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Note

A versatile plasmid biosensor useful to identify quorum sensing LuxR-family orphans in bacterial strains

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ABSTRACT

Eight *luxI*-family gene promoters (*luxI*, *cviI*, *ahlI*, *rhlI*, *cepI*, *phzI*, *traI* and *ppuI*) were cloned in tandem, upstream a promoterless *lacZ* gene in a promoter probe vector yielding pMULTIAHLPROM. This unique construct is useful in determining whether a bacterial strain not producing *N*-acyl homoserine lactone signal molecules (AHLs) possesses orphan LuxR type proteins able to respond to AHLs and activate transcription from quorum sensing target genes. Using pMULTIAHLPROM, it was demonstrated that *Enterobacter aerogenes* possibly contains a LuxR-family orphan able to activate *luxI*-family promoters independently from AHLs.

Many bacteria utilize a regulatory system known as quorum sensing (QS) to modulate gene expression as a function of their cell density (reviewed by Camilli and Bassler, 2006). These bacteria produce signaling molecules which at high cell density reach a threshold concentration/quorum that allows their detection resulting in the modulation of target gene expression. In Gram negative bacteria the most common QS system is modulated by the *N*-acyl homoserine lactone signaling molecules (AHLs). A typical AHL QS system involves two major components: an AHL synthase gene (belonging to the LuxI protein family) and a modular transcriptional response-regulator (belonging to the LuxR protein family) which detects and responds to the concentration of a specific AHL affecting gene expression of QS regulated genes by recognizing specific *lux*-boxes in their promoters (Fuqua, 2006). Recently some bacteria were found to possess in their chromosome only the *luxR*-transcriptional regulator devoid of their cognate *luxI*-family AHL synthase; consequently these were called orphan *luxR* genes. Orphan LuxR proteins may bind to exogenous AHLs produced by other neighboring bacteria or to endogenous signal molecules produced by other AHL QS systems, as some orphan *luxR* genes are present in bacteria which also possess complete AHL QS systems (Fuqua, 2006). The role of orphan LuxR proteins could be to extend the already existing AHL QS regulatory network or to enable bacteria to respond to signals produced by nearby cells.

Research in AHL QS has considerably benefited from the use of bacterial biosensors which are able to detect the presence of exogenous AHLs (reviewed by Steindler and Venturi, 2007). These biosensors do not produce AHLs and contain a LuxR-family protein regulating a target gene promoter (in most cases the promoter of the cognate *luxI* synthase) directing transcription of a reporter gene (Steindler and Venturi, 2007). In this study we have assembled a plasmid useful in identifying bacteria that possess orphan quorum sensing LuxR-family proteins able to affect transcription from promoters containing *lux*-boxes. This plasmid construct contained a series of 8 *luxI*-family gene promoters: *luxI*, *cviI*, *ahlI*, *rhlI*, *cepI*, *phzI*, *traI* and *ppuI*, cloned in tandem upstream a promoterlss *lacZ* gene yielding pMULTIAHLPROM. All these promoters are known to contain *lux*-boxes which are positively regulated by the cognate LuxR-family protein in the presence of the cognate AHL thus representing a wide range of LuxR-family regulated promoters. If pMULTIAHLPROM is harbored in a strain possessing an orphan LuxR-family protein, this can lead to transcription initiation from one or more of these promoters and can therefore be detected.

The different promoters were amplified by PCR using as template the corresponding genomic or plasmid DNA as listed in Table 1. The promoters were amplified using Vent DNA Polymerase (New England Biolabs) subsequently cloned in the pMOSBlue vector (Amersham-Pharmacia) and were then verified by DNA sequencing (Macrogen). Each primer was designed with the addition of a suitable restriction site at the 5′ end (Table 1) in order to clone the promoters sequentially in pBSIIKS (Stratagene). The series of AhlI–CviI–LuxI promoters were cloned sequentially in pBSIIKS generating pBSACL, then amplified with primer ACLf which contains a BamHI restriction site and primer

