosa* quorum sensing systems (*las* and *rhl*) which use two AHLs that are structurally different. In *P. aeruginosa*, the *las* and *rhl* quorum sensing systems regulate numerous genes, including *lasB*, which encodes LasB elastase (Gambello and Iglewski, 1991; Pearson et al., 1997; Schuster et al., 2004; Wagner et al., 2004). Both the *las* and *rhl* quorum sensing systems positively control *lasB* transcription through their respective cell to cell signals, *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C₁₂-HSL) and *N*-butyryl-L-homoserine lactone (C₄-HSL) (Pearson et al., 1994; Pearson et al., 1995; Pearson et al., 1997). These signals associate with, and thereby activate, the respective transcriptional activators, LasR and RhlR. Both LasR and RhlR can also be activated by acyl-homoserine lactone signals that are similar to their cognate signals (Ochsner and Reiser, 1995; Passador et al., 1996). Because of the differences in their cognate signals, we felt that an assay that used both LasR and RhlR would allow for the detection of a broad range of AHL signals. In this manuscript, we report the construction of a plasmid that contains *tacp-lasR*, *tacp-rhlR*, and *lasB'-lacZ*, and its ability to confer a non-signaling *P. aeruginosa* strain the ability to respond to a wide-range of AHL signals.

2. Materials and methods

2.1. Construction of bioassay plasmid pEAL01

Plasmid pEAL01 was constructed as follows (see Fig. 1). First, plasmid pECP16 was digested with *Hind*III (Invitrogen) and a 2120 bp fragment containing *tacp-lasR* was purified from an agarose gel using a GeneClean®III kit. Plasmid pECP16 was constructed by ligating the 1900 bp fragment from a *Sca*I and *Bam*HI digest of pECP8 (Wade et al., 2005), which contained *tacp-lasR*, into the *Sal*I digested plasmid pJPP8 (Pearson et al., 1997). The resulting 9.4 kb plasmid contains both *tacp-rhlR* and *tacp-lasR*. The plasmid pECP8 was constructed by ligating the PCR product of pKDT37 (Passador et al., 1996), containing the *lasR* coding region, into *Eco*RI-digested pEX1.8. The resulting plasmid contains a *tacp-lasR* fusion in which there

is optimal spacing (9 bp) between the ribosome binding site from pEX1.8 and the start codon of *lasR*. The *Hind*III fragment from pECP16 was then ligated into the *Hind*III site on pECP64 (Pearson et al., 1997). Plasmid pECP64 contains *tacp-lasR* and *lasB'-lacZ* fusions and is also a pEX1.8-based vector. The resulting plasmid, pEAL01, contained *tacp-lasR*, *tacp-rhlR*, *lasB'-lacZ*, *bla*, *lacI*, and ColE1 origin of replication for enteric bacteria, such as *Escherichia coli*, and the origin of replication for *Pseudomonads*. The plasmid was transformed by electroporation (Choi et al., 2006) into *P. aeruginosa* strain QSC105 (Chugani et al., 2001), which is a *lasI*, *rhlI*, *pqsH* mutant. This strain was chosen because it cannot synthesize AHL signals or the *Pseudomonas* quinolone signal, which eliminates any background from endogenously produced signals. *P. aeruginosa* strain QSC105 (pEAL01) was stored at -80°C in 10% skim milk (Fisher Scientific) and plated before each experiment.

2.2. β -Galactosidase assays

β -Galactosidase (β -Gal) assays were performed as follows. Cultures of *P. aeruginosa* strain QSC105 (pEAL01) were inoculated from freshly grown plates and grown overnight at 37°C with vigorous shaking (≥ 180 rpm) in 10 ml Peptone Tryptic Soy Broth (PTSB) (Ohman et al., 1980) supplemented with 200 μ g/ml carbenicillin (Sigma). The following day, a subculture was inoculated to a final OD₆₆₀ of 0.08 and allowed to grow for eight hours. The culture was then diluted into fresh media to a final OD₆₆₀ of 0.1 and 1 ml aliquots were transferred to tubes containing dried signals. The 1 ml cultures containing varying concentrations of signals were grown for 18 hours at 37°C with vigorous shaking and β -gal assays were performed as described by Miller (1972). All β -gal assays were performed in duplicate and data presented are the mean from at least three independent experiments. All signals were tested at 0.01, 0.1, 1, 10 and 50 μ M. The tested concentration does not exceed 50 μ M in order to maintain a concentration within the physiological concentration range found in nature.

