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Designing primers to fish auto-inducer synthase gene(s) of the quorum sensing system in γ -proteobacteria and their *in-silico* PCR validation

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Abstract
Quorum sensing is a well known phenomenon in bacteria that control diverse functions including colonization and formation of biofilm. The *luxI* gene, involved in quorum sensing of gram negative bacteria, codes for auto-inducer synthase/ acylhomoserine lactone synthase. As *luxI* homologues are sequence-diverse it is difficult to identify its loci by hybridization technique in different bacteria whose whole genome sequence(s) are unknown. We have used different bioinformatics tools taking the existing genome database into cognizance to design manually suitable degenerate primers for amplifying *luxI* gene homologues from diverse representatives of gamma-proteobacteria. Two primer pairs, Deg1F/Deg2R and Deg3F/Deg4R, were capable of in- silico PCR amplification from genome sequence(s) of *Halothiobacillus neapolitanus*, *Acinetobacter baumanii* ATCC 17978, *Acidithiobacillus ferrooxidans* ATCC 53993 and *Pseudomonas aeruginosa* PAO1 (with first primer pair); *Edwardsiella ictaluri*, *Edwardsiella tarda*, *Erwinia tasmaneinsis*, *Serratia proteamaculans*, *Pectobacterium wasabiae*, *Pectobacterium carotovorum*, *Pectobacterium atrosepticum*, *Dickeya zeae* and *Yersinia pestis* (with second primer pair). The phylogenetic trees derived from sequences of *luxI* homologues and 16S rRNA gene sequences of the respective genomes were almost identical showing two distinct clusters. The degenerate primer pairs were also found to be cluster specific.

Keywords: Degenerate primer, *in silico* PCR, acylhomoserine lactone synthase, *LuxI*, Seaview4

Bacteria communicates with each other and can collectively form a group with properties not expressed when they are alone (Greenberg 2000). Quorum sensing is the term used to define and quantify such behavior. Actually the individuals in a bacterial population senses the quanta of certain chemical signals called autoinducer (AI) produced by themselves in course of growth (Shaw *et al* 1997). N-acylated derivatives of L-homoserine lactone (acyl-HSLs) which are identified as AIs are the products of acyl-homoserine lactone synthase (Shaw *et al*.,1997). With few exceptions, these enzymes constitute an evolutionarily conserved family of homologues known as the *LuxI* family of autoinducer synthase (Gray and Garey 2001). AI-based sensing mechanisms have been widely studied in gram negative bacteria (Greenberg 2000; Parsek and Greenberg 2000; Swift *et al*.,1999) which in turn control several functions like exoenzyme synthesis, conjugation, antibiotic production, luminescence, metal tolerance and biofilm formation (Sarkar and Chakraborty 2008).

Nucleotide sequence analyses with several alignment tools have revealed wide diversity with infrequent short stretches of similarity in *luxI* gene homologues. These stretches are too small to design a universal primer for conducting PCR amplification. Identifying *luxI* homologues from species (whose complete genome sequences are not available) by DNA-DNA hybridization using a single probe would also not be possible. The most viable solution to the problem is to design degenerate primer set for the amplification of such sequences. A PCR primer is called degenerate if some of its positions have several bases (Kwok *et al*., 1994.) For example in a degenerate primer like bchY fwd (5'-CCNCARACNATGTGYCCNGCNTTYGG-3') (Yutin *et al*., 2009) the R , Y and N are degenerate that means primer is a mixture in which one contain either A/G at position of R, similarly C/T at position Y and A/T/G/C at position N.

As the whole genome sequences of many organisms are available in public domain it is possible to design gene specific primers for PCR amplification. A number of computer programs like Amplify (Jarmon 2004), simPCR (Rubin and Levy 1996), PCRAna (Nishigaki *et al*., 2000), PUNS (Boutros and Okey 2004), and Virtual PCR (Cao *et al*., 2005; Lexa *et al*., 2001) are available on-line which can predict the efficiency of PCR amplification with such designed primers. These programs however could not predict amplification with degenerate primer set (Cao *et al*., 2005).

