3 MATERIALS AND METHODS

3.1 Bacterial strains and plasmids used in the study

Bacterial strains and plasmids used in the study are mentioned in Table 6 and 7 respectively.

Table 6: List of bacterial strains used in the study

Strain	Characteristics	Source/ Reference
Ecoli DH5α	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Lab stock
Pseudomonas aeruginosa PAO1	Wild type	(Holloway 1955)
PAO1 *phrD*ΩGm	PAO1 with gentamicin disrupted *phrD*	This study
PAO1 *p18*ΩGm	PAO1 with gentamicin disrupted *p18*	This study

The names, relevant characteristics and source/reference are mentioned in the table

Table 7: List of plasmids used in the study

Plasmids	Features	Size (bp)	Source/Reference
pBluescriptKS (+)	Cloning vector ColE1 replicon; Ap^r	2961	Stratagene
pHERD30T	*E.coli-Pseudomonas* shuttle vector carrying inducible *pBAD* promoter, Gm^r	5216	Paolo Visca Roma Tre University, Rome, Italy
pBBRMCS5	broad host range cloning vector, Gm^r	4768	(Kovach et al. 1995)
pUCP18-RedS	λ recombinase, Cb^r	9659	(Lesic and Rahme 2008)
pME6013	Translational *lacZ* fusion vector; Tc^r	11600	Elisabeth Sonnleitner Max. F. Perutz Laboratories, Vienna, Austria
pBSKS*phrD*	$pBSKS^+$ with *phrD* gene, Ap^r	3077	This study
pHERD*phrD*	pHERD30T with *phrD* gene, Gm^r	5344	This study
pBSKS*p18*	$pBSKS^+$ with *p18* gene, Ap^r	3106	This study
pHERD*p18*	pHERD30T with *p18* gene, Gm^r	5376	This study
pphrD-GDC	pTZ57R with Gm disrupted *phrD*; Ap^r	5271	This study
pp18-GDC	pBSKS with Gm disrupted *p18*; Ap^r	4982	This study
pTZ57R	Cloning vector with TA overhangs; Ap^r	2886	TA cloning kit, Thermo scientific, USA
A-pME6013	*rhlR::lacZ* translational fusion with intact PhrD interaction region in pME6013	11860	This study
B-pME6015	*rhlR::lacZ* translational fusion with scrambled PhrD interaction region in pME6013	11809	This study

The names, features, size in base pairs and source/reference of the plasmids used in the study are mentioned in the table. Ap = ampicillin, Cb = carbenicillin, Tc = tetracycline, Gm = gentamicin, r = resistant

3.2 Media, chemicals, enzymes and kits

Media and general chemicals were bought from HiMedia Laboratories, India; Sisco Research Labs, India; and Qualigens, India. Analytical grade chemicals were bought from Merck, India, and Sigma-Aldrich, USA. All molecular biology grade chemicals, enzymes and kits were procured from Roche, Switzerland; Sigma-Aldrich, USA; Thermo Fisher Scientific, USA; Bangalore Genei, India; New England Biolabs, USA.

3.3 Media and culture conditions

Bacterial cultures were grown in readymade Luria broth procured from HiMedia Labs, India, that was used at a concentration of 20 g/L as per manufacturer's instructions. 1.5% agar was added into LB to make solid agar medium. For rhamnolipids measurement, the cultures were grown in M9 minimal medium: 5X M9 salts, 1 M $MgSO_4$, 20% glucose as carbon source, 1 M $CaCl_2$ made to final volume with water. 5X M9 salts contained $Na_2HPO_4.7H_2O$ 64 g/L, KH_2PO_4 15 g/L, NaCl 2.5 g/L, NH_4Cl 5 g/L. All the above media was autoclaved at 121 psi for 20 min before inoculations. For β-galactosidase assays, cultures were grown in Minimal medium P (Lesinger et al. 1972) (MMP: 20 mM glucose, 0.1% casamino acids, Na_2HPO_4 1.47 g/L, KH_2PO_4 0.648 g/L, $MgSO_4$ 0.2 g/L, $FeSO_4$ 0.001 g/L or phosphate limited peptone/glucose/ammonium salts medium (Medina et al. 2003) (PPGAS: 0.5% glucose (w/v), 1% peptone (w/v), NH_4Cl 20 mM, KCl 20 mM, Tris/HCl 120 mM pH 7.2, $MgSO_4$ 1.6 mM) or Luria broth.

