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1 **Penicillin V acylases from Gram-negative bacteria degrade *N*-acylhomoserine**
2 **lactones and attenuate virulence in *Pseudomonas aeruginosa***

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8

9 **Running head:** Penicillin V acylases as quorum quenching agents

10

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28 **Abstract:**

29 Virulence pathways in Gram-negative pathogenic bacteria are regulated by quorum-sensing
30 mechanisms, through the production and sensing of *N*-acylhomoserine lactone (AHL) signal
31 molecules. Enzymatic degradation of AHLs leading to attenuation of virulence (quorum
32 quenching) could pave the way for the development of new antibacterials. Penicillin V acylases
33 (PVAs) belong to the Ntn hydrolase superfamily, together with AHL acylases. PVAs are
34 exploited widely in the pharmaceutical industry, but their role in the natural physiology of their
35 native microbes is not clearly understood. This report details the characterization of AHL
36 degradation activity by homotetrameric PVAs from two Gram-negative plant pathogenic
37 bacteria, *Pectobacterium atrosepticum* (*PaPVA*) and *Agrobacterium tumefaciens* (*AtPVA*). Both
38 the PVAs exhibited substrate specificity for degrading long chain AHLs. Exogenous addition of
39 these enzymes into *Pseudomonas aeruginosa* greatly diminished the production of elastase and
40 pyocyanin, biofilm formation and increased the survival rate in an insect model of acute
41 infection. Subtle structural differences in the PVA active site that regulate specificity for acyl
42 chain length have been characterized, which could reflect the evolution of AHL-degrading
43 acylases in relation to the environment of the bacteria that produce them and also provide
44 strategies for enzyme engineering. The potential for using these enzymes as therapeutic agents in
45 clinical applications and a few ideas about their possible significance in microbial physiology
46 have also been discussed.

47 **Keywords:**

48 Penicillin V acylase, *N*-acylhomoserine lactone acylase, Ntn hydrolase, quorum quenching,
49 pathogenesis

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55 **Introduction:**

56 Penicillin acylases are microbial enzymes that cleave the amide bond of natural penicillins
57 (Arroyo et al. 2003), finding industrial application in the manufacture of the pharmaceutical
58 intermediate 6-aminopenicillanic acid (6-APA). Penicillin acylases can show substrate
59 preference for benzyl penicillin (Pen G, PGAs) or phenoxymethyl penicillin (Pen V, PVAs).
60 Although both enzymes belong to the Ntn hydrolase superfamily (Oinonen and Rouvinen 2000),
61 they differ in their catalytic *N*-terminal nucleophile residue (PGA-ser, PVA-cys) and their
62 subunit composition. While PGAs are heterodimeric enzymes, PVAs are homotetramers and are
63 evolutionarily related to bile salt hydrolases (BSHs) that deconjugate bile salts in the mammalian
64 gut (Kumar et al. 2006) forming the cholyglycine hydrolase (CGH) group. A recent study
65 (Panigrahi et al. 2014) has explored the phylogenetic clustering of CGHs from Gram-positive
66 and Gram-negative bacteria into two different groups.

67 Quorum sensing (QS) allows the bacteria to perceive their population density (Rutherford and
68 Bassler 2012) through the secretion of auto-inducer signal molecules and modulate gene
69 expression to trigger specific metabolic pathways. QS has been linked to bioluminescence,
70 bacterial virulence and swarming motility among other physiological processes (Li and Nair
71 2012). Bacterial pathogens including *Pseudomonas aeruginosa*, *Vibrio cholerae* and
72 *Acinetobacter baumannii* use QS to regulate virulence genes and formation of biofilms, thereby
73 increasing their persistence (Li and Tian 2012). Gram-negative proteobacteria use autoinducers
74 *N*-acylhomoserine lactones (AHLs) (Churchill and Chen 2011), with a homoserine lactone ring
75 linked via an amide bond to an acyl side chain (C₄-C₁₈) which may be saturated or unsaturated,
76 or with a hydroxy, oxo or no substituent on the carbon at the 3-position of the *N*-linked acyl
77 chain. Synthesized AHLs diffuse into neighbouring cells, where they modulate gene expression
78 through binding to the LuxR family of regulators. While *Pectobacterium carotovorum* and
79 *Agrobacterium tumefaciens* produce 3-oxo-C₆ and 3-oxo-C₈-HSLs, respectively (Uroz et al.
80 2009), *P. aeruginosa* utilizes C₄ and 3-oxo-C₁₂-HSLs as signals for auto-induction. Bacteria in
81 mixed-species communities have also been known to respond to structurally related non-cognate
82 AHLs produced by other bacteria (Winson et al. 1998).