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Table 1
Template DNAs and primers used for constructing pMULTIAHLPROM

Promoter	Template genomic or plasmid DNA[a]	Primers[b]	PCR fragment (bp)
luxI	pSB401	luxIPf gc**atcgat**cccatctctttatc luxIPr cgc**ctcgag**tcgactataaca	196
cviI	*Chromobacterium violaceum* ATCC31532	cvIPf ta**cccggg**acagccattgcgg cvIPr gc**atcgat**cggtccaacaccaa	246
ahlI	*P. syringae* pv. phaseolicola 1448A	ahlIPf cg**gagctc**ggagtcattattg ahlIPr aa**cccggg**aaccagtgtcaag	184
rhlI	*P. aeruginosa* PUPa3	rhlIPf cg**gaattc**tcggcgcgccctaccagatc rhlIPr cc**aagctt**tgtcgtgccggccgag	150
cepI	*B. cepacia* ATCC25416	cepIPf ggcc**aagctt**ggcacaacgacgcctatc cepIPr cc**atcgat**tgtatgtgcgcattacat	229
traI	*A. tumefaciens* NTL4	traIPf cgc**gtcgac**tgacgattaccgaatgttg traIPr cgctcgagcaagaaacgtagcggagag	248
ppuI	*P. putida* WCS358	ppuIPf ccg**ctcgag**agcttcatcgggaatcaa ppuIPr gg**ggtacc**cggcaggggtttgaat	134
phzI	*P. aureofaciens* 30–84	phzIPf cc**atcgat**ctctacgactacctgg phzIPr ac**gtcgac**tttatagaggacttg	168
luxI–cviI–ahlI	pBSACL	ACLf cg**ggatcc**ggagtcattattgtttgg ACLr gc**gaattc**tcgactataacaaaccat	Not applicable

[a] pSB401 contains the *luxI* promoter (Winson et al., 1998), *C. violaceum* ATCC31532 (McClean et al., 1997), *P. syringae* pv. phaseolicola 1448A (Joardar et al., 2005), *P. aeruginosa* PUPa3 (Kumar et al., 2005). *B. cepacia* ATCC25416 (Aguilar et al., 2003), *A. tumefaciens* NTL4 (Farrand et al., 2002). *P. putida* WCS358 (Geels and Schippers, 1983) and *P. aureofaciens* 30–84 (Wood and Pierson, 1996).
[b] Inserted restriction sites are shown in bold.

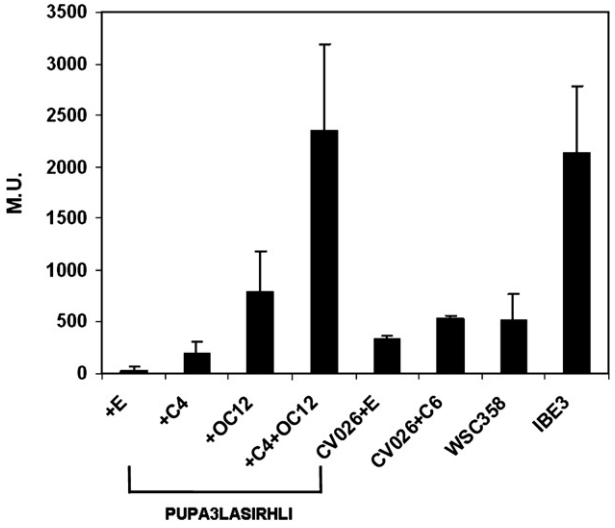

Fig. 2. pMULTIAHLPROM promoter activities in various bacteria. pMULTIAHLPROM was conjugated into various strains: *P. aeruginosa* strain PUPa3 *lasI* and *rhlI* double mutant (PUPA3LASIRHLI, Steindler et al., submitted for publication) without AHL addition or with added C4-AHL (2 μM) or C12-3oxo-AHL (2 μM) or both; in *C. violaceum* CV026 (McClean et al., 1997) without or with addition of C6-AHL, and in *P. putida* WSC358 wild type and the *rsaL* mutant IBE3 which overproduces AHLs (Bertani and Venturi, 2004). M.U. refers to β-galactosidase Miller Units as determined according to Miller et al. (Miller, 1972) and E refers to ethyl acetate (added as the same volume when added in other cultures in which it contains AHLs). The mean values of three replica experiments are shown.

