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ClpP1P2 peptidase activity promotes biofilm formation in Pseudomonas aeruginosa

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16 **ClpP1P2 peptidase activity promotes biofilm formation in *P. aeruginosa***

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41   **Summary**

42

43   Caseinolytic proteases (Clp) are central to bacterial proteolysis and control cellular physiology and
44   stress responses. They are composed of a double-ring compartmentalized peptidase (ClpP) and a
45   AAA+ unfoldase (ClpX or ClpA/ClpC). Unlike many bacteria, the opportunistic pathogen *P.
46   aeruginosa* contains two ClpP homologs: ClpP1 and ClpP2. The specific functions of these
47   homologs, however, are largely elusive. Here, we report that the active form of PaClpP2 is a part
48   of a heteromeric PaClpP1$_7$P2$_7$ tetradecamer that is required for proper biofilm development.
49   PaClpP1$_{14}$ and PaClpP1$_7$P2$_7$ complexes exhibit distinct peptide cleavage specificities and interact
50   differentially with *P. aeruginosa* ClpX and ClpA. Crystal structures reveal that PaClpP2 has non-
51   canonical features in its N- and C-terminal regions that explain its poor interaction with unfoldases.
52   However, experiments *in vivo* indicate that the PaClpP2 peptidase active site uniquely contributes
53   to biofilm development. These data strongly suggest that the specificity of different classes of ClpP
54   peptidase subunits contributes to the biological outcome of proteolysis. This specialized role of
55   PaClpP2 highlights it as an attractive target for developing antimicrobial agents that interfere
56   specifically with late-stage *P. aeruginosa* development.

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58

59   **Introduction**

60

61 Regulated protein degradation is central to bacterial physiology and development, playing critical

62 roles in establishing and maintaining appropriate levels of intracellular proteins. AAA+ (ATPases

63 associated with diverse cellular activities) proteases of the Clp family are macromolecular

64 complexes conserved in bacteria and in some eukaryotic organelles. They are composed of a self-

65 compartmentalized tetradecameric peptidase, ClpP, in complex with a hexameric AAA+ unfoldase

66 (ClpA or ClpX in *Escherichia coli* and *Pseudomonas aeruginosa*). Clp protease substrates include

67 proteins that regulate cellular stress responses, cell division, antibiotic resistance, motility, and

68 virulence (Gur et al., 2011; Culp and Wright, 2017). Substrates destined for degradation are

69 recognized and unfolded in an energy-dependent process by the unfoldase and then translocated

70 into the central chamber of ClpP, where they are degraded into short peptides (Sauer and Baker,

71 2011; Baker and Sauer, 2012) (Fig. 1 A, B).

72

73 Most Proteobacteria, including *E. coli*, contain a single *clpP* gene, which is transcribed, translated,

74 and processed to remove a propeptide. ClpP subunits assemble into inactive heptameric rings,

75 which then associate to form a functional homomeric tetradecamer. However, organisms from

76 phyla including Actinobacteria, Cyanobacteria, Firmicutes, and Chlamydiae frequently harbor two

77 or more *clpP* genes, which assemble into active heteromeric tetradecamers in some cases (Akopian

78 et al., 2012; Stanne et al., 2007; Dahmen et al., 2015; Pan et al., 2019). The specific biological

79 advantages provided by heteromeric ClpP complexes are poorly understood. Here, we investigate

80 the assembly, structures, activities, and biological roles of ClpP1 and ClpP2 from *P. aeruginosa.*

81 Our results provide insight into the potential biological benefits of having two ClpP isoforms.

82 The substrate specificity of Clp-family proteases is determined by the AAA+ unfoldase, which

83 recognizes specific peptide sequences (degron tags) on cognate substrates. After substrate

84 recognition, the AAA+ enzyme uses the energy of ATP hydrolysis to unfold and then translocate

85 the polypeptide into ClpP for degradation (Baker and Sauer, 2012). Binding of the AAA+ enzyme

86 to ClpP involves two key interactions: (i) docking of the IGF/IGL loops of ClpX/A into specific

87 hydrophobic pockets on the ClpP surface, and (ii) contacts between N-terminal axial loops of ClpP

88 and pore-2 loops of the unfoldase (Kim et al., 2001; Joshi et al., 2004; Bewley et al., 2006; Gribun

89 et al., 2005; Alexopoulos et al., 2012; Fei et al., 2020; Gatsogiannis et al., 2019; Ripstein et al.,

90 2020; Lopez et al., 2020). Unfoldase-ClpP interaction triggers expansion of the ClpP axial pores,

91   allowing polypeptides to enter the proteolytic chamber. In the ClpP chamber, active sites

92   containing a Ser-His-Asp catalytic triad cleave translocated polypeptides into ~8-10 amino acid-

93   long peptides (Thompson et al., 1994). Thus, through coordinated action, the Clp-family protease

94   complex chooses substrates, unfolds them, and cleaves them into short peptides (Fig. 1A, B).

95

96   The opportunistic pathogen *P. aeruginosa* is a Gram-negative bacterium that causes serious

97   chronic infections in immunocompromised patients and is a major factor in the mortality of

98   patients with cystic fibrosis (Lyczak et al., 2000; Gellatly and Hancock, 2013). The bacterium's

99   ability to switch from free-swimming planktonic cells to surface-attached biofilm communities

100   contributes to both antibiotic resistance and pathogenicity (Drenkard and Ausubel, 2002; Mah et

101   al., 2003). *P. aeruginosa* contains two *clpP* genes located at distinct genomic loci: *clpP1* (PA1801)

102   is in the same operon as *clpX*, as is common in many bacteria, whereas *clpP2* (PA3326) is at a

103   locus within a cluster of genes that appear to form a mobile genetic island. The *clpP1* and *clpP2*

104   genes also exhibit distinct expression patterns. The *clpP1* gene is expressed throughout growth

105   whereas *clpP2* is tightly controlled by quorum sensing signaling molecules and the LasR

106   transcription factor (Wagner et al., 2003; Schuster et al., 2003; Arevalo-Ferro et al., 2003; Hentzer

107   et al., 2003; Gilbert et al., 2009). Genetic studies implicate both *clpP1* and *clpP2* in virulence, and

108   deletion of either gene results in distinct cellular phenotypes (Qiu et al., 2008; Hall et al., 2017).

109   Purified *P. aeruginosa* ClpP1 is tetradecamer that is active both as a stand-alone peptidase that

110   cleaves very short peptides and as a protease when combined with ClpX or ClpA. ClpP2, however,

111   purifies as a catalytically inactive heptamer, and thus its active form has remained mysterious (Hall

112   et al., 2017).

113

114   Here, we demonstrate that a heptameric ring of PaClpP2 assembles with a heptameric ring of

115   PaClpP1 to form an active tetradecameric complex. Using biochemical, crystallographic, and

116   microbiological assays, we provide evidence that PaClpP1P2 hetero- and PaClpP1

117   homocomplexes have distinct peptidase and protease activities. Furthermore, we show that

118   PaClpP2 peptidase activity is specifically required for normal biofilm thickness, with only thin

119   biofilms forming in its absence. We discuss our findings in the context of models for how PaClpP1

120   and PaClpP2 contribute to pathogenicity and poor patient prognosis during *P. aeruginosa*

121   infections.

122

123   **Results**

124   ***P. aeruginosa* ClpP1 and ClpP2 form a heteromeric complex in vivo.**

125   In a previous study characterizing the isoforms encoded by *P. aeruginosa* genes *clpP1* (PA1801)

126   and *clpP2* (PA3326), PaClpP1 and PaClpP2 were purified after heterologous expression in *E. coli*

127   (Hall et al., 2017). PaClpP1 purified as an active tetradecamer, whereas PaClpP2 was an inactive

128   heptamer. We sought here to determine the active form of PaClpP2 by isolating it in native

129   complexes from *P. aeruginosa* cells.

130

131   We specifically tested the possibility that PaClpP2 exists in a co-complex with PaClpP1, as similar

132   heterocomplexes have been characterized from other bacterial species that carry multiple *clpP*

133   genes (Akopian et al., 2012; Stanne et al., 2007; Dahmen et al., 2015; Pan et al., 2019). To probe

134   for interactions between PaClpP1 and PaClpP2 in *P. aeruginosa* strain PAO1, we appended a

135   FLAG-tag to the chromosomal copy of *clpP2*. The cellular extract was made from stationary-phase

136   PAO1 and captured proteins were observed by stained SDS-PAGE (Fig. 1C). Two similarly sized

137   protein bands were evident: one consistent with the size of PaClpP2-FLAG and one consistent

138   with the size of native PaClpP1. The identity of PaClpP1 was verified by western blot following

139   SDS-PAGE using an antibody specific for PaClpP1, as well as directly by mass spectrometry (Fig.

140   1D and Fig. S2). We conclude that PaClpP1 and PaClpP2 interact in *P. aeruginosa* cells.

141

142   To purify larger amounts of the PaClpP1P2 co-complex for biochemical analysis, a dual-

143   expression plasmid encoding both ClpP forms (affinity-tagged PaClpP1-His$_6$ and PaClpP2-

144   StrepII) was used for co-expression in *E. coli*. The co-complex was purified using tandem Ni^{2+}-

145   NTA and streptavidin columns in series, followed by size-exclusion chromatography (Fig. S1).

146   The final column yielded a single peak, which eluted at the size expected for a tetradecamer (~320

147   kDa) and contained both PaClpP1-His$_6$ and PaClpP2-StrepII (Fig. 1E, F). Quantification of bands

148   in stained gels of the purified co-complex indicated that the two ClpP isoforms were present in an

149   approximate 1:1 ratio. Formation of PaClpP1P2 complexes strictly depended upon co-expression

150   and co-purification; as mixing of separately purified PaClpP1 and PaClpP2 did not result in

151   heterocomplex formation (Hall et al., 2017).