The following cell to cell signals or signal-like compounds were tested at various concentrations (see Fig. 2): *N*-3-oxo-dodecanoyl-homoserine lactone (3-oxo-C₁₂-HSL) (Quorum Sciences, Inc.), *N*-dodecanoyl-homoserine lactone (C₁₂-HSL) (Sigma/Fluka), *N*-decanoyl-homoserine lactone (C₁₀-HSL) (Sigma/Fluka), *N*-octanoyl-homoserine lactone (C₈-HSL) (Sigma/Fluka), *N*-heptanoyl-homoserine lactone (C₇-HSL) (Sigma/Fluka), *N*-3-oxo-hexanoyl-homoserine lactone (3-oxo-C₆-HSL) (gift from E. P. Greenberg), *N*-hexanoyl-homoserine lactone (C₆-HSL) (Sigma/Fluka), and *N*-butanoyl-homoserine lactone (C₄-HSL) (Sigma/Fluka). All signals were maintained in acidified ethyl acetate (Pesci et al., 1999) at -20°C until used.

2.3. Extractions

Extractions were performed on *P. aeruginosa* wild-type strain PAO1 grown in PTSB at 37°C, *Burkholderia cepacia* strain J2549 grown on Luria Bertani media (LB) at 37°C, *Erwinia carotovora* strain Ecc71 grown in LB at 30°C, and *Agrobacterium tumefaciens* strain R10 (pCF218) (Zhu et al., 1998) grown in LB supplemented with 2 μ g/ml tetracycline (this is a wild type strain that overproduces the 3-oxo-C₈-HSL). Overnight cultures from freshly streaked plates were sub-cultured to a final OD₆₆₀ of 0.08 and allowed to grow for eight hours. A 50 ml subculture was then inoculated to a final OD₆₆₀ of 0.1. This was grown for 18 hours at the appropriate temperature, with shaking, before the extraction with acidified ethyl acetate was performed. The cultures were centrifuged at 16,000 $\times$ g for 10 minutes and the supernatant was extracted twice with an equal volume of acidified ethyl acetate as described elsewhere (Pesci et al., 1999). After the addition of 10% sodium sulfate, the extract was dried and resuspended in 1 ml acidified ethyl acetate. For the *B. cepacia*, *E. carotovora*, and *A. tumefaciens* strains, we tested an amount of extract derived from 10 ml, 1 ml, or 0.1 ml of culture supernatant. For *P. aeruginosa* strain PAO1 we tested an amount derived from 1 ml or 0.1 ml of culture supernatant, as this was more than enough to activate the bioassay. As in the

previous bioassays, each extraction was repeated three separate times and the β -gal activity was measured.

3. Results and discussion

The purpose of this study was to develop a simple bioassay that could detect a large range of AHL signals. We constructed a plasmid (pEAL01) that would constitutively express *lasR* and *rhIR*, and contained a *lasB'-lacZ* transcriptional fusion. We tested the ability of *P. aeruginosa* strain QSC105 (pEAL01) to detect eight structurally diverse AHLs that had acyl chains ranging from four to twelve carbons. We found that this bioassay strain was able to detect the presence of each AHL signal tested (Fig. 3). The amount of signal required to activate the *lasB'-lacZ* fusion ranged from 0.1 μ M 3-oxo-C₁₂-HSL to 50 μ M C₁₂-HSL (Fig. 3). Not surprisingly, the signals that strain QSC105 (pEAL01) were most sensitive to, 3-oxo-C₁₂-HSL and C₄-HSL, are produced by *P. aeruginosa*, and are the cognate signals for the LasR and RhIR transcriptional activator proteins encoded on plasmid pEAL01. Although the signal concentration required to activate the *lasB'-lacZ* fusion differed greatly depending on the signal, strain QSC105 (pEAL01) was able to detect each signal at a physiologically relevant concentration. The ability of strain QSC105 (pEAL01) to detect the AHL signals with acyl chains ranging from four to twelve carbons makes this strain an efficient and simple AHL detection bioassay.

Because the plasmid can replicate in *Pseudomonads* and enteric bacteria, we also tested the ability of the plasmid to confer the ability to detect 3-oxo-C₁₂-HSL and C₄-HSL to *E. coli* (data not shown). As expected, *E. coli* strain DH5 α (pEAL01) was sensitive to both AHLs tested and could detect 3-oxo-C₁₂-HSL at concentrations as low as 10 nM, indicating the assay functions well in an *E. coli* background. These data also confirm that the detection of AHLs by this bioassay is a result of the plasmid and not due to our host *P. aeruginosa* strain.