The liquid cultures were grown with constant shaking at 120 rpm at 37°C. Growth of the culture was measured as the absorbance at a wavelength of 600 nm in Beckman Coulter DU® 720 general purpose UV/Vis Spectrophotometer. Antibiotics, inducers and other biochemicals were added to the media as and when required in concentrations mentioned in table 8.

Table 8: Working concentrations of antibiotics and other biochemicals used in the study

S. No	Name	Stock concentration	Final concentration	Organism
1	Ampicillin	100 mg/ml	100 µg/ml	*E. coli*
2	Carbenicillin	50 mg/ml	200 µg/ml	*P. aeruginosa*
3	Gentamicin	10 mg/ml	10 µg/ml	*E. coli*
			30 µg/ml	*P. aeruginosa*
4	Tetracycline	10 mg/ml	25 µg/ml	*E. coli*
		50 mg/ml	100 µg/ml	*P. aeruginosa*
5	X-Gal	40 mg/ml	40 µg/ml	
6	IPTG	100 mM	100 µM	
7	L-Arabinose	10%	0.2%	

3.3.1 L-Arabinose induction

Overnight grown cultures were diluted to an absorbance of 0.05 in fresh Luria broth and allowed to grow to the desired OD. The cultures were then induced with 0.2% L-Arabinose, harvested and processed further post 30 min induction.

3.4 Bioinformatics tools used in this study

A detailed bioinformatics analysis of the ncRNAs reported in *Pseudomonas aeruginosa* PAO1 by Livny *et al* 2006 and Moll *et al* 2008 was done. Sequences of ncRNAs were retrieved using Pseudomonas genome database (http://www.pseudomonas.com/). Secondary structures of the ncRNAs were elucidated using the Mfold Web Server (http://mfold.rna.albany.edu/). Putative targets of these ncRNAs were determined using Target RNA program (http://snowwhite.wellesley.edu/targetRNA) and RNA predator program (http://rna.tbi.univie.ac.at/RNApredator2/target_search.cgi).

3.5 Molecular Biology tools and techniques

3.5.1 Isolation of genomic DNA from *Pseudomonas aeruginosa*

1 ml of overnight grown culture was spun down at 10,000 rpm for 3 min and the culture pellet was resuspended in 567 µl of TE buffer with 25 µl lysozyme (10 mg/ml). Following incubation of 1 h at 37°C, 30 µl of 10% SDS along with 0.4 µl of 20 mg/ml proteinase K was added to the tube and further incubated at 55°C for 1 h. Equal volume of Tris-saturated phenol:chloroform:isoamyl alcohol was added in 25:24:1 ratio to the cell lysate and incubated at 65°C for 10 min with intermittent mixing. Post centrifugation at maximum speed for 10 min, the upper aqueous phase was collected in a fresh tube and equal volume of chloroform: isoamyl alcohol mixture (24:1) was added into it followed by mixing and centrifugation at 10,000 rpm for 10 min. As mentioned in the earlier step, the DNA containing upper aqueous phase was collected in a fresh tube and precipitated with $1/10^{th}$ volume of 3 M Sodium acetate pH 5.2 and 2.5 volumes of absolute ethanol. The thread of high molecular weight, pure genomic DNA was spooled out of the tube, dried and dissolved in appropriate volume of TE buffer. The quality of the genomic DNA was analyzed by electrophoresing on 0.7% agarose gel.

3.5.2 Isolation of plasmid DNA

Plasmid DNA was isolated by standard boiling lysis method from *E. coli* and alkaline lysis method from *Pseudomonas* overnight grown cultures (Sambrook and Russell 2001). Briefly, 1.5 ml of culture was spun and the cell pellet was resuspended in 250 µl of ice cold alkaline

lysis solution I. 400 µl of freshly prepared solution II was added to the tubes followed by an incubation on ice for 4 min. 350 µl of chilled alkaline lysis solution III was added and tubes were incubated on ice for 7 min. The tubes were then spun at maximum speed for 10 min and the plasmid containing supernatant was transferred in a fresh tube. DNA was recovered by centrifugation after adding two volumes of ethanol. The plasmid pellet was washed with 70% ethanol, dried and resuspended in appropriate volume of TE buffer. For boiling lysis method, cells were lysed by adding 3 µl of lysozyme (50 mg/ml) containing 750 µl TE followed by incubation of 40 sec in a boiling water bath. Post centrifugation, DNA was recovered from the supernatant by adding $1/10^{th}$ volume of sodium acetate and 2.5 volumes of absolute ethanol.