83 The disruption of AHL-directed signaling (termed “quorum quenching”, QQ) through inhibition
84 or enzymatic degradation is an attractive strategy for controlling bacterial pathogenesis and

85 biofilm formation (Dong et al. 2007). Enzymes that degrade AHL include lactonases (ring
86 cleavage) and acylases (amide bond cleavage), which have been characterized from a variety of
87 bacteria. An exhaustive list has been provided by Grandclément et al. (2016). Penicillin acylases
88 are known to share similar structural fold and mechanistic features with AHL acylases, and the
89 probability of substrate cross-reactivity has been suggested earlier (Kreszlak et al. 2007).
90 Although recent studies have demonstrated activity of *Kluyvera citrophila* PGA (Mukherji et al.
91 2014) and aliphatic penicillin acylase from *Streptomyces lavendulae* (Torres-Bacete et al. 2015)
92 on AHLs, both these enzymes are ser-Ntn hydrolases with heterodimeric structure. A new AHL
93 acylase from *P. aeruginosa* (HacB) (Wahjudi et al. 2011) cleaves Pen V to a small extent;
94 however, AHL degradation by PVA enzymes or any other cys-Ntn hydrolase has not been
95 explored in detail so far. Moreover, the role of PVAs in microbial physiology is not been clearly
96 understood till date, but a few possible links to quorum sensing and pathogenesis have been
97 suggested (Avinash et al. 2016b).

98 In earlier reports, we have characterized the unique biochemical (Avinash et al. 2015) and
99 structural (Avinash et al. 2016a) features of a highly active PVA from the Gram-negative
100 *Pectobacterium atrosepticum* (*PaPVA*). The present study describes the characterization of PVA
101 from another related plant pathogen *A. tumefaciens* (*AtPVA*, 62% sequence identity with
102 *PaPVA*) and elucidates the subtle structural differences between the enzymes. Further, we report
103 the promiscuous deacylation of AHLs by these PVAs, and explore the structural interactions
104 involved in AHL binding. The application of PVA enzymes also led to reduction in QS-
105 regulated biofilm formation in *P. aeruginosa* PAO1 culture and the attenuation of *P. aeruginosa*
106 virulence in *Galleria mellonella* infection models, making them attractive options for novel QQ-
107 based therapeutic formulations.

108

109 **Materials and Methods:**

110 **Bacterial strains and plasmids:**

111 The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* DH5a and BL21
112 star strains were maintained on Luria-Bertani (LB) medium supplemented on appropriate

113 antibiotics and cultured at 37°C. Antibiotics were added (100 µg/ml ampicillin, 35 µg/ml
114 kanamycin or 10 µg/ml tetracycline) as required.

115 **Preparation of *At*PVA and *Pa*PVA:**

116 The *pva* gene from *A. tumefaciens* (GenBank GI:159185562) was cloned in pET22b vector
117 between NdeI and XhoI restriction sites using the primers AtuF
118 (gcttgacatatgtgcacgcggttcggttatatag) and AtuR (ctgaatctcgagaagccccgagaaacttgaaag), and
119 expressed in *E. coli* BL21 star cells with a C-terminal His-tag. The enzyme was purified to
120 homogeneity using a HIS Select Ni²⁺ affinity column (Sigma) and ENrich™ 650 (BioRad) size
121 exclusion column. The protein was dialyzed against 10 mM Tris-Cl buffer pH 7.4 containing 100
122 mM NaCl and 1mM DTT and stored in aliquots at -20°C. *Pa*PVA was purified from
123 recombinant *E. coli* as described earlier (Avinash et al. 2015).

124 **PVA enzyme activity assay**

125 Pen V hydrolysis activity was estimated by studying the formation of Schiff's conjugate with the
126 product 6-APA and p-dimethyl amino benzaldehyde (Shewale et al. 1987). One unit (IU) of
127 enzyme activity was defined as the amount of enzyme producing 1 µmol 6-APA in 1 min.

128 **Biochemical characterization of *At*PVA**

129 The Pen V hydrolysis activity was assayed at different pH (4-9) and temperatures (20-70°C) to
130 ascertain the optimum conditions. *At*PVA stability was studied by incubating the protein in 10
131 mM Tris-Cl buffer pH 7.4 for 2 h at different temperatures between 30-90°C, and assaying for
132 PVA activity at 45°C after different time intervals. Effect of pH on enzyme stability was studied
133 by incubating the protein in 100 mM buffers of different pH (1-11) for 4 h at 25°C and assaying
134 the residual activity. Kinetic parameters were determined by assaying the enzyme activity with
135 increasing concentrations (5-240 mM) of penicillin V (potassium salt, Sigma) as substrate. The
136 data were fitted using non-linear regression as detailed for *Pa*PVA earlier (Avinash et al. 2015).

137 ***At*PVA crystallization and structure determination**

138 Crystallization trials were set up with *At*PVA (15 mg ml⁻¹) using the sitting drop vapour diffusion
139 technique. The protein crystallized in the 0.1M HEPES pH 7.5 and 12% PEG 3350 condition of

140 the PEG Rx crystallization screen (Hampton Research, USA). The crystals were frozen in liquid
141 nitrogen with 25% (w/v) 2, 5-hexanediol as cryoprotectant. Diffraction data were collected at
142 2.8 Å resolution at the SSRL-BL12-2 beamline at the Stanford Synchrotron Light Source (USA).
143 Investigation and scaling of the diffraction data was performed using XDS (Kabsch 2010) and
144 SCALA (Evans 2006). The *At*PVA structural model was built using molecular replacement on
145 Phaser ver. 2.5.6 (McCoy et al. 2007) and Autobuild (Phenix), with the refined structure of
146 *Pa*PVA (PDB ID: 4WL2) as the template. Further model building and refinement was done
147 using Coot and Refmac5 (CCP4 software suite) respectively. *At*PVA crystallized in P2₁2₁2₁
148 space group with a single tetramer per asymmetric unit (Table S1, Online Resource 1).