152

153   To explore the subunit arrangement within $PaClpP1_7P2_7$ complexes, we took two experimental

154   approaches. First, we carried out a glutaraldehyde crosslinking time course with three separate

155   samples: (i) $PaClpP1_{14}$, (ii) $PaClpP2_7$, and (iii) purified PaClpP1P2 co-complexes. Crosslinked

156   samples were then analyzed by Coomassie-stained SDS-PAGE. Interestingly, the time-dependent

157   crosslinking of PaClpP1P2 generated a gel pattern that resembled the sum of the gel patterns

158   observed during $PaClpP1_{14}$ crosslinking (rapid but incomplete dimer formation) and during

159    $PaClpP2_7$ crosslinking (a ladder of cross-linked species that continually formed over ~15 min)

160   (Fig. S3). This result is consistent with the model that the PaClpP1P2 tetradecamer is composed

161   of one homogenous PaClpP1 heptamer and one homogenous PaClpP2 heptamer, as the PaClpP1P2

162   crosslinking pattern highly resembled the sum of the behavior of the two subtypes.

163

164   To further probe the assembly architecture of PaClpP1P2, we took advantage of the previous

165   observation that tetradecameric ClpP can often be disassembled into its two constitutive heptamers

166   by incubation in certain salt solutions (Maurizi et al., 1998). Thus, after incubation of ClpP1P2

167   under these dissociation conditions, the resulting heptamers were isolated by size-exclusion

168   chromatography, subjected to StrepII-affinity purification, and analyzed by SDS-PAGE (Fig. S4).

169   This approach allows for selective capture of dissociated heptamers that contained PaClpP2-

170   StrepII. Notably, PaClpP1-His did not bind the Strep-tactin column and was recovered in the flow-

171   through and wash fractions (Fig. S4). In contrast, PaClpP2-StrepII initially bound to the column

172   but was clearly observed after biotin-specific elution. The discrete recovery of PaClpP1-His in the

173   wash fraction versus PaClpP2-StrepII in the elution fraction of this affinity column, combined with

174   their crosslinking patterns, indicate that the two heptameric rings in PaClpP1P2 are each composed

175   of a single detectable ClpP subtype: $PaClpP1_7$ or $PaClpP2_7$. These data, together with the ~1:1

176   stoichiometry of the two ClpP isoforms in the heterocomplex (Fig. 1), support a configuration of

177   two stacked homo-heptameric rings: one of PaClpP1 and one of PaClpP2. Hereafter, this complex

178   is referred to as $PaClpP1_7P2_7$. These results, together with the previous characterization of

179    $PaClpP1_{14,}$ allow us to conclude that *P. aeruginosa* makes two forms of ClpP tetradecamers during

180   its life cycle: $PaClpP1_{14}$ and $PaClpP1_7P2_7$.

181

182 ***Crystal structures of PaClpP1 and PaClpP2 reveal key distinguishing features.***

183 PaClpP1 and PaClpP2 share 74% and 40% sequence identity, respectively, with *E. coli* ClpP (Fig.

184 S5). To better understand the molecular differences between the PaClpP1and PaClpP2 peptidase

185 subunits, we solved crystal structures of PaClpP1 and PaClpP2 to resolutions of 2.6 Å and 2.0 Å,

186 respectively (Table S1; PDB: 5BQV and 5BQW). PaClpP1 crystallized as tetradecamer, one of its

187 biologically relevant assemblies. PaClpP2 crystallized as a heptamer. A crystallographic 2-fold

188 axis did generate a PaClpP2 14-mer, but we were unable to isolate a PaClpP2 tetradecamer from

189 cells or assemble this form *in vitro*, placing into question the biological relevance of a PaClpP2

190 tetradecamer. Furthermore, despite significant effort, we were unable to grow high-quality crystals

191 of the PaClpP1$_7$P2$_7$ complex. Nonetheless, analysis of our crystal structures revealed important

192 structural differences between PaClpP1 and PaClpP2.

193

194 As expected, PaClpP1 shared high structural similarity with *E. coli* ClpP (Cα RMSD = 0.43 Å)

195 and adopted the more extended conformation of ClpP barrel structures (~100 Å high, ~100 Å wide)

196 described previously (Kimber et al., 2010; Liu et al., 2014). PaClpP2 formed a tetradecamer by

197 crystallographic symmetry. Although we have no evidence that this protein

198 assembly/conformation is biologically relevant, it is nonetheless useful in visualizing the

199 similarities and differences between PaClpP1 and PaClpP2. The tetradecamer of PaClpP2

200 observed in the crystal adopted a more compressed barrel conformation than the active PaClpP1$_{14}$

201 (~90 Å high, ~102 Å wide) (Fig. 2A, B). Furthermore, although the overall conformations of

202 individual PaClpP1 and PaClpP2 subunits and rings were similar, three major differences were

203 notable.

204

205 First, residues 4-8 and 18-20 of PaClpP1$_{14}$ formed a β hairpin (residues 9-17 were disordered),

206 whereas residues 14-20 of PaClpP2$_7$ formed an α helix that pointed downwards into and closed

207 the axial pore (Fig. 2B, D). N-terminal β-hairpin loops are observed in numerous ClpP structures

208 (Kang et al., 2004; Gribun et al., 2005; Bewley et al., 2006), and their detailed conformations

209 determine the size of the axial ClpP pore. These loops also make contacts with ClpX and ClpA in

210 recent cryo-EM structures (Fei et al., 2020; Gatsogiannis et al., 2019; Ripstein et al., 2020; Lopez

211 et al., 2020). A second structural difference was observed near the hydrophobic pockets where

212 critical peptidase-unfoldase interactions occur. These pockets serve as docking sites for the

213   IGF/IGL loops of ClpX/ClpA, which are essential for complex formation with ClpP (Amor et al.,

214   2019; Fei et al., 2020; Lopez et al., 2020; Gatsogiannis et al., 2019). Interestingly, the most C-

215   terminal segment of PaClpP2 formed a single helical turn that protruded over its adjacent

216   hydrophobic pocket (Fig. 2E) in a position that would likely interfere with ClpX/ClpA binding.

217   Thus, structural differences near both the N- and C-termini of PaClpP2 suggest that it is unlikely

218   to dock stably with a partner Clp unfoldase in the absence of major conformational changes.

219

220   The third notable difference between the PaClpP1 and PaClpP2 structures was an alternative

221   positioning of the active-site residues. In the PaClpP1 structure, the Ser-His-Asp catalytic triad

222   appeared properly spaced and positioned for peptidase activity. In contrast, those in PaClpP2 were

223   not optimally positioned (Fig. 2F). In all subunits of PaClpP2, the active site Asp^{172} side chain was

224   too far (~10 Å) from the side chains of Ser^{98} and His^{123} to form an active catalytic triad (Fig. 2F).

225   This active-site misalignment in the crystal structure is consistent with the inactivity of the

226   PaClpP2 heptamer in solution.

227

ClpX engages the PaClpP1$_7$P2$_7$ complex through interactions with PaClpP1

229   The crystal structures reveal that the two main regions where ClpPs commonly interact with the

230   AAA+ unfoldases – the ClpP N-terminal loop and its hydrophobic binding pocket – are notably

231   altered in PaClpP2. These structural features suggest that PaClpP2 does not interact canonically

232   with unfoldases. Therefore, we investigated whether complexes containing ClpP1 and ClpP1P2

233   were active in degradation of protein substrates when partnered with the PaClpX or PaClpA

234   unfoldases. We performed kinetic experiments to compare the ability of PaClpX•P1$_{14}$ and

235   PaClpX•P1$_7$P2$_7$ to form proteolytic complexes and degrade a degron-tagged model substrate, GFP-

236   ssrA. In the presence of limiting concentrations of PaClpX, the K_M (~4.1 μM) and V_{max} (~1.7 min^{-1}

237   ClpX$_6^{-1}$) values for GFP-ssrA degradation by PaClpX•P1$_7$P2$_7$ were indistinguishable from those

238   by PaClpX•P1$_{14}$ (Fig. 3A). Likewise, the AAA+ unfoldase PaClpA functioned similarly in

239   complex with either ClpP1$_{14}$ or ClpP1$_7$P2$_7$ to degrade the model ClpAP substrate FITC-casein

240   (Fig. 3B). Thus, unfoldases can interact with ClpP1$_{14}$ and ClpP1$_7$P2$_7$ and function to degrade

241   protein substrates.

242

243   AAA+ unfoldases can dock to either one or both sides of many homomeric ClpP barrels, producing

244   singly-capped or doubly-capped proteolytic complexes (Grimaud et al., 1998). However, the

245   crystal structure of $PaClpP2_7$ suggests that interactions between ClpX/ClpA and PaClpP2 in the

246    $PaClpP1_7P2_7$ hetero-tetradecamer would be disfavored in the absence of major conformational

247   changes. To directly visualize the stoichiometry of PaClpX binding to $PaClpP1_7P2_7$, we mixed

248   PaClpX with $PaClpP1_{14}$ or $PaClpP1_7P2_7$ (in the presence of ATPγS, which stabilizes complexes)

249   and scored the number of singly-capped and doubly-capped ClpP barrels by electron microscopy.