3.5.3 Polymerase Chain Reaction (PCR)

Polymerase chain reactions were performed as per the standard details mentioned in (Sambrook and Russell 2001). The primers were designed using Primer3 web version 4.1.0 (http://primer3.ut.ee/) and purchased as deprotected, desalted oligos from Sigma-Aldrich or MWG Eurofins, Bengaluru. The PCR amplification reaction mixture contained 50 ng template, 1X Taq polymerase buffer with 1.5 mM $MgCl_2$, 0.5 µM of each primer, 0.2 mM of dNTPs and 1.5 units of High Fidelity Taq polymerase. Standard protocol was followed for PCR with denaturation temperature of 94°C, extension at 72°C and annealing temperature in the range of 55°C to 65°C depending upon the melting temperature of the primers used for amplification. Occasionally additives like 5% DMSO were added to the PCR mixture to facilitate amplification. A summary of PCR programs for different genes and a list of all the primers used in the present study is mentioned in the table 9 and 10 respectively.

3.5.4 Restriction enzyme digestion

DNA was mixed with 5 units of restriction enzyme per microgram of DNA, 1X supplied buffer and incubated at 28/37°C for 3 h. In case of double digestion, a compatible buffer was used whenever possible; otherwise sequential digestion was done.

3.5.5 Agarose gel electrophoresis

The required amount of DNA was mixed with 6 X bromophenol blue gel loading dye (0.25% bromophenol blue, 40% sucrose in water) to make it 1 X and loaded in 0.8-1.5% agarose gel. The gel was run through 0.5 X TBE (Tris-borate EDTA buffer pH 8.0) at 15 Volts/cm for 45 min to 2 h. Gel was stained in 0.5 µg/ml Ethidium Bromide solution in 0.5 X TBE for 20 min. Post staining, the gel image was documented in Bio-Rad's Gel Doc™ EZ Imager.

Table 9: PCR programs for genes amplified for this study

Gene	Initial denaturation 94°C	Denaturation temperature 94°C	Annealing temperature (°C), time (sec)	Extension temperature 72°C	Final extension 72°C
PhrD	4 min 45 sec	45 sec	54°C, 30 sec	30 sec	10 min
P18	4 min 45 sec	45 sec	63°C, 30 sec	30 sec	10 min
Gm	4 min 45 sec	45 sec	62°C, 30 sec	50 sec	10 min
PhrD-up	4 min 45 sec	45 sec	57°C, 30 sec	45 sec	10 min
PhrD-Dn	4 min 45 sec	45 sec	60°C, 30 sec	50 sec	10 min
PhrD-up with Gm*	2 min	45 sec	Touch down 67° to 58° in 6 cycles followed by 20 cycles at 58°C, 25 sec	1 min 35 sec	15 min
PhrD Up-Gm with PhrD -Dn*	3 min	45 sec	58°C, 25 sec	2 min 30 sec	15 min
P18-up	4 min 45 sec	45 sec	59°C, 30 sec	40 sec	10 min
P18-Dn	4 min 45 sec	45 sec	59°C, 30 sec	40 sec	10 min
P18-Dn with Gm*	3 min	45 sec	62°C, 30 sec	1 min 20 sec	15 min
P18-up with Gm-Dn*	3 min	45 sec	Touch down 63° to 59° in 6 cycles followed by 20 cycles at 59°C, 25 sec	2 min	15 min
A (P3 promoter with intact interacting region)*	4 min 45 sec	45 sec	59°C, 25 sec	30 sec	10 min
B (P3 promoter with scrambled interaction region)*	4 min 45 sec	45 sec	59°C, 25 sec	30 sec	10 min

*Elution required post amplification. Up=upstream, Dn=downsteam, Gm=gentamicin

3.5.6 Elution of DNA from agarose gels

DNA was resolved on 0.8% low melting agarose and the required band was cut out of the gel into a pre-weighed microfuge tube. DNA was eluted from the agarose piece either by *β*-agarase digestion and precipitation or by freeze thaw method of elution. Occasionally, gel elution kit with spin columns (Roche Diagnostics) was used. Small amount of the purified DNA was subjected to gel electrophoresis to confirm the purity and quantity of the eluted DNA.