In this work, we took advantage of certain output of SeaView version 4 program to manually design degenerate primers from the aligned sequences. SeaView is a multiplatform, graphical user interface for multiple sequence alignment and molecular phylogeny (Gouy *et al*., 2010) and is freely available at http://pbil.univ-lyon1.fr/software/SeaView. Another program

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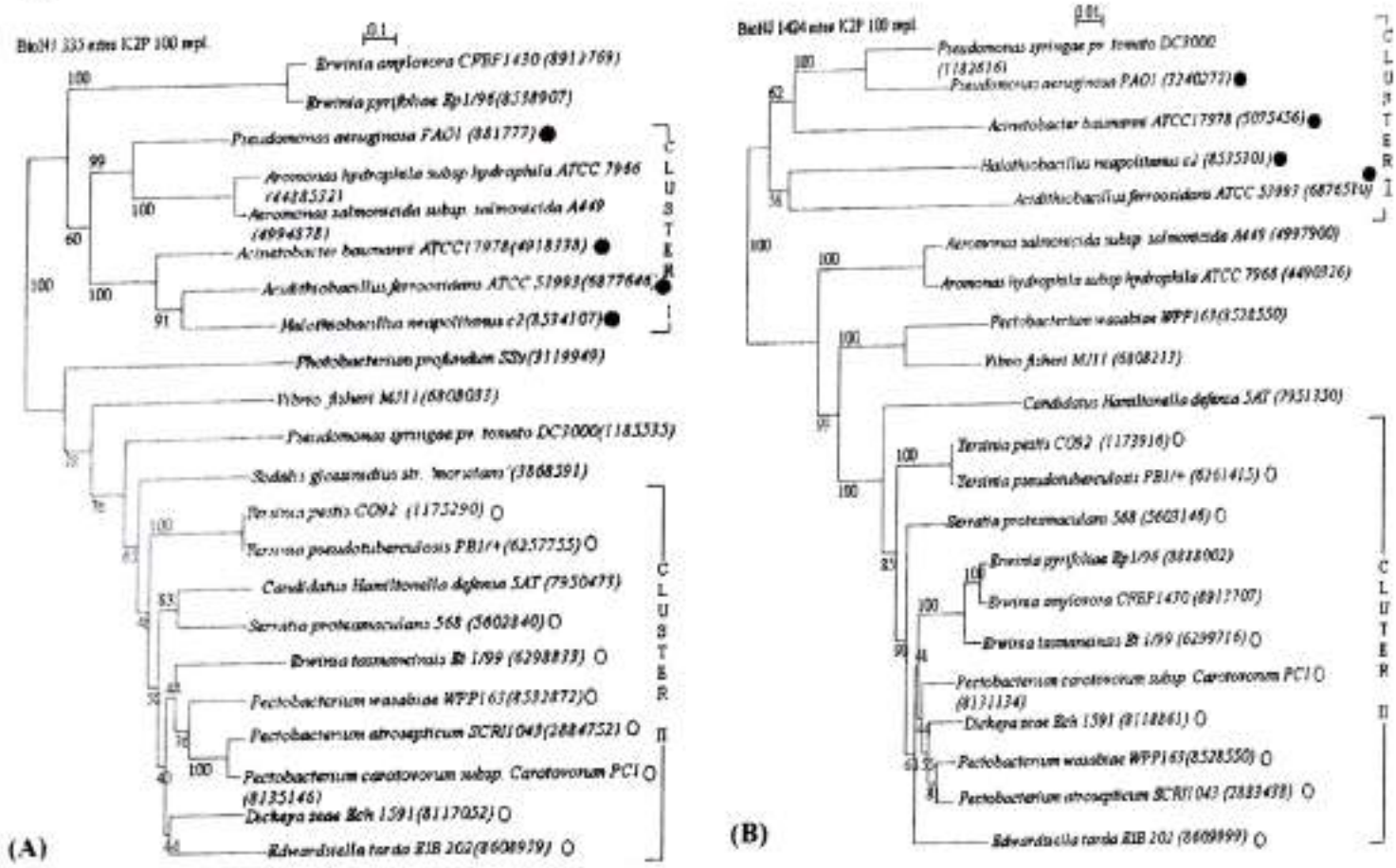

Figure. 1. Phylogenetic tree of (A) *luxI* homologue sequences, (B) 16S ribosomal RNA sequences from gamma proteobacteria. Dendogram generated in SeaView version 4 using distance method and BioNJ algorithm (Gascuel 1997). NCBI gene ID given in parenthesis. Nucleotide-level distances are observed divergence with Kimura's two-parameter. Bootstrapped to 100 replicates. (●) *luxI* homologue sequences amplified with degenerate primer pair Deg1F/Deg2R, (○) *luxI* homologue sequences amplified with degenerate primer pair Deg3F/Deg4R.