149 **Bioluminescence assay for detection of AHL degradation**

150 AHL degradation activity was monitored by employing biosensors that exhibit luminescence in
151 the presence of specific AHLs (Winson et al. 1998). Quenching of luminescence levels can be
152 used as an indication of AHL hydrolysis by the acylase enzymes (Steindler and Venturi 2007).

153 0.5 µL of 5 mM AHL stock solution in DMSO was spotted to a flat-bottom µClear white
154 microplate (Greiner Bio-One) and dissolved in 50 µL reaction mixture containing 5 µg enzyme
155 in 100 mM NaCl, 1 mM DTT and 25 mM Tris HCl buffer pH 7.4 (for *At*PVA) or 20 mM sodium
156 acetate buffer pH 5.2 (for *Pa*PVA). After 4 h incubation at 25°C, the enzyme was heat
157 inactivated (80°C for 15 min), and an equal volume of modified PBS (137 mM NaCl, 2.7 mM
158 KCl, 100 mM Na₂HPO₄, 1.8 mM KH₂PO₄) was added to each well, followed by 100 µl of 1:100
159 diluted overnight biosensor. Luminescence of the biosensors was measured at 30°C during a 12 h
160 time-course using FLUOstar Omega (BMG Labtech) as described previously (Papaioannou et al.
161 2009). Control reactions were performed in the same manner using heat-inactivated enzyme. *E.*
162 *coli* (pSB536) was used to analyze C₄-HSL degradation, *E. coli* (pSB401) for C₆- to C₈-HSL
163 (Swift et al. 1997), and *E. coli* (pSB1075) for C₁₀- to (3-OH- and 3-oxo-) C₁₂-HSL (Winson et al.
164 1998). To determine the enzyme activity on AHLs, the ratio of luminescence unit to biosensor
165 growth in OD₆₀₀ (relative luminescence unit, RLU) from active enzymes was compared to those
166 from inactive enzymes.

167

168

169 **HPLC analysis**

170 To confirm the deacylase activity of PVA enzyme on long chain AHLs, the reaction with C₁₀-
171 HSL was analyzed by HPLC (Uroz et al. 2008). The enzymes (25 µg in 3ml of same buffer as
172 the bioluminescence assay) were incubated with 0.4 mM C₁₀-HSL for 4 h at 25°C (heat-
173 inactivated enzyme was used as a control). Samples of 750 µl from time 0 and 4 h were
174 processed for detection of residual substrate, HSL and decanoic acid (Wahjudi et al. 2011).

175 For detection of the substrate, residual C₁₀-HSL in the reaction mixture was extracted twice with
176 an equal volume of acidified ethyl acetate. The free HSL released during the reaction was
177 dansylated with an equal volume of 2.5 mg ml⁻¹ dansyl chloride (in acetone) and incubated
178 overnight at 37°C (Lin et al. 2003). After SpeedVac evaporation, the sample was neutralized
179 with 50 µl of 0.2 M HCl and diluted with acetonitrile. Decanoic acid in the sample was extracted
180 thrice with an equal volume of hexane followed by drying under a nitrogen stream and
181 derivatization with 4-bromomethoxy-7-methyl coumarin (BrMMC) reagent was performed as
182 described previously (Wolf and Korf 1990).

183 HPLC was carried out in a Shimadzu LC-10AT VP system using a Phenomenex Luna C18
184 reverse-phase column (250 x 4.60 mm, 5 µm) coupled with a SPD-M10AVP PDA detector. The
185 column was washed with 5% acetonitrile in water (solvent A), and the sample was eluted in a
186 linear gradient to 100% acetonitrile (solvent B). C₁₀-HSL was detected at 219 nm, dansylated
187 HSL at 267 nm, and BrMMC-derivatized decanoic acid at 328 nm (Uroz et al. 2008). Reaction
188 control of reference substrate and products showed that the dansylation and BrMMC
189 derivatization was specific to HSL and decanoic acid, respectively (data not shown).

190 **Kinetics of AHL degradation by PVAs**

191 The kinetic behavior of *AtPVA* and *PaPVA* on 3-oxo-C₁₂-HSL was determined by an end-point
192 assay using ortho-phthalaldehyde (OPA) derivatization of the HSL product. Eight different
193 concentrations of 3-oxo-C₁₂-HSL in which the substrate was completely soluble (0.01-0.25 mM)
194 were prepared from DMSO stock. The reaction mixture consisted of 100 mM NaCl, 1 mM DTT
195 and 25 mM sodium phosphate buffer pH 7.4 (for *AtPVA*) or 20 mM sodium acetate buffer pH
196 5.2 (for *PaPVA*). The DMSO concentration was kept at 0.8% for each reaction. Enzyme (2 µg
197 *AtPVA* or 0.5 µg *PaPVA*) was added into the 1 ml reaction mixture; a 90 µL sample was taken

198 immediately and thereafter regularly at 1 min intervals. The enzyme was inactivated with 10 μ L
199 of 1M NaOH; this step did not interfere with the subsequent derivatization. After removal of
200 enzyme by centrifugation, 50 μ L was transferred into a black Fluotrac microplate (Greiner Bio-
201 One) and mixed with 50 μ L OPA reagent (Sigma-Aldrich), followed by 20 min incubation at
202 25°C. Fluorescence was measured on a FLUOstar Omega, BMG Labtech with an excitation at
203 355 nm and emission at 460 nm. A standard curve using 0-0.25 mM HSL standard prepared in
204 reaction mixture showed a straight line that can be fitted to the following equation: $y = 77290x +$
205 490.5 ($R^2=0.9996$). Initial velocity was limited in the range of 15% substrate conversion and
206 calculated from the standard curve. The enzyme kinetics model was analyzed by fitting the $v/[S]$
207 curves in GraphPad Prism software.