Table 10: **List of primers used in the study**

Primer	Sequence(5'-3')	Tm(℃)
Amplification of PhrD sRNA gene		
PhrDN(F)	GCTCTAGAATGCCAAGACTAGGAGCAG	55.56
PhrDN(R)	TGCACTGCAGAGCGGGGATTTACTATTTGT	55.45
Amplification of P18 sRNA gene		
P18(F)APM	GCTCTAGAGGCGGCGTCTACCTCAGC	64.40
P18(R)APM	TGCACTGCAGCCCAGGTAATGCCACTCAACA	63.14
Amplification of upstream flanking region of PhrD sRNA gene		
FupPhrD	GATCCGGGAGCGAACC	60.10
RupPhrD-Gm	CCGTTTCCACGGTGTGCGTCGCCATTTGTGACTGGAGCTG	61.81
Amplification of downstream flanking region of PhrD sRNA gene		
FdnPhrD-Gm	GTAAATTGTCACAACGCCGCCAGTCGTCTAGTCTCCTGTTTACG	59.53
PhrD-Dn-R	CAAACATCTTTCGCCCATTC	60.45
Amplification of upstream flanking region of P18 sRNA gene		
FupP18	CGCAACCAGATGGACTACAA	59.72
RupP18-Gm	CCGTTTCCACGGTGTGCGTCGCGTTTTCTGGATCAGCAC	59.39
Amplification of downstream flanking region of P18 sRNA gene		
FdnP18-Gm	GTAAATTGTCACAACGCCGCGACTGCGCCCTGTTCATTTT	62.46
RdnP18	GAGTTCGGCGATCTTCACCT	61.70
Amplification of Gentamicin marker		
F-Gm	GACGCACACCGTGGAAAC	61.18
R-Gm	CGGCGTTGTGACAATTTACC	61.31
Real time PCR		
PhrD RT fwd new	ATGCCAAGACTAGGAGCAGC	59.60
PhrD RT R	CAAACGTAAACAGGAGACTAGACG	59.42
RhlR NEW FWD	AGTTGCTGACCCAGAAGCTG	60.59
RhlR NEW REV	CTCAGGATGATGGCGATTTC	60.57
16S rRNA Ps F	CTCAGACACAGGTGCTGCAT	60.05
16S rRNA Ps R	CACCGGCAGTCTCCTTAGAG	60.01
P18 RT F	GGGTGCTGATCCAGAAAACG	62.89
P18 RT R	AAAATGAACAGGGCGCAGTC	62.46
AprE F	ATGTACATCGTGCCCAACAG	59.45
AprE R	GGTCTTGCTCTGGTTGAAGG	59.84
Protease IV F	CTGCTGAACAACGGCAACTC	61.97
Protease IV R	GCACTGGGTGGTGTTGTAGA	59.60
***lacZ* fusions of RhlR**		
AM_101	CG**GAATTC**GTCACAACCGCACAGTATCG	59.29
AM_102	TGCAGTAAGCCCTGATCGATGTTATGCCAGCACCGTTCAG	59.55
AM_103	ATCGATCAGGGCTTACTGCA	58.88
AM_104	AA**CTGCAG**GCGCCGCACTTCCTTTTC	60.13
AM_111	GCTGCCTGTTGGCCGAAAGGCGAGCCTATGACAACGTTCGACA	58.36
AM_112	CTCGCCTTTCGGCCAACAGGCAGCGCTGCGTCCTGAACGGTGCTGGCATAACATCGATCAGGGCTTACTGCA	

The sequence in bold are recognition sequence for restriction enzyme and those underlined are overlap sequences.

3.5.7 Ligation

Ligations were carried out with ligase supplied by either Fermentas or NEB as per the manufacturers' instructions at a concentration of 5 units of ligase per 50 ng of plasmid DNA. The molar ratio of vector to insert was usually 1:3 for cohesive end ligations and 1:10 for blunt end ligations.

3.5.8 Transformation of DNA into *E. coli* and *P. aeruginosa*

Ligation mixtures and plasmid DNA were transformed into either competent *E. coli* DH5α cells prepared by $CaCl_2$ method (Sambrook and Russell 2001) or by electroporation as per the manufacturer's instructions. *P. aeruginosa* cells were transformed by electroporation.