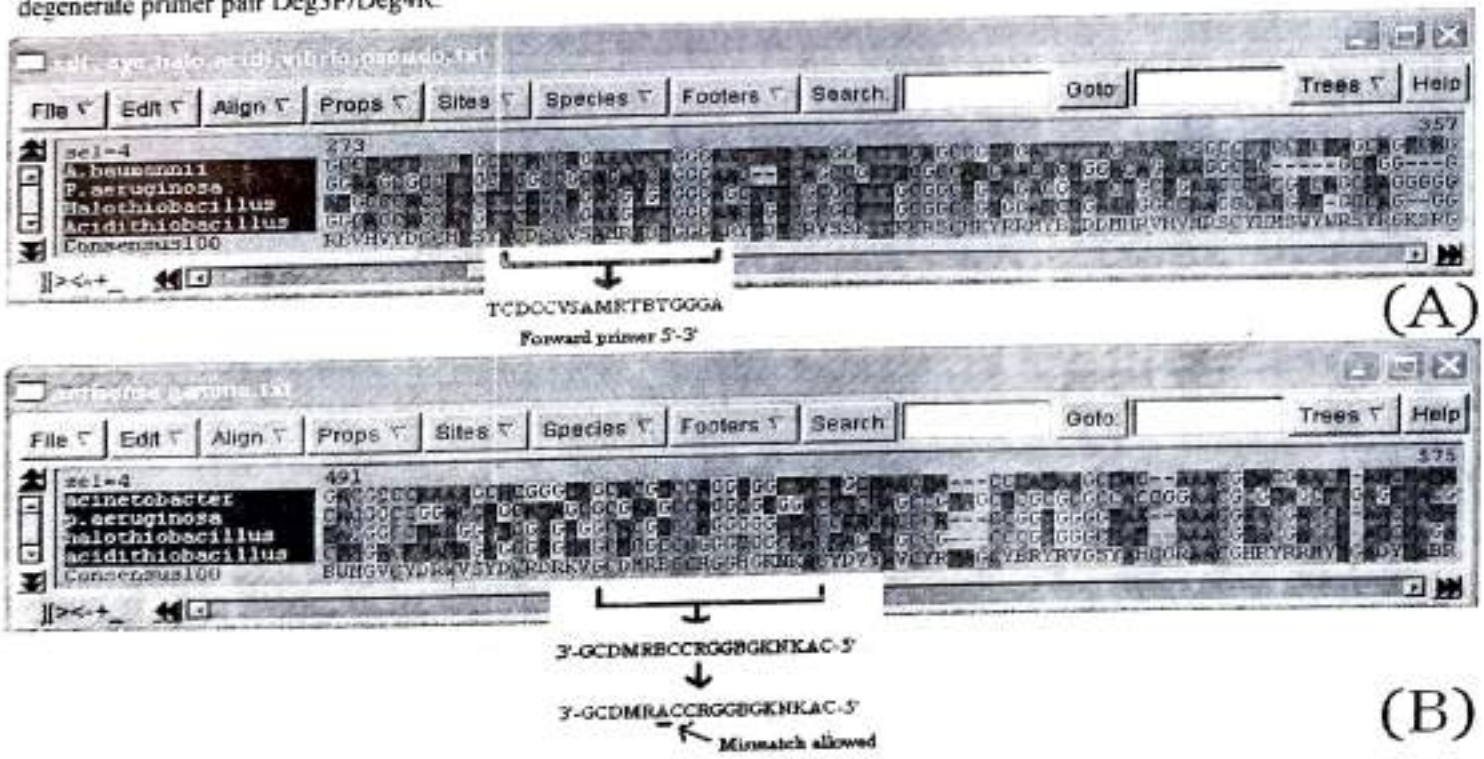

Figure. 2. Snapshot of SeaView window displaying ClustalW aligned *luxI* homologue sequences from gamma proteobacteria with degenerate letters just below the aligned sequences (A) forward primer, (B) reverse primer.

called *In silico* PCR (Bikandi *et al.*, 2004) (freely available at http://www.in-silico.com) was used to predict the possible *luxI* homologue amplification using degenerate primer pair(s) on template of whole genome sequences available in nucleotide databases.

Materials and Methods

Retrieval of luxI *homologous sequences:* Nucleotide sequences of *luxI* gene homologues in gamma-proteobacteria were retrieved from NCBI gene data bank (Table 1).