208 **Docking of AHLs to *PaPVA* and *AtPVA***

209 The 3D structures of C₆-HSL, C₁₀-HSL and 3-oxo-C₁₂-HSL used in the docking study were
210 obtained from PubChem compound database. Partial atomic charges of each ligand atom were
211 determined from OPLS_2005 all-atom force field using *LigPrep*. Grid based ligand docking
212 program *Glide* was used for docking these ligands in the binding site of *PaPVA* and *AtPVA*. The
213 binding site was defined as a grid box of dimension 26x26x26 Å, centered on the Cys1 residue.
214 Receptor grid generation was followed by ligand docking where the ligands were docked flexibly
215 using *Glide*'s extra precision. Free energy of binding was roughly estimated by using an
216 empirical scoring function called *GlideScore*, which includes electrostatic, van der Waals
217 interaction and other terms for rewarding or penalizing interactions that are known to influence
218 ligand binding. All structural figures were prepared using *PyMol* or *CCP4MG*.

219 **Disruption of quorum sensing in *Pseudomonas aeruginosa* PAO1 by PVAs**

220 Purified *AtPVA* (0.08 mg ml⁻¹) or *PaPVA* (0.4 mg ml⁻¹) was added to a 1:100 diluted overnight
221 culture of *P. aeruginosa* PAO1 in 100 ml LB. Samples were taken at 6 and 24 h post inoculation,
222 centrifuged for 5 min and supernatant was stored at -20°C until further analysis.

223 *(i) AHLs measurement.* The levels of 3-oxo-C₁₂-HSL and C₄-HSL were measured by
224 bioluminescence assay using biosensor *E. coli* pSB1075 and pSB536 respectively (Winson et al.
225 1998; Swift et al. 1997). Cell-free supernatant was filtered through a 0.2 μ m pore filter, and 20

226 μL of the sample was mixed with 180 μL of 1:100 diluted overnight biosensor culture. Light
227 production was monitored at 30°C for 12 h.

228 **(ii) Elastase assay.** Cell-free supernatant (100 μL) was added to 900 μL of elastase buffer (100
229 mM Tris HCl pH 7.5; 1 mM CaCl_2) containing 20 mg of Elastin Congo Red (ECR, Sigma
230 Aldrich) (Ohman et al. 1980). After 2h at 37°C, elastase activity of the supernatant was measured
231 as A_{495}/A_{600} .

232 **(iii) Pyocyanin assay.** Cell-free supernatant (5 ml) was extracted with 3 ml chloroform, and re-
233 extracted with 1 ml of 0.2 M HCl (Essar et al. 1990). After centrifugation, the absorbance of HCl
234 layer was measured at 520 nm. Production of pyocyanin ($\mu\text{g ml}^{-1}$ culture) was calculated as
235 $(A_{520}/A_{600}) \times 17.072$.

236 **(iv) Biofilm formation assay.** The static biofilm assay was performed in a round-bottom
237 polystyrene 96-well plate (Greiner Bio-One) using a method by Merrit et al. (2005) with
238 modification. 0.5 mg ml^{-1} *AtPVA* or 0.66 mg ml^{-1} *PaPVA* was added to an overnight culture of
239 *P. aeruginosa* PAO1 (0.01OD) in M9 medium (47.7 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$; 22 mM KH_2PO_4 ; 8.5
240 mM NaCl; 18.7 mM NH_4Cl ; 2 mM MgSO_4 ; 0.1 mM CaCl_2 ; 0.01 mM glucose). A minimum of
241 20 wells per treatment were used with an aliquot of 110 μL in each well. Biomass quantification
242 was performed using a crystal violet method (Chow et al. 2014) after 18 h at 30°C.

243 **(v) Galleria mellonella killing assay.** Larvae of *G. mellonella* were obtained from Frits Kuiper
244 (Groningen, The Netherlands) and kept in a dark container at 15°C. Animals of 2.5-3 cm size
245 were selected for the assay, with a minimum of 15 animals per treatment. An overnight culture of
246 *P. aeruginosa* PAO1 was diluted 1:100 in LB medium, grown into an early logarithmic phase
247 (A_{600} 0.3-0.4), and the CFU count was determined from a standard curve of CFUs/ A_{600} . The
248 cells were then washed with sterile 10 mM MgSO_4 , and diluted into 10^3 CFU/mL. Afterwards,
249 100 μL of enzyme (0.5 mg ml^{-1} *AtPVA* or 0.66 mg ml^{-1} *PaPVA*) or reaction buffer was added to
250 900 μL of bacteria and incubated at 30°C for 1 hour. An insulin pen (HumaPen Luxura; Lilly
251 Nederland) was used to inject 10 μL of the culture to the last proleg of the larvae. Animals
252 injected with 10 mM MgSO_4 served as a control for physical trauma. Infection development was
253 followed for 24 hours at 30°C (Beeton et al. 2015; Koch et al. 2014b). The animals were
254 considered dead when not reacting to touch or have turned black.