250   Approximately half of the PaClpX•$ClpP1_{14}$ particles had PaClpX bound on both sides the ClpP

251   core (Fig. 3C). In contrast, when PaClpX and $PaClpP1_7P2_7$ were mixed, only 2% of the particles

252   had observable PaClpX on both faces, whereas 98% of the complexes were singly-capped, and the

253   small number of doubly-capped complexes may represent PaClpX bound to low concentration of

254   contaminating $ClpP1_{14}$ present in the preparation. Regardless, doubly-capped $PaClpP1_7P2_7$

255   peptidases either do not assemble or are a very rare species.

256

257   To further probe interactions between PaClpP1 and PaClpP2 and the PaClpX unfoldase, we used

258   site-directed mutagenesis. Arg^{192} of EcClpP is required for stable docking with EcClpX (Fei et al.,

259   2020). We mutated the equivalent residue in PaClpP1, Arg^{194}, to lysine (^{RK}ClpP1). ^{RK}ClpP1

260   supported very little degradation of GFP-ssrA in the presence of PaClpX (Fig. 3D) and did not

261   bind PaClpX in electron-microscopy experiments (Fig. S6). Thus, Arg^{194} of $PaClpP1_{14}$ plays an

262   important role in assembly of the PaClpX•$P1_{14}$ complex. Moreover, purified $PaClp^{RK}P1_7P2_7$

263   heterocomplexes also did not significantly degrade GFP-ssrA (Fig. 3D), supporting a model in

264   which the $PaClpP2_7$ face of $PaClpP1_7P2_7$ does not interact stably with PaClpX. We hypothesize

265   that the $ClpP2_7$ face is also defective in binding to the ClpA unfoldase, which uses a very similar

266   docking mechanism to ClpX (Grimaud et al., 1998; Gribun et al., 2005; Bewley et al., 2006).

267

268   Rates of GFP-ssrA degradation were similar under conditions that favored formation of doubly-

269   capped $(PaClpX_6)_2$•$PaClpP1_{14}$ complexes or twice as many singly-capped $PaClpX_6$•$PaClpP1_{14}$

270   complexes (Fig. 3E). This observation strongly suggests that both ClpX hexamers in these doubly-

271   capped complexes are fully active during degradation. Similar results have been reported for *E.

272   coli* ClpXP and ClpAP (Ortega et al., 2002; Maglica et al., 2009). Because only one PaClpX

273   hexamer can bind $ClpP1_7P2_7$, a bivalent protease assembly would not be expected to form with

274 the PaClpP1P2 heterocomplex. Thus, this distinct unfoldase-binding property may contribute to

275 the functional specialization of PaClpP1$_{14}$ and PaClpP1$_7$P2$_7$ complexes in *P. aeruginosa*.

276

ClpP2 active sites are essential for normal biofilm development in P. aeruginosa

278 As shown previously, deletion of *clpP1* in *P. aeruginosa* results in several cellular phenotypes

279 including defects in cell attachment, motility, pyocyanin and pyoverdine production, and

280 pathogenicity in a mouse model (Fernández et al., 2012; Shanks et al., 2006; Hall et al., 2017;

281 Zhao et al., 2016). In contrast, deletion of *clpP2* specifically impairs the microcolony-formation

282 stage of biofilm development in a flow-cell assay (Hall et al., 2017). The importance of PaClpP2

283 in biofilm assembly/maturation was also observed under static (non-flow) growth conditions. In

284 this assay, biofilms formed by Δ*clpP2* cells were thinner than those formed by wild-type PAO1 as

285 measured by confocal microscopy using GFP-labeled strains (Fig. 4). Thus, the *clpP2* deletion

286 changes the three-dimensional architecture of *P. aeruginosa* biofilms, perhaps through alteration

287 of the biofilm extracellular matrix, as regulation of alginate biosynthesis is under the control of

288 ClpP-dependent proteolysis in some strains (Qiu et al., 2008). In contrast to the inhibitory effect

289 of the *clpP2* mutation, deletion of *clpP1* slightly increased biofilm thickness, as reported

290 previously (Fig. 4B; Shanks et al., 2006).

291

292 We next tested whether the peptide cleavage activity of PaClpP2 (presumably within a

293 PaClpP1$_7$P2$_7$ heterocomplex) was important for the observed role of PaClpP2 in biofilm

294 growth/morphology. Rather than using a strain with a *clpP2* deletion, a *clpP2^{S98A}-flag* allele, which

295 encodes protein lacking the catalytic serine, was integrated at the *clpP2* locus and the biofilm

296 growth assay was repeated (Fig. 4). This *clpP2^{S98A}* strain exhibited a similar but more severe

297 biofilm-growth defect compared to the Δ*clpP2* strain, whereas the *clpP2-flag* control strain

298 developed biofilms of normal thickness (Fig. 4B). This result indicates that the biofilm defect

299 resulted from mutation of the catalytic serine residue in *clpP2*. We also confirmed through co-

300 immunoprecipitation that PaClpP1 interacted well with PaClpP2^{S98A}-FLAG *in vivo*, strongly

301 suggesting that expression, stability, and assembly into a heterocomplex occurred normally (Fig.

302 1C). These results lead us to propose that cleavage of a peptide or protein by the PaClpP2 active

303 site influences biofilm thickness.

304

305 *PaClpP2 has a distinct cleavage specificity that contributes to ClpP1$_7$P2$_7$ activity*

306 To characterize the peptidase activity of PaClpP1$_7$P2$_7$ and compare it to that of PaClpP1$_{14}$, the

307 purified enzymes were first assayed for activity against a panel of 18 short peptides each linked to

308 a C-terminal fluorogenic AMC (7-amino-4-methylcoumarin) leaving group. Cleavage between the

309 C-terminal amino acid and AMC results in a quantifiable fluorescent signal (Fig. 5A, left). ClpP

310 tetradecamers without an associated AAA+ unfoldase can hydrolyze only short peptides that

311 diffuse into the ClpP degradation chamber through the constricted axial pores (Maurizi et al.,

312 1994). Both PaClpP1$_{14}$ and PaClpP1$_7$P2$_7$ heterocomplexes had substantial and generally similar

313 cleavage activity against seven of the eighteen substrate peptides tested and exhibited weak

314 cleavage activity against the remaining peptides (Fig. 5B).

315

316 However, the activities against some peptides indicated that the two complexes had different

317 substrate preferences. Among the eight peptides robustly cleaved by the ClpP complexes, most

318 were cleaved at a faster rate by PaClpP1$_{14}$ than PaClpP1$_7$P2$_7$, but two of them, GGL-AMC and LLL-

319 AMC, were cleaved more rapidly by the heterocomplex (Fig. 5B, C). One of the weakly-cleaved

320 substrates, LEHD-AMC, was also preferentially cleaved by PaClpP1$_7$P2$_7$. To confirm that faster

321 GGL-AMC and LLL-AMC cleavage was mediated by PaClpP2$_7$ active sites, we purified and assayed

322 PaClp^{SA}P1$_7$P2$_7$, a variant with the active-site serine residues in PaClpP1$_7$ replaced by alanine.

323 PaClp^{SA}P1$_7$P2$_7$ degraded GGL-AMC and LLL-AMC well, confirming the PaClpP2$_7$ active sites

324 within the heterocomplex contribute substantially to the higher cleavage rates of these substrates

325 (Fig. 5C, D). Conversely, PaClp^{SA}P1$_7$P2$_7$ displayed poor activity against IETD, AAN, and LLE

326 and was nearly inactive against the AAA, DEVD, and IIW substrates. We note, however, that the

327 basal activities of both wild-type complexes were also low against IETD, LLE, and DEVD (Fig.

328 5B). These experiments demonstrate that the active sites of both PaClpP1 and PaClpP2 contribute

329 to the peptide-cleavage activity of PaClpP1$_7$P2$_7$ and that the PaClpP1 and PaClpP2 rings exhibit

330 differences in their preferred cleavage sites.

331

332 *PaClpP1$_{14}$ and PaClpP1$_7$P2$_7$ display distinct protease cleavage patterns*

333 We next sought to compare the cleavage specificities of the PaClpP isoforms during protein

334 degradation (Fig. 5A, right) by analyzing the activity of the following proteolytic complexes:

335 PaClpX•P1$_{14}$, PaClpX•P1$_7$P2$_7$, PaClpX•Clp^{SA}P1$_7$P2$_7$ and PaClpX•ClpP1$_7$^{SA}P2$_7$. We used LC-

336   MS/MS to identify more than 13,000 cleaved peptides from four different protein substrates for

337   each protease complex, allowing us to determine product peptide lengths and cleavage sequences.

338   Although all four complexes generated peptides that averaged 8-11 residues in length (Fig. S7),

339   the complexes that each contained only one active ClpP ring (PaClpX•Clp^{SA}P1$_7$P2$_7$ and

340   PaClpX•ClpP1$_7$^{SA}P2$_7$) generated a statistically significantly larger pool of longer peptides (from

341   12-28 residues) (P value > 0.05; Fig. S7B). This observation is consistent with a previous report

342   that inactivating the catalytic triads of EcClpP$_{14}$ through chemical modification leads to a shift in

343   the product-size distribution towards longer peptides (Thompson et al., 1994).