In order to prepare electrocompetent cells of *E. coli,* overnight grown cultures of *E.coli* were diluted into fresh 100 ml LB in 500 ml flasks and allowed to grow till the OD_{600} reached 0.3-0.4. Cells were harvested and centrifuged at 4000xg for 15 min. Pellet was washed with equal volume of cold 10% glycerol. Two more washes of chilled 10% glycerol with half and one-fourth volume of glycerol were given. The cells were finally resuspended in 0.3 ml of 10% glycerol and used for electroporation. Cells were mixed with purified DNA and added into a pre-chilled 2 mm electroporation cuvette. Cells were given pulse at 2000 Volts, 200 Ω and 25 µF in a BTX Harvard ECM 630 electroporator. The electroporated cells were immediately diluted with 1 ml LB and incubated at 37°C for 2 h with constant shaking followed by plating on LA plates containing suitable antibiotic.

For preparation of *Pseudomonas* electrocompetent cells, 6 ml cells with an optical density of 0.5 were washed thrice with 300 mM Sucrose and resuspended in a final volume of 0.1 ml 300 mM Sucrose (Choi et al. 2006).

3.6 Northern Blot

3.6.1 Total RNA extraction

10-15 µg of total RNA was extracted from bacterial culture of required optical density by acid-guanidium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi 2006). Briefly, 10^7 cells were harvested from the cultures grown to the desired growth phase by centrifugation and the pellet was resuspended in 20 µl (50 mg/ml) lysozyme containing 500 µl TE buffer. Lysozyme was allowed to act for 10 min at 37°C. 1 ml of solution D was added and the samples were thoroughly pipetted to fragment the DNA followed by an incubation of 20 min. To 1 ml of lysate, 0.1 ml 2 M sodium acetate pH 4.0, 1 ml water saturated phenol and 0.2 ml chloroform/ isoamyl alcohol (49:1) were added sequentially and

the tube was shaken vigorously. Samples were incubated on ice for 15 min and spun at 10,000 rpm at 20°C for 20 min. The upper aqueous phase containing the RNA was transferred to a fresh tube and precipitated with equal volume of isopropyl alcohol. RNA was precipitated overnight at -20°C. Following centrifugation the pellet was washed with 70% ethanol, dried and dissolved in 50 µl TE buffer. At the time of electrophoresis RNA was denatured by mixing with equal volume of 2X Formamide gel loading dye (95% de-ionized Formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, 5 mM EDTA pH 8.0, 0.025% SDS) and heating at 65°C for 10 min followed by snap cooling. RNA was separated on a 6% polyacrylamide gel (composition given in table 11) containing 6 M urea in 0.5X TBE at 5 Volts/cm till bromophenol blue dye front reached the bottom of the gel. RNA was then electroblotted on neutral Hybond nylon membrane at 300 mA for 2 h in 1X TBE. RNA thus transferred to the membrane was UV-crosslinked at 254 nm in a UV crosslinker for recommended time and then processed further.

Table 11: **Composition of 6% polyacrylamide 6 M urea gel**

Components	**Volume**
30% acrylamide:bisacrylamide mix	7 ml
Urea	12.6 gm
10X TBE	3.5 ml
Water	Make the volume to 35 ml
10% ammonium persulfate	350 µl
TEMED	35 µl
Total	**35 ml**

3.6.2 Probe labeling

DIG High Prime labeling and detection kit from Roche Diagnostics that uses Digoxygenin (DIG), a steroid hapten for DNA probe labeling, was used. 1 µg of PCR amplified gel purified DNA fragment in 16 µl was denatured by heating in a boiling water bath for 10 min followed by snap cooling. It was then mixed with 4 µl 5X labeling mixture containing optimal concentrations of random primers, nucleotides, dUTP (alkali-labile), Klenow enzyme, and buffer components and incubated at 37°C for 16 h. The resultant probe was then quantified by the dot blot method described in the same kit and stored at -20°C until further use.

3.7 Real time PCR assays

Real time assays were performed using total RNA isolated from early stationary phase cultures of OD_{600} of 2 as per the manufacturer's instructions (High Pure RNA isolation kit, Roche). cDNA first strand synthesis was done using Genei RT-PCR kit. Real time PCR was performed and fold expression was determined by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The results were normalized to those obtained for the 16S rRNA gene. The wild type strain consisting of empty vector was used as the calibrator.