255 **Accession code:**

256 The structural coordinates for *AtPVA* have been deposited in the PDB under the accession codes
257 **5J9R**.

258

259 **Results:**

260 **Biochemical characterization of *AtPVA***

261 *AtPVA* was expressed as a tetramer of molecular mass 148 kDa; the enzyme exhibited a specific
262 activity of 205 $\mu\text{molmin}^{-1}\text{mg}^{-1}$ with high specificity for Pen V over bile salts and other β -lactam
263 antibiotics (Fig. S1, Online Resource 1). Maximum Pen V hydrolysis was observed at 45°C in
264 optimum pH 6 - 7 (Fig. 1). *AtPVA* was stable in the pH range 5-8, while *PaPVA* (Avinash et al.
265 2015) was more stable in acidic pH (3-6). There was also a drastic reduction in *AtPVA* activity
266 and loss of tertiary structure at 60°C (Fig. 1).

267 *AtPVA* was observed to exhibit complex kinetic behaviour similar to *PaPVA*, showing positive
268 cooperativity and substrate inhibition with Pen V and modulation of PVA activity in the presence
269 of bile salts (Fig. 2a). The major difference between *AtPVA* and *PaPVA* lies in the extent of
270 substrate inhibition; *AtPVA* showed a K_i of 47.2 mM, compared to 163.1 mM for *PaPVA*. Near
271 complete reduction of *AtPVA* activity was observed at 240 mM Pen V, while *PaPVA* still had
272 considerable activity (20% of V_{max}) at the same concentration (Avinash et al. 2015). Drastic
273 reduction in Pen V hydrolysis with *AtPVA* was also observed in the presence of high GDCA
274 (glycodeoxycholate, a bile salt) concentration (Fig. 2b).

275 **Structural analysis of *AtPVA***

276 The structural features of *AtPVA* closely resemble the *PaPVA* structure (PDB ID 4WL2) with a
277 few subtle differences. Although the *AtPVA* tetramer (Fig. 3) possesses a similar non-planar
278 orientation and distance between subunits as *PaPVA* (Avinash et al. 2016a), the angle between
279 the opposite subunits (169.6°) was closer to the planar shape of the PVA from *Bacillus*
280 *sphaericus* (171°) than *PaPVA* (158°). *AtPVA* shares many similar active site residues with
281 *PaPVA* including the nucleophilic N-terminal cysteine (C1), and the presence of two Trp

282 residues (W21, W80) in the active site participating in substrate binding. Superposition of the
283 two structures revealed that *AtPVA* (and other PVAs) lack the 5-residue insertion in the loop
284 region (61-74) near the active site in contrast to *PaPVA* (Avinash et al. 2015). It is possible that
285 the length of this loop might play a role in modulating the substrate inhibition in PVAs from
286 Gram-negative bacteria. Finally, *AtPVA* and *BtBSH* (BSH from Gram-negative *Bacteroides*
287 *thetaitotamicron*, PDB ID 3HBC) also lack a solvent-exposed loop covering the region 228-239
288 that is present in *PaPVA*.

289 **AHL degradation by PVAs**

290 The ability of PVAs from Gram-negative bacteria (*PaPVA* and *AtPVA*) to hydrolyze AHL
291 signals was evaluated to explore their possible association with quorum sensing. Incubation (4 h)
292 of long chain AHLs with pure PVA enzymes showed reduction in bioluminescence compared to
293 the heat-inactivated control, indicating AHL degradation. Activity of *PaPVA* was restricted to
294 C₁₀ and C₁₂-HSL. *AtPVA* was active on a broader substrate spectrum (C₆ to C₁₂-HSL), although
295 significant quenching was observed with the long chain AHLs, with moderate activity on C₆ and
296 C₈-HSLs (Table 2). Both enzymes were observed to be distinctly more active on straight chain
297 AHLs, with only moderate quenching in case of oxo- or hydroxy- substituted AHLs. The activity
298 of the PVA enzymes on long chain AHLs was further confirmed by monitoring the degradation
299 of C₁₀-HSL using HPLC (Fig. 4).

300 **Kinetics of AHL degradation**

301 For kinetic analysis, 3-oxo-C₁₂-HSL was chosen as a representative substrate as it is a highly
302 studied signal produced by *P. aeruginosa* and has significant clinical relevance (Cooley et al.
303 2010; Miyari et al. 2006). *PaPVA* (18.9 $\mu\text{molmin}^{-1}\text{mg}^{-1}$) exhibited 4-fold higher activity over
304 *AtPVA* (4 $\mu\text{molmin}^{-1}\text{mg}^{-1}$) with 0.2 mM 3-oxo-C₁₂HSL as substrate, similar to the trend for Pen
305 V as substrate (Avinash et al. 2015).

306 *AtPVA* and *PaPVA* showed sigmoid $v/[S]$ curves with increasing concentrations of 3-oxo-C₁₂-
307 HSL, exhibiting a better fit for allosteric behaviour. However, saturation could not be achieved
308 for both the enzymes as the low solubility of 3-oxo-C₁₂-HSL in aqueous buffer did not permit
309 rate measurements at concentrations higher than 0.25 mM. A reasonable estimate of kinetic
310 parameters calculated by applying initial values as constraints to the allosteric sigmoidal

311 equation revealed similar $K_{0.5}$ values but a significantly higher V_{\max} for *PaPVA* (Fig. 5).
312 Apparent k_{cat}/K_m values for *PaPVA* ($13.5 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) and *AtPVA* ($2.68 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) were
313 comparable to the available value for HacB acylase ($7.8 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) (Wahjudi et al. 2011) and
314 10 fold higher than PvdQ acylase ($5.8 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$) (Koch et al. 2014a).