Table 1. List of gamma proteobacteria having *luxI* homologue sequences

Gamma proteobacteria		NCBI Gene ID[1]	NCBI Gene ID[2]
Aeromonadales	*Aeromonas salmonicida subsp. salmonicida* A449	4994878	4997900
	A. hydrophila subsp hydrophila ATCC 7966	4488532	4490326
Pseudomonadales	*Pseudomonas syringae pv. tomato* DC3000	1185535	1182616
	Pseudomonas aeruginosa PAO1	881777	3240272
	Acinetobacter baumanni ATCC17978	4918338	5075456
Enterobacteriales	*Edwardsiella tarda* EIB 202	8608939	8609999
	Yersinia pestis CO92	1175290	1173916
	Yersinia pseudotuberculosis PB1/+	6257755	6261415
	Erwinia amylovora CFBP1430	8912769	8913707
	Erwinia pyrifoliae Ep1/96	8538907	8888002
	Erwinia tasmaneinsis Et 1/99	6298833	6299716
	Serratia proteamaculans 568	5602840	5603146
	Candidatus Haniltonella defensa 5AT (Acyrthosiphon pisum)	7950473	7951350
	Pectobacterium atrosepticum SCRI1043	2884752	2883438
	Pectobacterium carotovorum subsp. Carotovorum PC1	8135146	8131134
	Pectobacterium wasabiae WPP163	8532872	8528550
	Sodalis glossinidius str. 'morsitans'	3868591	3866445
	Dickeya zeae Ech 1591	8117052	8118861
Vibrionales	*Vibrio fisheri* MJ11	6808033	6808213
	Photobacterium profundum SS9	3119949	3120506
Acidithiobacillales	*Acidithiobacillus ferrooxidans* ATCC 53993	6877646	6876510
Chromatiales	*Halothiobacillus neapolitanus c2*	8534107	8535301

[1] *Coding for Acylhomoserine lactone/ Autoinducer synthase;* [2] *Coding for 16S Ribosomal RNA*

Table 2. Properties of designed degenerate primers

Degenerate primers 5' to 3'	Length	Degener-acy	TM in °C		%GC
			Oligo analysis[a]	Oligocalc[b]	
Deg1F-TCDCCVSAMRTBTGGGA	17	216	58.8	42-54(B),47-60(SA)	58.8
Deg2R-CAKNKGBGGRCCARMDCG	18	1152	64.4	46-64(B),51-70(SA)	64.4
Deg3F-AARGAYMGDCTNCAHTGG	18	288	56.8	41-55(B),47-61(SA)	56.8
Deg4R-AAYHYBCCADCCDGADC	17	972	57.1	40-57(B),45-62(SA)	57.1

a=freely available at www.operon.com/technical/toolkit.aspx, b=freely available at www.basic.northwestern.edu/biotools/oligocalc.html, B=Basic, SA= Salt Adjusted

Multiple Sequence Alignment: Sequences from the selected genera were aligned using ClustalW multiple alignment program in SeaView 4.

Designing Degenerate Primer: A phylogenetic tree of *luxI* sequences was obtained using distance method in SeaView 4 software (Figure 1A). This was used as the guide tree to select genera of closely related sequences for designing degenerate primers. Sequences of each cluster in the tree were separately aligned with ClustalW in SeaView 4. Most similar stretches of nucleotides having minimum 10 A+T+C+G in the multiple alignments were tentatively chosen to test the properties close to an ideal primer for PCR reaction (Fig.2A & B). Fast PCR (www.biocenter.helsinki.fi/bi/programs/fastpcr.htm) was used to get the complementary sequence for making the reverse primer. The primers were basically analyzed for melting point, %GC, hairpin, self complementarities and primer dimer check using OligoCalc (www.basic.northwester.edu/biotools/oligocalc.html) and Oligo Analysis Tool (www.operon.com/technical/toolkit.aspx). Attention was also paid to have 3□ GC clamps at 3□ end for increasing the specificity of the primer (Rouchcka *et al.*, 2005). Using 100% consensus, degenerate sequences (letter codes were followed according to the recommendations of Nomenclature Committee of the International Union of Biochemistry and Molecular Biology) were constructed from the aligned sequence.

In silico *PCR Amplification:* The designed degenerate primer pair was fed to the blank primer box followed by selection of bacterium (whose whole genome sequence is available in the database) in the interacting page of the online software, *In silico* PCR, for the output.