344

345   In Figures 5E-H, the preferred sequences surrounding the cleavage sites produced by each enzyme

346   variant are displayed as WebLogos (Crooks et al., 2004). Substrate residues surrounding the

347   cleavage site are labeled as positions P3-P2-P1-P1′-P2′-P3′, where the scissile bond is between

348   P1-P1′ (Schechter and Berger, 1967). For all four enzyme variants, the residue at P1 was most

349   conserved (~0.54 to ~0.61 bits). However, the identity of the most preferred amino acid(s) at this

350   position differed. For the enzyme variants that had only PaClpP1 active sites, Ala, Gly, and Leu

351   appear equally favored (Fig. 5E, F). In contrast, cleavage by the PaClpP2 active site revealed a

352   substantially stronger preference for Leu at the P1 site, with Phe being a less-preferred second

353   choice (Fig. 5G). Both Ala and Gly, preferred residues at P1 for the PaClpP1 active site, were also

354   notably less represented in sequences cleaved by PaClpP2 (Fig. 5G). The specificity for Leu at the

355   P1 position for PaClpP2 during protein degradation is consistent with our findings from peptide

356   degradation experiments, as GGL and LLL, which also contain Leu at the P1 position, was also

357   preferentially cleaved by enzymes carrying active PaClp2 subunits.

358

359   Additionally, the cleavage specificity of the PaClpP2 active site appears to be more stringent than

360   that of PaClpP1, as the residue at the P1 position dominated cleavage-site selection to a greater

361   extent for PaClpP2 than it did for PaClpP1. This is shown by the WebLogo for

362   PaClpX•Clp^{SA}P1$_7$P2$_7$ (Fig. 5G), with the P1 position contributing ~0.64 bits of sequence

363   information compared to only ~0.23 bits for each of the other positions (P3, P2, etc.). In contrast,

364   for PaClpX•ClpP1$_{14}$ and PaClpX•ClpP1$_7$^{SA}P2$_7$, which have only PaClpP1 active sites, other

365   positions contributed as much as 0.32 bits (Fig. 5E and 5F). Importantly, the cleavage signature of

366   PaClpP2 is evident in the WebLogo of PaClpX•ClpP1$_7$P2$_7$, as Leu is larger and above Gly and

367   Ala, which are preferred residues for PaClpP1. Thus, recognition/cleavage by PaClpP1$_7$P2$_7$
368   appears to reflect the preferences of both rings of the heterocomplex, thereby revealing that ClpP2
369   changes the overall specificity of the PaClpP1P2 heterocomplex. In depth analysis of the four
370   WebLogos revealed additional differences between PaClpP1$_{14}$ and PaClpP1$_7$P2$_7$ at positions P2
371   and P1′, which are highlighted in Fig. S8.

372

373   Our experiments probing peptide-cleavage specificity and biofilm formation lead to two related
374   observations. First, PaClpP1$_{14}$ and PaClpP1$_7$P2$_7$ have distinct (although partially overlapping)
375   cleavage preferences. Second, there is a unique requirement for functional ClpP2 actives site for
376   proper biofilm development. These results support a model in which preferential cleavage of a
377   peptide or protein by ClpP2 within the heterocomplex promotes properly structured and clinically
378   deleterious biofilm development during late stages of *P. aeruginosa* growth.

379

380   **Discussion**

381   ***P. aeruginosa makes two distinct functional ClpP peptidases during development.***

382   The opportunistic pathogen *P. aeruginosa* encodes and expresses two distinct homologs of ClpP
383   peptidase subunits (Hall et al., 2017). We previously determined that PaClpP1 is expressed
384   throughout different growth phases, assembles into functional tetradecamers, is an active
385   peptidase, and forms a critical part of functional proteases when partnered with the PaClpX or
386   PaClpA AAA+ unfoldase (Hall et al., 2017). In this study, we identify PaClpP2 as an active
387   component of a functionally specialized proteolytic complex. By purifying and characterizing
388   PaClpP2-containing complexes from native *P. aeruginosa* cultures and from *E. coli* expression
389   strains that co-overproduce *clpP1* and *clpP2*, we identified the active form of PaClpP2 as part of
390   a PaClpP1$_7$ClpP2$_7$ enzyme and found that this atypical ClpP homolog has distinct activities from
391   those of ClpP1. Based on these observations, as well as expression studies showing that *clpP2* (but
392   not *clpP1*) is induced approximately 10-fold during stationary phase when biofilms develop
393   (Waite et al., 2006), we conclude that *P. aeruginosa* makes two distinct forms of the ClpP
394   peptidase, PaClpP1$_{14}$ and PaClpP1$_7$ClpP2$_7$, during its life cycle, and these different peptidases play
395   substantial roles in determining developmental outcomes during bacterial growth.

396

397   ***PaClpP2 has distinct structural features and is related to L. monocytogenes ClpP1***.

398   PaClpP1 is a canonical member of the ClpP-peptidase family with high sequence identity and very

399   similar biochemical properties to *E. coli* ClpP (Hall et al., 2017). PaClpP2 is more divergent in

400   sequence and is on a distinct branch in phylogenetic trees, where it clusters with ClpP2s from close

401   relatives of *P. aeruginosa* and with ClpP1 from the firmicute *Listeria monocytogenes* (Fig. S9).

402   This observation suggests that PaClpP2 and LmClpP1 were likely acquired via horizontal gene

403   transfer from a common ancestor. Indeed, PaClpP2 shares 50% identity with LmClpP1, 42%

404   identity with *E. coli* ClpP, and only 40% identity with PaClpP1 (Fig. S5). *L. monocytogenes*, like

405   *P. aeruginosa*, makes two types of functional ClpP enzymes: a canonical ClpP tetradecamer

406   (LmClpP2$_{14}$) and a LmClpP1$_7$P2$_7$ heterocomplex (Dahmen et al., 2015).

407

408   Our structural and biochemical experiments demonstrate that the active form of PaClpP2 is a

409   heptamer that stacks with a heptamer of PaClpP1 to form the PaClpP1$_7$P2$_7$ tetradecamer. An

410   interesting feature of the ClpP2$_7$ side of this complex is that it does not interact stably with AAA+

411   unfoldases such as PaClpX or PaClpA. The basis for this lack of interaction is suggested by our

412   crystal structure of PaClpP2$_7$, in which both the N- and C-terminal regions of each subunit adopt

413   conformations that are predicted to be incompatible with ClpX or ClpA binding. Like

414   PaClpP1$_7$P2$_7$, LmClpP1$_7$P2$_7$ binds asymmetrically to ClpX, with LmClpX binding to the

415   LmClpP2$_7$ side (Dahmen et al., 2015), suggesting that failure to bind AAA+ unfoldases may be a

416   property of this alternative ClpP subclass.

417

418   ***Models for the roles of two ClpP forms: cleavage specificity as a regulatory function?***

419   Our new insights into *P. aeruginosa* ClpP2, together with previous studies, illuminate possible

420   models for the biological roles of the two distinct ClpP peptidases during bacterial growth and

421   development (Fig. 6). ClpP1$_{14}$ is a canonical family member: the *clpP1* gene is in an operon with

422   *clpX* and is constitutively expressed throughout exponential and stationary phase growth. As an

423   enzyme, PaClpP1$_{14}$ is an active peptidase with broad cleavage specificity and interacts with both

424   PaClpX and PaClpA to form active proteases. These proteases can be singly- or doubly-capped by

425   an unfoldase, and ClpP$_{14}$ is twice as active when doubly-capped. PaClpP1$_{14}$ is thus a typical

426   component of ClpXP and ClpAP enzymes that function in growing bacteria to identify and degrade

427   protein-quality-control substrates, such as ssrA-tagged failed translation products, transcription

428   factors, and other regulatory/metabolic proteins. For example, PaClpA, PaClpP1, and the ClpA-

429   adaptor protein (ClpS) are reported to control levels of the transcription factor CdpR, which helps

430   to regulate production of pyocyanin, biofilm development, and pathogenicity in a mouse model

431   for infection (Zhao et al., 2016). Interestingly, *clpP1*-defective strains show increased pyocyanin

432   production and altered biofilm structure (thicker and fluffier), suggesting that loss of ClpP1

433   activity causes changes in early regulatory pathways that contribute to pathogenesis (Hall et al.,

434   2017; this study).

435

436   The *clpP2* gene and the PaClpP1$_7$P2$_7$ enzyme exhibit several distinctive features compared to

437   PaClpP1. Expression of *clpP2* is activated by LasR, a quorum-sensing transcription factor (Gilbert

438   et al., 2009). As a consequence, PaClpP2 is undetectable during exponential growth but becomes

439   abundant in stationary phase and biofilms. Furthermore, instead of clustering on the genome with

440   other protein quality control enzymes like *clpP1*, *clpP2* maps to a gene cluster called *aze* that

441   appears to be a mobile genetic island. Genes at this locus are also controlled by quorum sensing

442   and have recently been shown to synthesize a modified nonribosomal peptide called

443   azetidomonamide B or azabicyclene (Hong et al., 2019; Patteson et al., 2019). Genes homologous

444   to *clpP2* and *aze* are clustered in several bacterial genomes, which suggests a potential functional

445   relationship that remains to be explored.