315 **Binding of long chain AHLs to *AtPVA* and *PaPVA***

316 Docking studies were performed to understand the structural interactions responsible for the
317 selective activity of PVAs on long chain AHLs. The mode of binding was almost identical in
318 both PVAs, with the AHLs ($\text{C}_6\text{-HSL}$, $\text{C}_{10}\text{-HSL}$ and $3\text{-oxo-C}_{12}\text{-HSL}$) binding to the active site
319 with similar amide bond orientation and favourable binding energy (Fig. S2, Online Resource 1).
320 However, the extent of interaction of enzyme residues with the substrate molecule seemed to
321 increase with the increase in length of acyl chain of the AHL molecule.

322 The lactone ring was housed in the same pocket where the β -lactam moiety was bound in the
323 case of Pen V (Avinash et al. 2016a) with an Asn residue (N250 in *AtPVA* or N271 in *PaPVA*)
324 involved in hydrogen bonding with the NH group of the amide bond. The AHL acyl chain fits
325 into a hydrophobic pocket lined primarily by the two Trp residues in the active site (W23, W87
326 in *AtPVA* and W21, W80 in *PaPVA* respectively) and residues from loop 2 and loop3
327 surrounding the active site (Fig. 6). It appears that longer hydrophobic chains in $\text{C}_{10}\text{-HSL}$ and $3\text{-oxo-C}_{12}\text{-HSL}$
328 enable greater number of hydrophobic interactions with the enzyme. The loop
329 residues (Y61, L137, A138 in *AtPVA* and F63, M69, L146 and A147 in *PaPVA* respectively)
330 form additional interactions with the hydrophobic acyl chain in these substrates, probably
331 enhancing the strength of binding and favourably orienting the AHL molecule in the active site.
332 Better binding affinity values (estimated as glidescores) and smaller nucleophilic attack distances
333 from the N-terminal catalytic cysteine (C1) to the carbonyl carbon of the substrate were also
334 observed in $\text{C}_{10}\text{-HSL}$ and $3\text{-oxo-C}_{12}\text{-HSL}$ over $\text{C}_6\text{-HSL}$ (Table 3). The presence of a (oxo- or
335 hydroxy-) substituent did not effect a significant change in binding orientation, although a
336 reduction in activity was observed (Table 3). It is possible that a change in polarity due to the
337 presence of a 3' substituent might have caused a binding impediment. A preference for
338 unsubstituted AHLs has also been observed in AHL acylases from *Shewanella* sp. (Morohoshi et
339 al. 2008) and *Acinetobacter* sp. (Ochiai et al. 2014).

340 **Quorum quenching in *P. aeruginosa* by PVAs**

341 Exogenous addition of the PVAs into *P. aeruginosa* PAO1 culture was followed by
342 measurement of AHL levels and monitoring of QS-regulated virulence factors and biofilm
343 formation, to study their quorum quenching activity. Decrease in 3-oxo-C₁₂-HSL levels was
344 apparent 6h post incubation (Fig. 7a), but the accumulation of C₄-HSL was unaffected (data not
345 shown). This result corroborates the finding that both PVAs hydrolyze only long chain AHLs.

346 Elastase and pyocyanin levels were also negatively influenced at 6 h after acylase addition (early
347 stationary phase) (Fig. 7b). Interestingly, *AtPVA* almost completely blocked the production and
348 pyocyanin and elastolytic activity even at 5-fold lower concentration than *PaPVA*, despite
349 *PaPVA* exhibiting higher activity on AHLs *in vitro*. This might be explained by a probable loss
350 in enzyme (*PaPVA*) activity at pH 7 required for *P. aeruginosa* growth, or proteolytic
351 degradation of the enzymes in bacterial culture. Although there was comparable decrease in 3-
352 oxo-C₁₂-HSL levels in both cases after 6 h, the insufficient stability of *PaPVA* might have
353 caused a delay in AHL degradation, giving the bacteria time to activate the QS circuit. Decrease
354 in QQ-mediated attenuation of virulence over time has been observed earlier in the case of AhIM
355 from *Streptomyces* sp. (Park et al. 2005).

356 PVA-mediated AHL degradation also led to a moderate reduction in biofilm formation by *P.*
357 *aeruginosa* (Fig. 7c). Weakening of biofilm structure in *P. aeruginosa* has been linked to the
358 disruption of the 3-oxo-C₁₂HSL regulated *lasI/R* QS system (DeKievit et al. 2001). In addition,
359 the therapeutic effects of PVAs in attenuation of *P. aeruginosa* virulence could be ascertained by
360 studies on *G. mellonella* larvae. Simplicity of use and a positive correlation between *P.*
361 *aeruginosa* virulence patterns in insects and mice make *G. mellonella* an attractive alternative
362 infection model for anti-virulence experiments (Papaioannau et al. 2013; Jander et al. 2000). In
363 the present study, preincubation of *P. aeruginosa* culture (10 cfu) with PVAs was observed to
364 increase the survival rates of *G. mellonella* larvae after 24 h from only 10.3±7.2% in untreated
365 infection to 73±5% (*AtPVA*) or 53.7±11% (*PaPVA*) (Fig. 7d). Control injection with only
366 MgSO₄ only did not affect the survival of the larvae. Here too as in the *in vitro* assay, *AtPVA*
367 turned out to be more efficient in attenuating virulence. Regardless, these results establish the
368 potential efficacy of PVAs as QQ therapeutic agents.