ACLr having an EcoRI restriction site (Table 1). The resulting fragment was therefore cut with BamHI and EcoRI and cloned upstream of the series of CepI–PhzI–TraI–PpuI previously cloned sequentially in pBSIIKS, yielding plasmid pBS8PROM. The complete multiple promoter was sequenced for verification, excised from pBS8PROM as BamHI–KpnI fragment and cloned in the BglII–KpnI sites of the promoter probe vector pMP220, upstream of the promoterless *lacZ* reporter gene generating the pMULTIAHLPROM (Fig. 1).

In order to verify the functionality of this multiple promoter construct, we tested the response of pMULTIAHLPROM by conjugating it into some of the bacterial strains from which the *luxI*-gene promoters originated. The plasmid was therefore introduced into (i) a

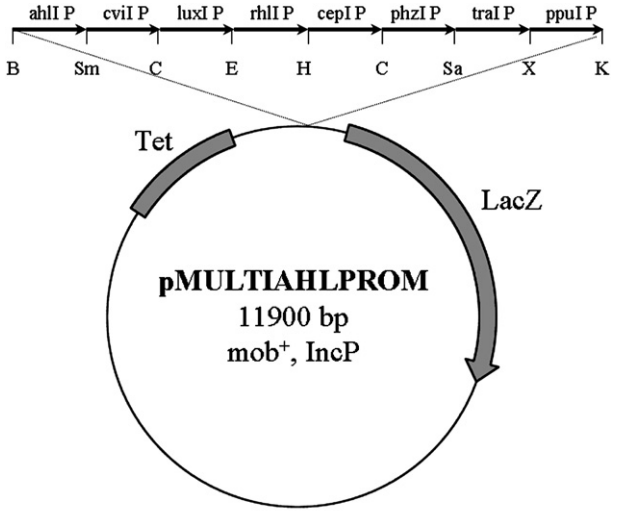

Fig. 1. Graphic representation of the MULTIAHLPROM construct. The position of the eight *luxI*-type promoter clones upstream the promoterless *lacZ* gene is shown represented by the name of the promoter followed by a "P" meaning promoter. Restriction enzymes are: B-BamHI, Sm-SmaI, C-ClaI, E-EcoRI, H-HindIII, Sa-SalI, X-XhoI, K-KpnI.

mutant derivative of *P. aeruginosa* unable to produce AHLs (C4-AHL and C12-3-oxo-AHL), hence at least the *rhlI* and *lasI* promoters should respond upon exogenous addition of the cognate AHL, (ii) *P. putida* WCS358 wild type and a mutant derivative in the *rsaL* gene which overproduces AHLs and (iii) in *Chromobacterium violaceum* CV026, which is unable to produce AHLs. We then determined promoter functioning by measuring β-galactosidase activities as follows; overnight bacterial cultures were diluted in LB medium to an A_{600} of 0.1, if required the AHL was added (see Figure for details) and after 6 h of growth β-galacotsidase activity was measured according to Miller, 1972. As depicted in Fig. 2, it was observed that the *lasI*, *rhlI*, *ppuI* and *cviI* promoters in pMULTIAHLPROM were able to respond in *P. aeruginosa*, *P. putida* and *C. violaceum* respectively since β-galactosidase activities increased significantly upon addition of cognate AHLs. This result indicated that 4 *luxI*-type promoters of pMULTIAHLPROM responded well to their cognate LuxR-AHL complex and thus it was hypothesized that most probably all other 4 *luxI*-family promoters were also functional in this unique multipromoter construct.