446

447   At the level of enzyme function, two differences between PaClpP1$_7$P2$_7$ and PaClpP1$_{14}$ stand out.

448   First, PaClpP2 cannot form an active protease on its own and has evolved structural features that

449   prevent efficient AAA+ unfoldase interaction. Thus, to function as a protease, PaClpP2 must co-

450   assemble with PaClpP1 to form a PaClpP1$_7$P2$_7$ heterocomplex. Degradation studies reveal that

451   only the ClpP1 ring of this complex can bind ClpX, but that both the PaClpP1 and PaClpP2

452   peptidase active sites participate in protein degradation. Second, PaClpP1 and PaClpP2 have

453   distinct amino-acid signatures in their preferred polypeptide cleavage sites. For example, we found

454   that PaClpP1$_7$P2$_7$ preferentially cleaved substrates with Leu at the P1 position, whereas ClpP1$_{14}$

455   favored Ala and Gly (Fig. 5). The differential cleavage specificities of PaClpP1 and PaClpP2 at

456   the P1 position likely reflect the structural properties of the PaClpP1 and PaClpP2 S1 binding

457   pockets (Hedstrom, 2002; Szyk and Maurizi, 2006). Indeed, the S1 binding pocket of PaClpP2

458   contains a Phe residue (position 154) that could aid in better hydrophobic packing of substrate

459   side-chains during peptide positioning and catalysis (Fig. S10).

460

461 These results indicate that PaClpP2 in complex with PaClpP1 imparts an altered substrate-

462 processing activity, both to the $PaClpP1_7P2_7$ tetradecameric peptidase and to the associated AAA+

463 protease complex. Our *in vitro* results demonstrate that ClpP2 modulates both ATP-dependent and

464 ATP-independent processing of substrates by $PaClpP1_{14}$, whereas the experiments *in vivo*

465 demonstrate that growth of a normally-structured thick biofilm is dependent on PaClpP2

466 enzymatic activity. Taken together, these results support models that involve $PaClpP1_7P2_7$

467 specifically producing a cleavage product that has a downstream function in enhancing biofilm

468 formation (Fig. 6B).

469

470 *Multiple peptidase isoforms*

471 While genomes encoding multiple alternative peptidase subunits for AAA+ proteases are

472 continuing to be discovered, the immunoproteasome of jawed vertebrates is one of the best-

473 characterized proteases containing alternative peptide isoforms and reveals a biologically

474 important and developmentally regulated example of cells inducing expression of alternative

475 peptidase subunits to help deal with cellular stress. In this case, in response to a challenge such as

476 viral infection, cells induce expression of three alternative β subunits for the 20S peptidase particle.

477 The difference in cleavage preference, especially of the alternative β1 subunit, results in the

478 generation of a pool of peptides that is biased away from cleavage after acidic residues and

479 enhanced in production of peptides with N-terminal hydrophobic residues. This latter family of

480 peptides is better suited to be displayed by the peptide-binding receptors of the immune system.

481 This example, together with our observations indicating a distinct cleavage specificity for

482 PaClpP2, emphasizes that the peptidase subunits of AAA+ proteases, not just the ATPase

483 regulatory particles, can play a key and perhaps widespread role in determining the biological

484 outcomes of protein/peptide degradation.

485

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495

496   **Author Contributions**

497   Individual authors have contributed as follows: (i) GDM, BMH, RTS, & TAB, the conception or design

498   of the study, (ii) GDM, BMH, GC-O, RAG, JJZ, JRK, KR, RTS, & TAB, the acquisition, analysis, or

499   interpretation of the data; and (iii) GDM & TAB, major contribution to writing of the manuscript; (iv)

500   GDM, RTS, JRK, JJZ, & TAB, critical revisions to the manuscript.

501

502   **Experimental Procedures**

503   **Strains and plasmids**

504

505   PCR was carried out on *Pseudomonas aeruginosa* PAO1 genomic DNA provided by the American

506   Type Culture Collection (ATCC). Plasmids were selected using 100 μg mL^{-1} ampicillin, 50 μg

507   mL^{-1} kanamycin, 10 μg mL^{-1} gentamicin (*E. coli*) or 30 μg/ml gentamicin (*P. aeruginosa)* as

508   appropriate. Deletion or allelic-exchange strains of *Pseudomonas aeruginosa* PAO1 were made

509   with homologous recombination as described using vector pMQ30, yeast strain *S. cerevisiae*

510   InvSc1 and *E. coli* mating strain ST18 (Hmelo et al., 2016). Briefly, pMQ30 deletion constructs

511   were obtained by co-transforming cut pMQ30 plasmid and PCR-amplified regions flanking *P.*

512   *aeruginosa* genes of interest into *S. cerevisiae* strain InvSc1. Reconstituted deletion plasmids were

513   isolated, purified at greater concentration and transformed into *E. coli* strain ST18 for mating with

514   *P. aeruginosa* PAO1. Genetic knock outs and allelic-exchange strains were confirmed with colony

515   PCR. PaClpP1-His$_6$ and PaClpP2-StrepII were expressed in pET23b (Novagen). PaClpP1-His$_6$

516   and PaClpP2-StrepII were overexpressed from pETDuet1 vector (Novagen). PaClpX was

517   expressed with an N-terminal thrombin-cleavable poly-histidine tag. Tagmaster Site-Directed

518   Mutagenesis (GM Biosciences) was used to introduce point mutations. Generation of *clpP2-FLAG*

519   (WT and S98A) variant alleles were constructed by homologous recombination as described above

520   using the following primer sequences (5´-3´): CGCTATTACGCCAGCTGGCGAAAGGGGGA-

521   TGTGCTGCAAGCTGCATGGCTATGCTTCAC (GM13_For) and TCGGTGTGATGAA-

522 GCGGATC (GM14_Rev) for amplifying the 5′ upstream region of *clpP2* with 40 base pair

523 homology to pMQ30, TATGAAAACCGATGACAAGGACC (GM15_For) and TTACTTGT-

524 CGTCGTCGTCCTTATAG (GM16_Rev) for amplifying *clpP2*-FLAG from a separate plasmid,

525 GCTACCTGGTCAGGACTATAAGGACGACGACGACAAGTAAGCCGGATAAATACGAA

526 GGC (GM17_For) and GAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGCCA-

527 GGGATTCCTACGAACC (GM19_Rev) for amplifying the 3′ downstream region of *clpP2*, with

528 homology to FLAG and pMQ30.

529

530 **Protein expression and purification**

531 For protein induction, PaClpP1, PaClpP2 and PaClpP1ClpP2 variants were expressed in *E. coli*

532 strain JK10 (*clpP*-) grown at 37°C in Luria-Bertani (LB) broth supplemented with antibiotics. At

533 OD_{600} of 0.6, temperature was reduced to either 30ºC (for PaClpP1-His$_6$ and PaClpP2-StrepII) or

534 20ºC (for coexpression of PaClpP1-His$_6$ and PaClpP2-StrepII), isopropyl β-D-1-

535 thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM in 1 L cultures and cells

536 were harvested after 3 h (PaClpP1-His$_6$ and PaClpP2-StrepII) or overnight (coexpression of

537 PaClpP1-His$_6$ and PaClpP2-StrepII). For purification of PaClpP1-His$_6$/P2$_7$-StrepII, cell pellets

538 were resuspended in Buffer S (50 mM sodium phosphate pH 8.0, 100 mM NaCl, 10% glycerol, 5

539 mM imidazole), lysed with French press, and cell lysate was mixed with 1% w/v CHAPS detergent

540 for 30 min at 4ºC. Lysate was then centrifuged for 20 min at 30,000 RCF, and the supernatant was

541 applied to Ni-NTA resin equilibrated in Buffer S, washed extensively with Buffer S plus 20 mM

542 imidazole, and protein was eluted with Buffer S plus 300 mM imidazole. The eluate was diluted

543 1:3 into Buffer W (100 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol) and applied to Strep-

544 Tactin resin (IBA) equilibrated in Buffer W, and washed extensively with Buffer W. Protein was

545 eluted with 2.5 mM Desthiobiotin (IBA) in Buffer W and concentrated by ultracentrifugation with

546 a 30 kDa centrifugal filter (Amicon) to ~500 µL. This material was further purified using a

547 Superdex200 16/600 HR gel-filtration column (GE Healthcare) equilibrated in Buffer X (25 mM

548 Tris (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, 10% glycerol). Fractions containing

549 protein at >95% purity as judged by SDS-PAGE were combined, concentrated, flash frozen in

550 aliquots, and stored at –80ºC. For purification of PaClpP1-His$_6$ or PaClpP2$_7$-StrepII variants, the

551 same experimental procedure as above was followed, except only Ni-NTA resin was used for

552 PaClpP1-His$_6$ and only Strep-Tactin resin was used for PaClpP2-StrepII, followed by size-

553   exclusion chromatography (see above). *P. aeruginosa* ClpA was overexpressed and purified with

554   Ni-NTA, as described for PaClpP1-His. Protein was then incubated for 2 h at 30°C with His-tagged

555   Ulp1 to cleave between the SUMO domain and ClpA, eluted from Ni-NTA resin, dialyzed into

556   buffer Q50 (50 mM Tris (pH 8.0), 1 mM DTT, 10 mM $MgCl_2$, 10% glycerol, 50 mM KCl), and

557   purified on a Mono Q column (GE Healthcare). Selected fractions were further purified by size

558   exclusion chromatography in Buffer Q200 (50 mM Tris (pH 8.0), 1mM DTT, 10 mM $MgCl_2$, 10%

559   glycerol, 200 mM KCl). GFP with a C-terminal ssrA tag (*E. coli*) was expressed and purified as

560   described (Dougan et al., 2002). *Note:* The C-terminal ssrA tag on GFP is the *E. coli* sequence (-

561   AANDENYALAA). The *P. aeruginosa* ssrA sequence is very similar (-AANDDNYALAA).

562   **Enzymatic assays and crosslinking**

563

564   Protein and peptide degradation assays were carried out in 25 mM HEPES-KOH (pH 7.6), 5 mM

565   KCl, 5 mM $MgCl_2$, 0.032% NP-40, and 10% glycerol (PD buffer) at 30°C. Peptidase assays used

566   2.85% dimethyl sulfoxide (DMSO), 100 µM fluorogenic peptide (Bachem, Peptides International,

567   or Enzo Life Sciences), and 0.4 µM ClpP tetradecamer ($PaClpP_{14}$), and cleavage was monitored

568   by fluorescence with excitation/emission 380/460 nm at 30ºC. Degradation of GFP-ssrA for

569   Michaelis-Menten analysis was monitored as described using 0.54 µM $PaClpP_{14}$, 0.1 µM $ClpX_6$,

570   and an NADH-coupled ATP regeneration system (Nørby 1988; Oliveras et al., 2014). Enzymatic

571   assays were monitored on a SpectraMax M5 microplate reader (Molecular Devices) in a black

572   384-well flat-bottom plate (Corning). For crosslinking of PaClpP complexes, 0.4 µM $PaClpP_{14}$

573   was mixed with 0.125% glutaraldehyde at room temperature, and reactions were stopped by flash-

574   freezing after indicated time points and analyzed by SDS-PAGE. Casein fluorescein

575   isothiocyanate (FITC-casein) was purchased from Sigma-Aldrich.