370 Discussion:

371 Enzymes active on AHLs hold great potential for application as QQ agents in clinical therapy as
372 they can reduce virulence without affecting the growth of the bacteria, thereby diminishing the
373 chance for emergence of resistant strains. Apart from the many AHL acylases and lactonases
374 characterized so far, it has recently come to light that other related enzymes can promiscuously
375 degrade the AHL signals as well, effecting QQ albeit at a lower rate. Examples include
376 mammalian paraoxanases (Dong et al. 2007), porcine acylase (Xu et al. 2003) and PGA from
377 *Kluyvera citrophila* (KcPGA) (Mukherji et al. 2014). Although PVAs and PGAs come under the
378 same functional ambit, they show significant differences in sequence and structural composition.
379 While AHL acylases are generally homologous to heterodimeric PGAs and share similar active
380 sites including an N-terminal catalytic serine, bacterial PVAs are homotetrameric and
381 evolutionarily related to BSHs with cysteine at the N-terminal. The heterodimeric acylase from
382 *Streptomyces avendulae* (SPVA) active on aliphatic penicillins and Pen V has been recently
383 hinted to degrade AHLs (Torres-Bacete et al. 2015), but it shares significant sequence and
384 structural homology with the ser-Ntn hydrolases. In the present study, the ability of cys-Ntn
385 PVAs from Gram-negative bacteria to degrade long chain AHLs and attenuate QS-mediated
386 virulence in *P. aeruginosa* has been described for the first time. Both the organisms employed in
387 this study are also well-known plant pathogens that produce AHLs and employed as model
388 systems to study AHL-based QS mechanisms (Steindler and Venturi 2007). The AiiB (Liu et al.
389 2007) and BlcC/AttM (Carlier et al. 2003; White et al. 2009) lactonases from *A. tumefaciens*
390 have been implicated in QQ; however, no acylase active on AHLs has been reported so far from
391 these bacteria.

392 Acylases active on AHLs have been observed to vary in their substrate specificities, and separate
393 into different phylogenetic clusters (Ochiai et al. 2014). Enzymes of the AAC group (including
394 AAC from *Shewanella* sp., PvdQ from *P. aeruginosa*, AhlM from *Streptomyces* sp. and AiiD
395 from *Ralstonia* sp.) degrade only long chain AHLs, while some members of the penicillin G
396 acylase group (including QuiP and HacB from *P. aeruginosa*, and AiiC from *Anabena* sp.) group
397 can act on both long and short chain AHLs. A newly characterized AHL acylase AmiE of the
398 amidase family (Ochiai et al. 2014) possesses an activity preference for long chain unsubstituted

399 AHLs similar to PVAs. However, the PVA enzymes shared little sequence similarity (<15%)
400 with any of the known acylases active on AHLs (Fig. S3, Online Resource 1). In addition, both
401 the PVAs explored in this study did not act on the AHL signals secreted by the bacteria that
402 produce these enzymes – 3-oxo-C₈-HSL of *A. tumefaciens* and 3-oxo-C₆-HSL of *P.*
403 *atrosepticum*. It would be however, interesting to study whether the substrate spectrum of
404 penicillin acylases would include the non-canonical aryl HSLs (Ahlgren et al. 2011) as well,
405 given that penicillins also possess aryl side chains.

406 Docking analysis showed that the AHLs bind to PVA enzymes at the same site as Pen V, with
407 the acyl chain housed in a hydrophobic pocket lined by Trp residues and loop 2 and 3 while the
408 lactone ring interacts with residues from loop 4. Accommodation of the AHL acyl chains in the
409 active site hydrophobic pocket has been illustrated in the AHL acylase PvdQ (Bokhove et al.
410 2010) and KcPGA (Mukherji et al. 2014), while the *S. lavendulae* acylase also contains a long
411 hydrophobic pocket to bind aliphatic penicillins that can accommodate AHLs. The size of the
412 hydrophobic pocket and the conformational variations of a few critical residues in the binding
413 site have been suggested to modulate the activity of different PGAs on AHLs (Chand et al.
414 2015). Moreover, it has been demonstrated in PvdQ that mutagenesis of two residues (L α 146W,
415 F β 24Y) in the active site could change the size of the hydrophobic binding pocket thus effecting
416 a change in substrate specificity from long chain to medium chain AHLs (Koch et al. 2014a).