In a set of rice rhizosphere isolates (Kumar et al., 2005; Velusamy et al., 2006; Vlassak et al., 1992; Steindler et al., submitted for publication), we carefully determined that 29 of these do not produce AHLs (Steindler et al., submitted for publication). Briefly, these 29 isolates were part of a group of 51 and were assayed for AHL production using four different AHL biosensors which cover the most common structurally different AHLs. Verification of production of the signal molecules was performed both via assays on solid growth media and via purification of AHL from spent supernatants and analysis via TLC. It was therefore hypothesized that their genome could harbor an "orphan *luxR*" gene encoding a LuxR-family protein able to bind AHL signaling molecules produced by other bacteria or to AHL mimic agonist compounds produced by the plant itself (Bauer and Mathesius, 2004; Degrassi et al., 2007; Ferluga et al., 2007). In order to test for the possible presence of "orphan *luxR*" genes in the 29 non-AHL producing rice rhizosphere strains, we conjugated pMULTIAHLPROM into all of them. β-galactosidase activities were then determined for each strain in four culture conditions: (i) LB media, (ii) LB media containing 1 μM of each C4-, C6-, C7-, C8-, C10- and C12-

AHLs, (iii) LB media containing 1 μM of each C6-3oxo, C8-3oxo, C10-3oxo and C12-3oxo-AHLs and (iv) LB media containing 1 μM of each C6-3OH, C8-3OH, C10-3OH and C12-3OH-AHLs. It was postulated that if a LuxR-family orphan was present, in any of these strains, which could bind any of these AHLs it could then possibly initiate transcription from one or more of the *luxI*-family gene promoters present in pMULTIAHLPROM. In 27 strains we did not detect any significant increase in β-galactosidase activities in all four growth conditions. In two strains we detected very strong promoter activities (approx. 2000 mU) in all four growth conditions at comparable levels. In fact, the promoter activity was present independently of the presence/absence of added AHLs. It was therefore concluded that these two strains possibly contain a regulatory protein (most probably a LuxR-family protein) which was able to activate transcription from one or more *luxI*-family promoters in pMULTIAHLPROM independently of AHLs. For the remaining 27 strains (which are fluorescent pseudmonads, Steindler et al., submitted for publication) it was concluded that they either did not possess LuxR-family proteins or that if they did, they were unable to respond to the provided AHLs and/or to recognize one of the 8 *luxI*-gene promoters.

The two strains which displayed strong β-galactosidase activities when they harbored pMULTIAHLPROM were both classified as *Enterobacter aerogenes* following some basic bacteriological tests and 16rDNA sequencing (LMG, Ghent, Belgium; Accession numbers AM933750 and AM933751). For both strains, spent supernatants from 100 ml LB cultures were extracted for AHL purification and analyzed via TLC analysis utilizing 4 different AHL bacterial biosensors (*C. violaceum* CV026, *E. coli* pSB401 and pSB1075, and *A. tumefaciens* NTL4) as described previously (Steindler and Venturi, 2007). These four AHL detectors used have different specificity towards AHLs thus ensuring the screening of a wide range of structurally different molecules. Results have demonstrated that both strains do not produce AHLs, hence it was concluded that rhizosphere *E. aerogenes* most probably possesses a LuxR-family protein able to activate transcription from *luxI*-type promoters independently of AHLs. However, it cannot be excluded that they are synthesizing signal molecules different from AHLs which activate the LuxR type proteins which then bind to activate one or more of the *luxI*-type promoters. Other non-AHL producing bacteria reported to possess functional orphan quorum sensing LuxR proteins include *E. coli* and *Salmonella enterica* which possess SdiA which is able to detect AHLs produced by other species (Janssens et al., 2007; Smith and Ahmer, 2003).

In summary, we have constructed and showed the functionality and utility of a unique promoter probe construct containing 8 *luxI*-type promoters in tandem. This plasmid is particularly useful in determining whether a bacterial strain possesses orphan LuxR type proteins able to respond to AHLs and activate transcription from quorum sensing target promoters.

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