576

577   **Western blotting and co-immunoprecipitation**

578   PAO1 or *clpP2-FLAG* cultures for co-immunoprecipitation were grown overnight at 37ºC shaking

579   in LB. The next day, cells from 1 mL of culture were harvested by centrifugation (10 mins at

580   2,350xg), and cell pellets were lysed using B-PER bacterial protein extraction reagent

581   (ThermoFisher). For co-immunoprecipitation of ClpP2-FLAG from *P. aeruginosa* strains, anti-

582   FLAG M2 magnetic beads (Sigma-Aldrich) were incubated with cell lysate containing either

583   *clpP2-1xFLAG* (+) or untagged *clpP2* (-) alleles at the genomic loci, and eluted with excess

584   3xFLAG peptide (Sigma-Aldrich). The eluate was separated by SDS-PAGE and proteins were

585   stained with SYPRO orange (Sigma-Aldrich). The eluate was analyzed by Western blot for

586   PaClpP1 (1:15,000 anti-PaClp1) and PaClpP2 (1:7,500 anti-PaClpP2) as described (Hall et al.,

587   2017). Secondary antibody was anti-rabbit IgG-alkaline phosphatase (AP) conjugate (Bio-Rad)

588   used at a 1:10,000 dilution. Blots were incubated with AP ECF substrate (GE Healthcare) and

589   imaged with a Typhoon FLA 9500 scanner (GE Healthcare).

590

591   **Crystallography and negative-stain electron microscopy**

592   For crystallization of PaClpP1, hanging drops with 0.5 µL of PaClpP1P2 protein at 6.6

593   mg/ml in storage buffer (0.05 M Tris HCl (pH 7.5), 0.05 M NaCl, 5 mM EDTA, 25 mM DTT, 5%

594   glycerol) were mixed with 0.5 µL well solution (0.1 M sodium malonate, 53.5% MPD) over a

595   reservoir of 500 mL well solution at 20°C. For crystallization of PaClpP2, hanging drops with

596   1.5 µL of 32.6 mg/ml PaClpP2-StrepII in storage buffer (100 mM Tris (pH 8.0), 150 mM NaCl,

597   10% glycerol) and 1.5 µL of precipitant (1.8 M sodium phosphate monobasic monohydrate,

598   potassium phosphate dibasic, pH 6.9) were incubated over a reservoir of 500 ml precipitant at

599   20°C. Crystals were soaked in precipitant plus 10% ethylene glycol and frozen in liquid N_2. X-ray

600   diffraction data were collected at the Advanced Photon Source (APS) beamline 24-ID-E with X-

601   ray wavelength 0.9792 Å. For the PaClpP2 crystal, diffraction images were indexed, integrated

602   and scaled using HKL2000 (Otwinoski and Minor, 1997). The structure was solved by molecular

603   replacement with Phaser using a portion of a *S. aureus* ClpP monomer (PDB 3QWD) as the search

604   model (McCoy et al., 2007). The asymmetric unit in the MR solution consisted of seven monomers

605   in a heptameric ring. The PaClpP2 amino-acid sequence was inserted with Autobuild in Phenix

606   (Adams et al., 2010), and the resulting model was refined using Coot (Emsley et al., 2010) and

607   Phenix. For the PaClpP1 structure, diffraction data were processed using XDS (Kabsch et al.,

608   2010) and solved by molecular replacement, using a heptameric ring derived from the *E. coli* ClpP

609   structure (PDB 1YG6) as a search model. The model was refined by iterative rebuilding in Coot

610   and refinement using Phenix. Coordinates for both structures were deposited in the Protein Data

611   Bank (5BQV and 5BQW). For electron microscopy, 0.5 µM $PaClpX_6$ and 0.2 µM $PaClpP_{14}$ were

612   diluted in 25 mM Hepes (pH 7.6), 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM TCEP, 2 mM ATPγS to

613   achieve 18 ng/µL total protein. 5 µL of this mixture was spotted per copper grid (Electron

614 Microscopy Sciences) and stain with 2% uranyl acetate. Images were obtained using a FEI Technai

615 Spirit Transmission Electron microscope.

616

617 **Mass spectrometry and data analysis**

618 Protein digestion samples were resuspended to 1 mM in 2% formic acid. 1 μL was analyzed for

619 each sample. The sample was loaded with an autosampler directly onto the column, which was

620 made from a 50 μm ID 360 μm OD capillary (PicoFrit, New Objectives, Woburn, MA) filled with

621 25 cm of 1.9 μm Reprosil-Pur C18 AQ. Peptides were eluted at 200 nL min^{-1} from the column

622 using an EasyNanoLC (Thermo Scientific, San Jose, CA) with a gradient of 5% buffer B (80%

623 acetonitrile, 0.1% formic acid) over 3 min, followed by 5% buffer B to 25% buffer B over 20 min.

624 The gradient was then switched from 25% to 100% buffer B over 5 min and held constant for 3

625 min. Finally, the gradient was changed from 100% buffer B to 100% buffer A (100% water, 0.1%

626 formic acid) over 0.5 min and then held constant for 13.5 min. The application of a 2.0 kV distal

627 voltage electrosprayed the eluting peptides directly into the Thermo Orbitrap QEHFX mass

628 spectrometer equipped with a Flex Source (Thermo Scientific). Mass spectrometer-scanning

629 functions and HPLC gradients were controlled by the Xcalibur data system (Thermo Scientific).

630 The mass spectrometer was set to scan MS1 at 60,000 resolution with an AGC target set at 3e6.

631 The scan range was m z^{-1} 375-1500. For MS2, spectra were acquired with an AGC target of 1e6

632 and a maximum IT at 200ms at 30,000 resolution. The top 5 peaks were analyzed by MS2. Peptides

633 were isolated with an isolation window of m z^{-1} 1.2 and fragmented at 28 CE. Only ions with a

634 charge state of 2 and 3 were considered for MS2. Dynamic exclusion was set at 10 sec. Mass

635 spectrometry data was analyzed with ProteomeDiscoverer 2.3 and searched against a database

636 comprising of the four protein sequences (FNR_ECOLI, GFP_ssrA, RARC_BPP22,

637 HTitin_HUMAN) added to an *E. coli* database (released May 14, 2019) containing common

638 contaminant proteins. No enzyme was specified, and oxidized methionine and N-terminal

639 acetylation were allowed as protein modifications. Only high confidence peptides from the PSM

640 table were analyzed further. Peptides not from the four proteins analyzed were eliminated.

641

642 **Biofilm assays**

643 *P. aeruginosa* strains were fluorescently tagged at an intergenic neutral chromosomal locus

644 downstream of the *glmS* gene with *egfp* in mini-Tn*7* constructs as described (Lambertsen et al.,

645   2004; Choi et al., 2006). Overnight culture of GFP-labeled strains were prepared in LB broth with

646   shaking at 37°C. Overnight cultures were diluted in ABTGC medium (Chua et al., 2015) to an

647   initial OD_{600} of 0.01, added to a glass-bottom 96-well plate, and incubated for 48 h at 37°C. Image

648   acquisition was performed using a confocal laser scanning microscope (LSM 800; Zeiss) equipped

649   with a 63×/1.4 NA oil immersion or a 100×/1.4 NA oil immersion objective. Images were analyzed

650   with Zeiss ZEN 2.1 imaging software (Thornwood, NY, USA). The excitation wavelength for GFP

651   was 488 nm. At least five stacks were recorded for each well and at least three independent wells

652   were analyzed for each condition. Images were acquired with a step size of 0.5 µm. Biofilm

653   quantification was performed using COMSTAT (Heydorn et al., 2000).

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863 **Figure Legends**

864

865 **Figure 1: PaClpP1 and PaClpP2 co-immunoprecipitate from *P. aeruginosa* and *E. coli*.**

866 A) Schematic of ATP-dependent proteolysis by $PaClpXP1_{14}$. $PaClpX_6$ is shown in purple, PaClpP1 is
867 shown in blue.

868 B) Schematic of ATP-dependent proteolysis by $PaClpP1_7P2_7$. Color scheme is the same as in A), except
869 PaClpP2 is shown in cyan.

870 C) ClpP2-FLAG was isolated from cell lysates of PAO1 containing either *clpP2-FLAG* (lane C), untagged
871 *clpP2* (lane A), or *clpP2^{S98A}-FLAG* alleles using anti-FLAG antibody-conjugated beads and elution
872 with excess FLAG peptide (Sigma-Aldrich). Eluate was separated by SDS-PAGE and proteins were
873 stained with SYPRO orange (lanes E-F). Molecular weight standards are shown in lane A. Molecular
874 weights of monomers: ClpP2-FLAG, 23,237 Da; ClpP1, 21,879 Da.