417 PVAs occur in a diverse range of bacteria and some fungi (Avinash et al. 2016b), and are usually
418 expressed constitutively. It has been demonstrated in *V. cholerae* (Kovacikova et al. 2003) that
419 the PVA expression is reduced during the induction of virulence genes by the AHL-based
420 AphA/HapR QS system and expressed more at high cell densities. Moreover, long chain AHLs
421 have been known to antagonize QS in organisms that use C₆-C₈ HSLs as signals, including
422 *Chromobacterium violaceum* (McClellan et al. 1997) and *Aeromonas hydrophila* (Swift et al.
423 1997). It is therefore possible that the PVAs could be employed in the environment to gain a
424 competitive advantage in a mixed species community (Roche et al. 2004), while not interfering
425 with the bacterium's own QS system. Further genomic and knockout analyses of PVA producing
426 strains could help shed some light on the relevance of their QQ ability in microbial physiology.
427 Nevertheless, the recent additions of many novel acylases to the list of AHL-degrading enzymes

428 seem to go hand in hand with the complexity of AHL-based signaling mechanisms in Gram-
429 negative bacteria.

430 Importantly, the knowledge of AHL-hydrolysis activity of penicillin acylases adds them to the
431 list of QQ enzymes that can be developed for clinical applications. PVA enzyme formulations
432 could have great potential for the biocontrol of *P. aeruginosa* pulmonary infection in cystic
433 fibrosis patients. A dry powder formulation of the enzyme could not only be directly delivered
434 into the lungs, but also increases its shelf life (Wahjudi et al. 2011). With their broad spectrum
435 activity, PVAs can also help attenuate virulence in *Acinetobacter baumannii* (Chow et al. 2014)
436 and co-infections by other pathogens whose QS mechanisms are at least partly dependent on
437 long chain AHLs. QQ enzymes have also been applied to disrupt bacterial biofilms on silicone
438 surfaces (Ivanova et al. 2015). Sustained QQ activity can be ensured for clinical application by
439 enhancing protein stability (via directed evolution) and the use of stabilizing excipients. It is also
440 advantageous that many penicillin acylases have been already optimized for industrial use with
441 methods for their production on large scale; this could help in reducing development times for
442 their clinical application in QQ systems. However, their activity levels and specificity for AHL
443 acyl chain length should also be studied to direct their application to specific pathogens. With the
444 recent expansion in the volume of information about QS systems in pathogenic bacteria, the
445 development of a battery of enzymes acting on a broad range of AHLs would definitely prove
446 beneficial in tackling bacterial virulence. In addition to its potential clinical application, this
447 result also encourages the further exploration of possible link between QQ and the natural role of
448 PVAs for the bacteria.

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456 Conflict of interest: All the authors declare that they have no conflict of interest.

457 Ethical approval: All applicable international, national, and/or institutional guidelines for the care
458 and use of animals were followed.

459

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617

618 **List of Figures:**

619

620 **Fig. 1** *AtPVA* (a) pH and (b) temperature optima, stability at increasing (c) pH (after 4 h) and (d)
621 temperature. Maximum activity was taken as 100%

622 **Fig. 2** (a) $v/[S]$ plot of *AtPVA* with Pen V as substrate. Kinetic parameters are given in inset. (b)

623 Relative PVA activity in the presence of increasing concentrations of GDCA. Pen V
624 concentration was kept constant at 50 mM

625 **Fig. 3** (a) Tetramer structure of *AtPVA*. Subunits are shown in different colours. (b)
626 Superposition of monomer structures of *AtPVA* and *PaPVA*. The loop extensions in *PaPVA* are
627 shown in green (residue numbering according to *PaPVA*). N-terminal cysteine (stick
628 representation) is shown in yellow

629 **Fig. 4** HPLC analysis of residual C₁₀-HSL and released HSL and decanoic acid, for *AtPVA*
630 (upper panels) and *PaPVA* (lower panels) after 4h incubation with C₁₀-HSL at 25⁰C. Reduction
631 of C₁₀-HSL levels was corroborated with the occurrence of free HSL and decanoic acid,
632 confirming the acylase activity of PVAs on C₁₀-HSL

633 **Fig. 5** v/[S] curves for (a) *AtPVA* and (b) *PaPVA* showing sigmoid kinetics with 3-oxo-C₁₂-HSL
634 as substrate. Kinetic parameters are given in inset

635 **Fig. 6** Mode of binding of 3-oxo-C₁₂-HSL in the binding site pocket of (upper) *AtPVA* and
636 (lower) *PaPVA*. The hydrophobic pocket in which the alkyl side chain fits is shown as mesh

637 **Fig. 7** Influence of *AtPVA* or *PaPVA* on *P. aeruginosa* PAO1 culture: (a) 3-oxo-C₁₂-HSL level,
638 (b) Elastolytic activity and pyocyanin production 6 h after exogenous addition of enzyme, (c)
639 Biofilm formation, (d) Survival rate in *G. mellonella* 24h after infection with *P. aeruginosa*
640 PAO1. Larvae injected with MgSO₄ were taken as control. Error bars indicate standard deviation
641

642 **List of Tables:**

643
644 **Table 1** Bacterial strains and plasmids used in this study

645 **Table 2** Specificity of purified *AtPVA* and *PaPVA* for different AHL substrates. Remaining
646 AHLs after degradation assay were detected by suitable Lux-based biosensor at 30°C for 12h.
647 Bioluminescence (%RLU) is expressed relative to heat-inactivated enzyme (taken as 100%).
648 Results are displayed as Mean ± SD from three independent experiments.

649 **Table 3** Properties of different AHL substrates and results of docking with *AtPVA* and *PaPVA*
650 structures (AlogP = hydrophobicity, SA = surface area, Nadist = Nucleophilic attack distance
651 between SH group of cys1 and carbonyl carbon atom of AHL)