We then set out to learn whether our bioassay could be used to detect signals from culture supernatants. To test this, β -gal activity was determined after *P. aeruginosa* strain QSC105 (pEAL01) was exposed to a culture supernatant extract from four different species of AHL-producing bacteria. Supernatants taken from *P. aeruginosa* strain PAO1, *B. cepacia* strain J2549, *E. carotovora* strain Ecc71, and *A. tumefaciens* strain R10 (pCF218) were extracted with acidified ethyl acetate as described above. Fig. 4 shows that strain QSC105 (pEAL01) detected the presence of AHL signals in the extracts from all four bacteria. As would be expected, the addition of strain PAO1 extract resulted in the highest β -gal activity (Fig. 4a). An extract derived from 1 ml of strain PAO1 culture caused a very large activation of the assay ($46,063 \pm 4,590$ Miller Units), which is most likely caused by a combination of the two AHL signals, 3-oxo-C₁₂-HSL and C₄-HSL, produced by *P. aeruginosa* (Brint and Ohman, 1995). The bioassay also detected the presence of high concentrations of AHLs or AHL-like compounds in 1 ml extracts of *B. cepacia* strain J2540 ($2,360 \pm 1,100$ Miller units) and 10 ml extracts of *A. tumefaciens* strain R10 (pCF218) ($20,546 \pm 4,808$ Miller units; Fig. 4c). Interestingly, the bioassay had decreased responsiveness to the 10 ml extracts of *B. cepacia* strain J2540 as compared to the 1.0 ml extracts (Fig. 4b). This could be attributed to the 10 ml extract having a high concentration of a compound that inhibits *P. aeruginosa* responsiveness to AHL signals, and indicates the necessity for testing a range of extract concentrations. The bioassay was also able to detect an AHL or AHL-like molecules in extracts derived from 10 ml of supernatant from *E. carotovora* strain Ecc71 ($1,888 \pm 710$ Miller units; Fig. 4d). This strain utilizes the CarI and ExpI synthases (Andersson et al., 2000) to produce 3-oxo-C₆-HSL as its major AHL signal. This AHL signal is the most structurally divergent AHL tested as compared to those recognized by RhIR and LasR. Although the bioassay did not readily detect 3-oxo-C₆-HSL in the 1 or 0.1 ml strain Ecc71 extracts, the fact that it was able to detect the

signal in the extract derived from 10 ml of culture supernatant shows that the bioassay would be of practical use for detection of AHLs unrelated to those produced by *P. aeruginosa*.

The AHLs tested are produced by many different bacteria and differ in their structure, from 3-oxo-C₁₂-HSL to C₄-HSL. A bioassay based on one quorum sensing system would have a limited detection range and may not respond to AHLs that greatly differ by acyl side chain length or in the presence or absence of an oxy group. Previous work (Zhu et al, 1997) has shown that overexpression of the LuxR homolog, TraR, allowed for the detection of numerous AHLs besides its cognate signal. Our data are consistent with these findings and the overexpression of both LasR and RhlR in plasmid pEAL01 further broadens the potential detection of AHLs by our bioassay. The expression of both LasR and RhlR in plasmid pEAL01 gives this bioassay the ability to recognize a range of structurally diverse AHLs.

Taken together, the data support the notion that strain QSC105 (pEAL01) can be used to detect a broad range of AHL signals. Our data also shows that the plasmid could be used to transform *E. coli* and potentially any *Pseudomonad* or enteric bacteria into an AHL bioassay strain if so desired. This method provides a simple alternative for previously described bioassays that have a limited detection range or require more complicated experimentation. To our knowledge, this is the first AHL bioassay that has been shown to recognize all AHLs produced by *P. aeruginosa* with high sensitivity as well as the other AHL signals tested.

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Fig. 1.

Construction of pEAL01. Plasmids pECP64 and pECP16 were digested with *HindIII* and the resulting 2,120 bp fragment from pECP16, containing the *tacp-rhlR* gene, was ligated into the *HindIII* site of pECP64. The coding regions are shown as large bold arrows. *ori*, *E. coli* origin of replication; *P. a. ori*, *P. aeruginosa* origin of replication. For further details, see Section 2.1.

Fig. 2.
Structure of *N*-acyl homoserine lactones used in this study.

Fig. 3.

β-gal activity of *P. aeruginosa* strain QSC105 (pEAL01) grown in the presence of various *N*-acyl homoserine lactones. Cultures were grown in PTSB media supplemented with the indicated concentration of *N*-acyl homoserine lactone. After 18 hours of growth, cultures were assayed for β-gal activity, which is presented in Miller units as the mean ± SD of results from duplicate assays from at least three independent experiments.

Fig. 4. Detection of *N*-acyl homoserine lactones in culture extracts by *P. aeruginosa* strain QSC105 (pEAL01). Cultures were grown in PTSB media supplemented with different concentrations of culture supernatant extracts from: (A) *P. aeruginosa* strain PAO1, (B) *B. cepacia* strain J2540, (C) *A. tuelfaciens* strain R10 (pCF218), and (D) *E. carotovora* strain Ecc71. After 18 hours of growth, each culture was assayed for β-gal activity. β-gal activity is presented in Miller units as the mean ± SD of results from duplicate assays from at least three independent experiments. Extract concentrations are represented as follows: 10X, derived from 10 ml of culture supernatant; 1X, derived from 1 ml of culture supernatant; 0.1X, derived from 0.1 ml of culture supernatant. β-gal activity of *P. aeruginosa* strain QSC105 (pEAL01) grown in the

presence of 50 μM of synthetic *N*-acyl homoserine lactones that would be produced by the bacterium being tested are included for comparison. Abbreviation: no addition, NA.