875 D) The eluate was analyzed by Western blot for PaClpP1 (1:15,000 anti-PaClp1) and PaClpP2 (1:7,500
876 anti-PaClpP2). Secondary antibody: goat anti-rabbit (1:10,000).

877 E) Gel filtration profile of $PaClpP1_7P2_7$. Peak fractions corresponding to tetradecamers (58-64 mL, as
878 indicated by dotted lines) were pooled and concentrated for biochemical analysis.

879 F) ClpP1 and ClpP2 co-associate during purification. SDS-PAGE of elution fractions. Lanes 1-10
880 correspond to samples taken from 1 mL fractions eluting from 56-66 mL. Elution fractions
881 corresponding to lanes 3-9 were pooled and concentrated for further characterization. Molecular
882 weights of monomers: ClpP1-His$_6$, 23,063 Da; ClpP2-StrepII, 23,326 Da.

883

884 **Figure 2: Crystal structures of $PaClpP1_{14}$ and $PaClpP2_7$ complexes.**

885 A) Side and top-down views of homomeric PaClpP1 (PDB code: 5BQV, blue, with a single monomer in
886 purple).

887 B) Side and top-down views of homomeric PaClpP2 (PDB code: 5BQW, deep teal, with a single monomer
888 in yellow).

889 C) Overlay of PaClpP1 (blue) and PaClpP2 (cyan) monomers with insets showing select structural
890 differences.

891 D) N-termini of PaClpP1 and PaClpP2. The first 13 residues of PaClpP2 and the first four residues of
892 PaClpP1 are missing electron density.

893 E) The C-terminus of PaClpP2 adopts a single helical turn, while the C-terminus of PaClpP1 is
894 unstructured. The side chains of the amino acids making up the docking site for AAA+ unfoldases
895 (hydrophobic binding pocket) are represented as black sticks.

896   F) Active sites. The active site residues of PaClpP1 are properly aligned (distances in angstroms are

897   labeled on figure), while the residues of ClpP2 are not (the distances from PaClpP2 S98 to H123 and

898   H123 to D172 are 6.5Å and 10.1Å, respectively). Residue numbers in blue refer to the mature amino

899   acid sequence of PaClpP1, and residue numbers in cyan refer to the amino acid sequence of PaClpP2.

900

901 **Figure 3: Both PaClpP1$_7$ and PaClpP1$_7$P2$_7$ form active proteases.**

902   A) Degradation of GFP-ssrA by PaClpX$_6$ (0.1 µM) and PaClpP1$_{14}$ (filled circles) or PaClpP1$_7$P2$_7$ (open

903   triangles) (0.54 µM). Curves are fit to the Michaelis-Menten equation. Solid line refers to

904   PaClpX•ClpP1$_{14}$ (K$_M$ = 4.2 ± 0.5 µM, V$_{max}$ = 1.6 ± 0.08 min^{-1} ClpX$_6^{-1}$) and dotted line refers to

905   PaClpX•ClpP1$_7$P2$_7$ (K$_M$ = 4.1 ± 0.52 µM, V$_{max}$ = 1.7 ± 0.09 min^{-1} ClpX$_6^{-1}$).

906   B) Degradation of 50 µM FITC-casein (Casein fluorescein isothiocyanate) by 0.4 µM PaClpP1$_{14}$ or

907   PaClpP1$_7$P2$_7$ and 0.2 µM PaClpA$_6$. For both (A) and (B), data points are averages of three independent

908   replicates performed in duplicate ± SD.

909   C) Negative-stain microscopy images of PaClpX$_6$ (0.5 µM) with PaClpP1$_{14}$ or PaClpP1$_7$P2$_7$ (0.2 µM).

910   Approximately half of the PaClpX•P1$_{14}$ complexes were doubly-capped by PaClpX (left; 46% doubly-

911   capped, 54% singly-capped), whereas most PaClpX•ClpP1$_7$P2$_7$ complexes were singly-capped (right;

912   98% singly-capped). Representative doubly- and singly-capped complexes are circled with dotted and

913   solid lines, respectively.

914   D) Efficient degradation of GFP-ssrA (8 µM) requires productive interaction of PaClpX$_6$ (0.5 µM) with

915   the PaClpP1$_7$ ring of PaClpP1$_7$P2$_7$. Wild-type PaClpP1$_{14}$ or PaClpP1$_7$P2$_7$ (2 µM) with PaClpX degrades

916   GFP-ssrA, whereas the PaClpX-binding-defective variant of PaClpP1$_{14}$ (R194K) was not able to

917   support degradation of GFP-ssrA in a heterocomplex with PaClpP2$_7$ (PaClp^{RK}P1$_7$P2$_7$). Data points are

918   averages of three independent replicates performed in triplicate ± SD.

919   E) Violin plots of GFP-ssrA degradation by PaClpX•ClpP1$_{14}$ and PaClpX•ClpP1$_7$P2$_7$. The rate of GFP-

920   ssrA (8 µM) degradation is dependent upon the amount of PaClpP$_7$-bound PaClpX$_6$. 2:1 complex

921   formation was favored under conditions containing 1.0 µM PaClpX$_6$ and 0.5 µM of PaClpP1$_{14}$, while

922   1:1 stoichiometries were favored under conditions containing 1.0 µM PaClpX$_6$ and 1.0 µM PaClpP1$_{14}$,

923   0.5 µM PaClpP1$_7$P2$_7$, or 1.0 µM PaClpP1$_7$P2$_7$.

924

925 **Figure 4: The active site of PaClpP2 is essential for robust biofilm development in *P. aeruginosa***

926 **PAO1.**

927   A) Representative side-view confocal images of GFP-labeled PAO1 biofilms grown in 96-well glass-

928   bottom plates under static conditions for 48 hours. Strains are either wild type, Δ*clpP1*, Δ*clpP2*, or

929   contain a FLAG-tagged catalytic-null allele of *clpP2* in lieu of the wild-type gene (*clpP2^{S98A}-flag*).

930 B-C) Quantifications are averages of three independent wells, with five z-stacks taken per well. Error bars

931   are ± SEM. Biofilm quantification was performed using COMSTAT software (Heydorn et al., 2000).

932   * p < 0.0001 as analyzed by one-way ANOVA.

933

934 **Figure 5: The PaClpP1$_7$ and PaClpP2$_7$ active sites of PaClpP1$_7$P2$_7$ contribute uniquely to peptide**

935 **cleavage.**

936 A) Schematic of assays used for the determination of cleavage-site specificity by PaClpP$_{14}$ complexes.

937 B) Cleavage of a panel of 18 fluorescent peptide substrates (100 µM) by PaClpP1$_{14}$ or PaClpP1$_7$P2$_7$ (0.4

938   µM) at 30ºC. Substrates are ordered by the properties of the amino acid at position P1´ (from left to

939   right: small to large, and non-polar to charged). Data points are averages of three independent replicates

940   performed in duplicate ± SD.

941 C) The PaClpP2$_7$ active sites contribute to peptide hydrolysis. PaClp^{SA}P1$_7$P2$_7$ is a partially-inactive variant

942   of PaClpP1$_7$P2$_7$ in which the active serine of PaClpP1 is mutated to alanine. Peptide substrates are

943   ordered as in (B). Peptidase activity is normalized to PaClpP1$_{14}$.

944 D) Percent contribution of PaClpP2$_7$ activity to total peptidase activity in PaClpP1$_7$P2$_7$. Relative peptidase

945   activities of PaClp^{SA}P1$_7$P2$_7$ for each substrate were divided by the relative peptidase activities of the

946   wild-type heterocomplex for that substrate. Substrates are ranked from left to right based on decreasing

947   sensitivity to PaClpP2$_7$ cleavage.

948 E-H) More than 13,000 proteolytic products spanning four substrates (GFP-ssrA, FNR-ssrA, arc-GCN4p1-

949 st11-ssrA, and Halo-(V15P titin^{I27})$_4$-ssrA) were sequenced by mass spectrometry and compiled to create

950 WebLogos. (E) Motif generated from PaClpX•ClpP1$_{14}$ complexes. (F) Motif generated by

951 PaClpX•ClpP1$_7^{SA}$P2$_7$ complexes. (G) Motif generated by PaClpX•Clp^{SA}P1$_7$P2$_7$ complexes. (H) Motif

952 generated by PaClpX•ClpP1$_7$P2$_7$ complexes.

953

954 **Figure 6: Summary and models for PaClpP1 and PaClpP2 assemblies and functions.**

955 A) Graphical summary of the oligomeric states and biochemical activities of PaClpP1$_{14}$, PaClpP2$_7$, and

956   PaClpP1$_7$P2$_7$. Whereas PaClpP2$_7$ is an inactive heptamer, PaClpP1$_{14}$ and PaClpP1$_7$P2$_7$ are active

957   tetradecamers that can interact with PaClpX and PaClpA to degrade model substrates. PaClpP1$_7$P2$_7$

958   binds asymmetrically to PaClpX (right panel), whereas PaClpP1$_{14}$ can bind two equivalents of PaClpX$_6$

959   (i.e., can be doubly-capped). PaClpP1$_7$P2$_7$ promotes biofilm thickness, whereas PaClpP1 impacts

960   motility, biofilm formation, and phenazine production *in vivo* ([1]Hall et al, 2017; [2]Zhao et al., 2016;

961   [3]This study, and Shanks et al., 2006).