3.8 Rhamnolipid measurement

Supernatant from bacterial cultures grown for 72 h in M9 minimal medium was adjusted to pH 2.5±0.2 using 1 N HCl. The acidified sample was then extracted with 5 volumes of chloroform. 4 ml of chloroform extract was allowed to react with freshly prepared methylene blue solution containing 200 µl 1 g/L of methylene blue (prepared in 10 mM borax buffer pH 10.5 and stabilized by adjusting pH to 5.5) and 4.9 ml distilled water. The samples were mixed vigorously for 4 min followed by a standing time of 15 min. The absorbance of the chloroform phase was read at 638 nm with chloroform as blank with Beckman Coulter DU® 720 spectrophotometer. and normalized with Abs 600 of the cultures (Pinzon and Ju 2009). Each sample was analyzed in duplicates and results are average of three independent experiments.

3.9 Pyocyanin assay

5 ml culture grown to stationary phase was harvested and pyocyanin was extracted with 3 ml chloroform from cell-free supernatant. The organic phase was acidified with 1 ml of 0.1 N HCl and absorbance was estimated at 520 nm. Pyocyanin levels were expressed as A_{520}/A_{600} of culture supernatant (Sonnleitner et al. 2011). The experiment was done thrice in duplicates.

3.10 *β*-galactosidase assay

β-galactosidase activity was estimated as described by Miller (Miller 1972) using cells permeabilized by 5% chloroform. Overnight grown cultures were diluted to an initial absorbance of 0.05 into fresh Luria broth. The cultures were allowed to grow at 37°C with constant agitation. 200 µl culture aliquots were periodically withdrawn and *β*-galactosidase activity was determined. The Miller units are represented as mean of three independent experiments.

$$\text{Specific } \beta\text{-galactosidase activity} = \frac{A420\ x\ V1}{(V2\ x\ T\ x\ 0.00486\ x\ mg\ protein)}$$

Where A_{420} is the absorbance at 420 nm, V1 is the final volume in ml of the assay (1.7 ml), V2 is the volume in ml of the culture used for the assay (0.2 ml), and T is the reaction time in min. The molar extinction coefficient of 2-nitrophenol is 4860/M/cm at pH 10.

3.11 Alkaline protease assay

Alkaline protease activity was determined using azocasein as a substrate in Tris-HCl buffer at pH 9 as per the protocol mentioned by Towatena and group with certain modifications (Hutadilok-Towatana et al. 1999). 75 µl of culture supernatant of an 18 h grown culture was incubated with 125 µl of azocasein (2% in 50 mM Tris-HCl with 2 mM $CaCl_2$, pH 9) for 30 min at 37°C. 600 µl of 10% TCA was added and the samples were allowed to stand for 15 min to ensure complete precipitation of undigested material after thorough vortexing. Samples were centrifuged at 10,000 rpm for 10 min after which 600 µl supernatant was mixed with 700 µl of 1 M NaOH. Absorbance of the samples was measured at 440 nm and specific activity of the enzyme was calculated as:

$$\text{Specific activity} = \frac{A440\ x\ V1}{(V2\ x\ T\ x\ 32\ x\ mg\ protein)}$$

Where A_{440} is the absorbance at 440 nm, V1 is the final volume in ml of the assay (1.5 ml), V2 is the volume in ml of the culture used for the assay (0.075 ml), and T is the reaction time in min. The molar extinction coefficient of azo dye is 32/M/cm.

3.12 Protease IV assay

Activity of the protease IV enzyme of *Pseudomonas aeruginosa* was determined by measuring 4-nitraniline released after the breakdown of a protease IV specific chromogenic substrate, Chromozym PL (tosyl-glycyl-polyl lysine-4-nitranilide-acetate). Briefly, 15 µl of culture supernatant from the cultures grown to OD_{600} 4, was allowed to react with 40 µg of Chromozym PL in 50 mM Tris-HCl, 150 mM NaCl pH 8 to a total volume of 100 µl for 30 min. Absorbance at 405 nm was measured after an incubation of 30 min at 37°C (Conibear et al. 2012). Protease IV activity/ml/min/mg protein was determined as follows:

$$\text{Protease IV activity/ml/min/mg protein} = \frac{A405\ x\ V1}{(V2\ x\ T\ x\ 10.4\ x\ 0.53\ x\ mg\ protein)}$$

Where A_{405} is the absorbance at 405 nm, V1 is the final volume in ml of the assay (0.1 ml), V2 is the volume in ml of the culture used for the assay (0.015 ml), T is the reaction time in min, 10.4 = extinction coefficient, 0.53 = path length of 100 µl sample in a microtitre plate.