Results and Discussion

Phylogenetic analysis of the retrieved *luxI* homologue sequences from gamma proteobacteria has revealed two distinct clusters (Fig. 1A) which also correlates with the phylogenetic tree drawn from their 16S rRNA gene sequences (Fig. 1B).

Two degenerate primer pairs were designed using SeaView 4 and were designated as Deg1F/Deg2R and Deg3F/Deg4R and their properties are shown in Table 2. Using the *in silico* PCR tool (allowing one nucleotide mismatch but in one nucleotide at 3' end) it was found

Table 3. *In silico* PCR amplification using degenerate primer pair Deg1F/Deg2R (*) and Deg3F/Deg4R (#)

Organism	Amplicon	Sequence Name
Acinetobacter baumannii	238 bp*	Homoserine lactone synthase
Acidithiobacillus ferrooxidans	225 bp*	Autoindducer synthesis protein
Halothiobacillus neapolitanus c2	228 bp*	Acyl-homoserine-lactone synthase
Pseudomonas aeruginosa	213 bp*	Autoinducer synthesis protein *LasI*
Pectobacterium atrosepticum SCRI1043	390 bp#	Acylhomoserine lactone synthase
Erwinia tasmaniensis	390 bp#	Acylhomoserine lactone synthase
Pectobacterium carotovorum subsp. carotovorum PC1	390 bp#	Acyl-homoserine-lactone synthase
Pectobacterium wasabiae WPP163	390 bp#	Acyl-homoserine-lactone synthase
Edwardsiella tarda	393 bp#	AHL synthase
Edwardsiella ictaluri	393 bp#	Autoinducer synthase putative
Dickeya zeae	390 bp#	Acylhomoserine lactone synthase
Dickeya dadanti	390 bp#	Acylhomoserine lactone synthase
Serratia preteamaculans 568	390 bp#	Autoinducer synthesis protein
Yersinia pestis	390 bp#	N-acylhomoserine lactone synthase
Yersinia entercolitica	390 bp#	N-acylhomoserine lactone synthase
Yersinia pseudotuberculosis	390 bp#	N-acylhomoserine lactone synthase

All amplicons were of single band

that the degenerate primer pair, Deg1F/Deg2R was capable of amplifying *luxI* homologue sequences from *Acinetobacter baumanii, Pseudomonas aeruginosa, Halothiobacillus neapolitanus,* and *Acidithiobacillus ferrooxidans* and Deg3F/Deg4R enabled to amplify *luxI* homologue sequences from several bacterial species of the genera *Erwinia, Yersinia, Serratia, Pectobacterium, Edwardsiella* and *Dickeya* (Table 3).

The degenerate primer was manually designed using SeaView 4 since it allowed display of degenerate letters just below the aligned sequence (Fig 1A&B) and has an easy to use graphical interface (Gouy *et al* 2010). This has enabled us to select short nucleotide sequence containing of at least 10 A+T+G+C which is the prime requisite for in-silico PCR amplification. The *in silico* PCR software program linked with NCBI genome data bank was found to be important software tool which allowed amplification using degenerate primer. The output of this program showed the nucleotide positions of the genome region which have undergone amplification, length of the amplicon and a simulation of the electrophoretic mobility on agarose gel. Further information of each amplicon may be obtained following the corresponding links: DNA sequences, list of ORFs that are included in the amplicon, and a link to the NCBI site, which displays a map of the chromosome around the amplicon (Bikandi *et al*., 2004).

Functional ability of the designed primers to amplify the target gene(s) could therefore be worked out in silico by accessing whole genome sequence (including plasmids when available) in the database. In all cases it would be mandatory to consider primers have been designed correctly, so that they do not form dimers, hairpin or any other aberrant structures preventing amplification.

Identification of two clusters in the phylogenetic tree constructed with *luxI* homologues has helped us to select the bacterial species for preparing the degenerate primers since the task has become easier because of lesser degeneracy among sequences of individual clusters. The applicability of the degenerate primers could be further enhanced if more mismatches are allowed under real wet lab conditions. To our observation in-silico PCR amplification with Deg1F/Deg2R failed with *Aeromonas salmonicida* genome template (component of Cluster1, Figure 1A) as the present version of the software, *In silico* PCR, did not allow more than two mismatches.

Hence an exercise to design degenerate primers (when necessary in situations like *luxI* sequences) for amplification of gene homologue of interest with the help of a combination of SeaView 4 and *In Silico* PCR programs would be very useful for the researchers in real wet lab situations.

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