962 B) Models for *in vivo* functions of PaClpP1$_7$P2$_7$. The left panel shows an ATPase-independent model for

963   PaClpP1$_7$P2$_7$ activity, in which a small propeptide can diffuse into the axial pore of the heterocomplex

964   and be cleaved by PaClpP1$_7$P2$_7$ to produce an active peptide product. The right panel depicts an

965   ATPase-dependent function of PaClpP1$_7$P2$_7$, where a biologically important proteolytic product is

966   generated by unfoldase-coupled PaClpP1$_7$P2$_7$. Both (A) and (B) represent models in which a currently

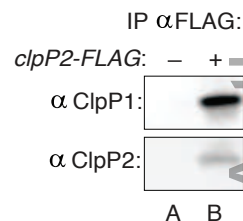967   unknown target with a functional role in biofilm architecture is produced via cleavage by PaClpP1$_7$P2$_7$.
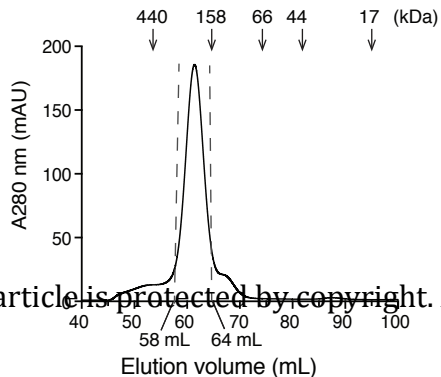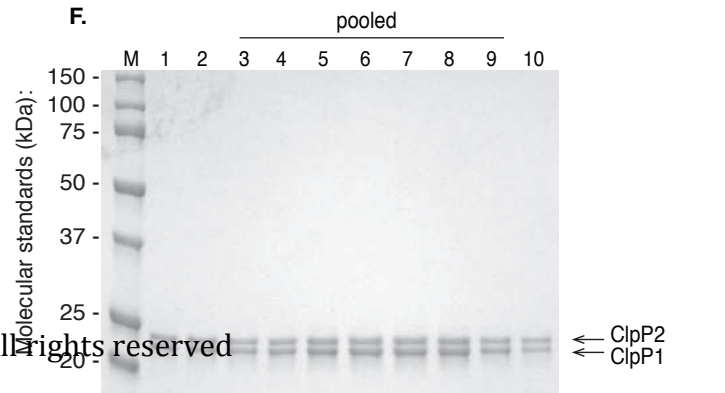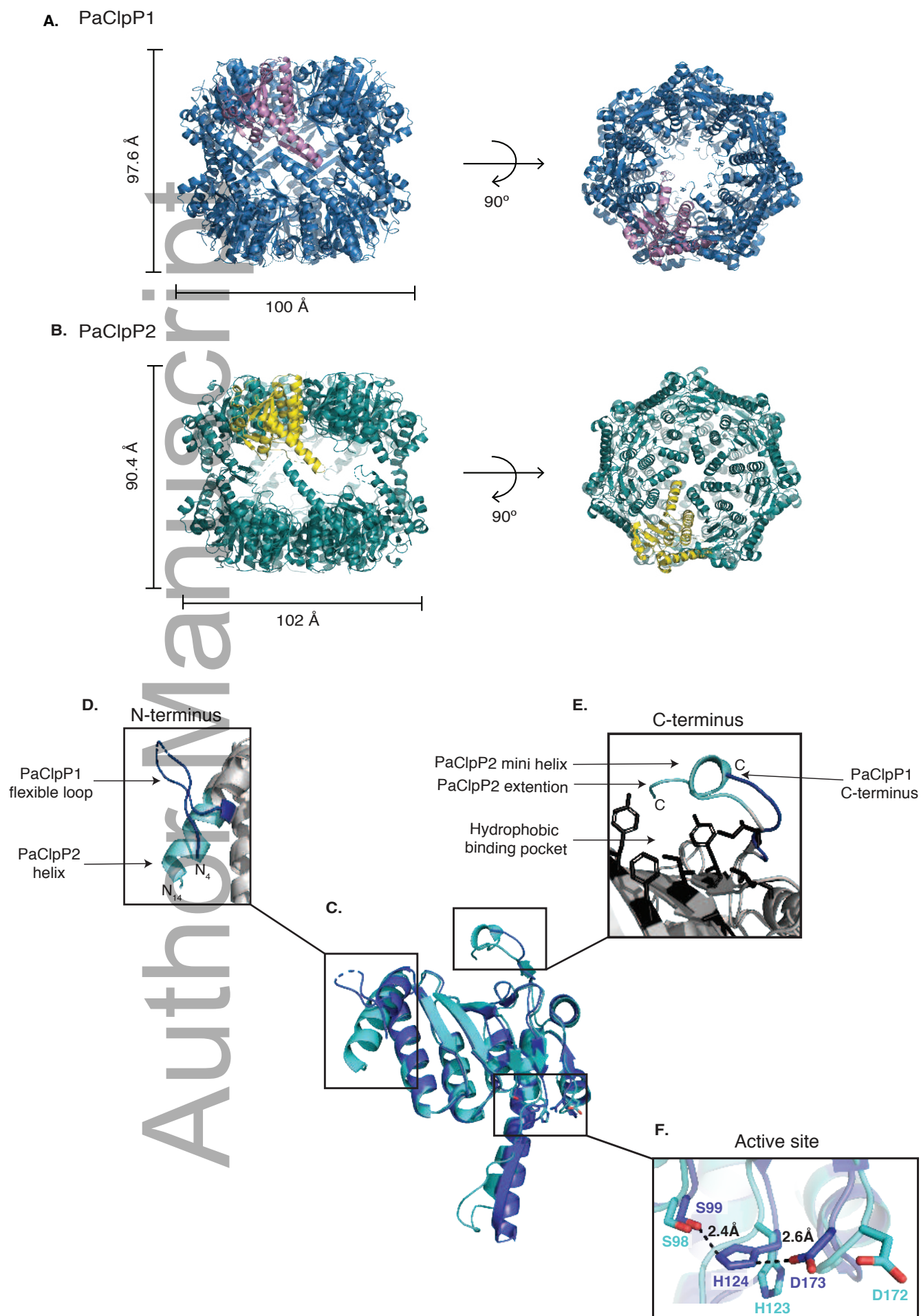
A.

PaClpX
PaClpP1$_{14}$

ATP / ADP

ATP / ADP

B.

PaClpX
PaClpP1$_7$
PaClpP2$_7$

ATP / ADP

ATP / ADP

C.

| | lysate | | | IP αFLAG | | |

Molecular standards (kDa):

150
100
75
50
37
25
20

Lanes: A B C D E F G

PAO1 *clpP2-flag* *clpP2^{S98A}-flag* PAO1 *clpP2-flag* *clpP2^{S98A}-flag*

→ ClpP2-FLAG
→ ClpP1

D.

IP αFLAG:
clpP2-FLAG: − +

α ClpP1:
α ClpP2:

A B

E.

440 158 66 44 17 (kDa)

A280 nm (mAU)

200
150
100
50

40 50 60 70 80 90 100
58 mL 64 mL
Elution volume (mL)

F.

pooled

M 1 2 3 4 5 6 7 8 9 10

Molecular standards (kDa):

150
100
75
50
37
25
20

→ ClpP2
→ ClpP1

A. PaClpP1

97.6 Å

100 Å

B. PaClpP2

90.4 Å

102 Å

D. N-terminus

PaClpP1 flexible loop

PaClpP2 helix

N_4

N_{14}

E. C-terminus

PaClpP2 mini helix

PaClpP2 extention

C

C

Hydrophobic binding pocket

PaClpP1 C-terminus

C.

F. Active site

S99

S98

2.4Å

2.6Å

H124

H123

D173

D172

A.

B.

C.

PaClpX•ClpP1_{14}   PaClpX•ClpP1_{7}P2_{7}

50 nm

46% doubly-capped   2% doubly-capped
54% singly-capped   98% singly-capped

D.

E. X_{6}:P_{14} complex ratio:

All reactions contain 1.0 μM PaClpX_{6}

A.

clpP2^SA ΔclpP2 ΔclpP1 Wildtype

20 µM

mmi_14649_f4.pdf

B.

Relative average thickness
(Entire area) (µM)

0.50 0.75 1.00 1.25

PAO1

ΔclpP1 *

ΔclpP2 *

clpP2-flag

clpP2^S98A-flag *

A. Peptidase assay / Protease assay

B.

C.

D.

E. PaClpXP1$_{14}$

F. PaClpXP1$_7$^{SA}P2$_7$

G. PaClpX^{SA}P1$_7$P2$_7$

H. PaClpXP1$_7$P2$_7$

A. Summary

	PaClpP1$_7$P2$_7$	PaClpP1$_{14}$	PaClpP2$_7$
Oligomeric state:	14-mer	14-mer	7-mer
Active?:	Yes	Yes	No
Stoichiometry of ClpX$_6$:ClpP$_{14}$:	1:1	2:1	0
Phenotype:	Biofilms	Motility[1,2]	Biofilms[3] Pyocyanin production[1,2] Pyoverdine production[1,2]

Interaction with PaClpX:

B. Models

Peptide processing event by PaClpP1$_7$P2$_7$ (ATPase-independent)

Biofilm-promoting factor

Diffusable peptide

Proteolytic product generation by PaClpP1$_7$P2$_7$ (ATPase-dependent)

Protein substrate

Biofilm-promoting factor