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2 **Title:** “Cell surface associated LapA of *Pseudomonas fluorescens* is anchored inside its Type-

3 1 Secretion TolC-like Pore”

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24 **Abstract.**

25 The type-1 secretion system (T1SS) of gram-negative bacteria enables a one-step
26 translocation strategy known to move functionally diverse proteins from the cytoplasm
27 into the extracellular environment without a periplasmic intermediate. LapA of
28 *Pseudomonas fluorescens* Pf0-1 is a giant type-1 secreted (T1S) adhesin that facilitates
29 biofilm formation only when displayed at the cell surface. A LapA-targeting periplasmic
30 protease, LapG, connects intracellular cyclic diguanylate (c-di-GMP) levels with cell surface-
31 associated LapA by cleaving and absolving LapA from the cell surface under conditions
32 unsuitable for biofilm formation. Here, we demonstrate that LapA contains a novel N-
33 terminal element, called the retention module (RM), which prohibits classical one-step T1S
34 of LapA. We provide evidence that the RM of LapA tethers LapA at the cell surface through
35 its outer membrane TolC-like pore, LapE, where LapA is accessible to the periplasmic
36 protease LapG. We also demonstrate that this unusual retention strategy is likely
37 conserved among LapA-like proteins and represents a new subclass of T1SS ABC
38 transporters exclusively involved in transporting LapA-like adhesins.

39 **Significance Statement.**

40 Bacteria have evolved multiple secretion strategies to interact with their environment. For
41 many bacteria, the secretion of cell surface associated adhesins is often key for initiating
42 contact with a preferred substrate to facilitate biofilm formation. Our work demonstrates
43 that *P. fluorescens* uses a previously unrecognized secretion strategy to retain the giant
44 adhesin LapA at its cell surface. Further, we identify likely LapA-like adhesins in various
45 pathogenic and commensal Proteobacteria and provide phylogenetic evidence these
46 adhesins are secreted by a new subclass of T1SS ABC transporters.

47 **Text.**

48 The biofilm lifestyle is profoundly consequential to human health and industry, for
49 better and worse (1, 2). Although bacteria initiate surface contact and biofilm formation
50 through a variety of strategies, many microbes need cell surface-associated protein
51 adhesins to bind a surface. The family of giant (>200 kDa) T1S repeats-in-toxin (RTX)-
52 containing adhesins is known to be critical for biofilm formation or surface binding by a
53 variety of organisms, including *Pseudomonas*, *Bordetella*, *Legionella*, *Vibrio*, *Shewanella* and
54 *Marinomonas* (3–8).

55 Mechanistic studies from our lab on the giant RTX adhesin, LapA, of *Pseudomonas*
56 *fluorescens* Pf0-1 identified a novel regulatory node, LapDG, that links cell surface
57 associated LapA levels to intracellular c-di-GMP levels. Here, the activity of the LapA-
58 targeting periplasmic protease, LapG, is inhibited when its effector, LapD, a transmembrane
59 protein, is bound to cellular c-di-GMP. Conversely, when c-di-GMP levels decrease in the
60 cell, LapD releases LapG. Free LapG in turn cleaves LapA from the cell surface, releasing the
61 adhesin into the supernatant where it is inoperative for biofilm formation (9, 10).

62 Homologs of the *lapDG* genes are found through the Proteobacteria, suggesting that this
63 poorly understood surface display strategy may be quite common (11, 12).

64 Like other T1S proteins, such as HlyB of *E. coli* and CyaA of *B. bronchiseptica*, LapA's C-
65 terminal secretion signal and cognate T1S machinery (LapEBC) are required for LapA
66 secretion and thus biofilm formation (6, 13). T1SS is considered a one-step secretion
67 strategy that lacks any periplasmic intermediate (14), and it is currently unclear how LapA
68 localizes to the cell surface with an N-terminal dialanine cleavage motif that is accessible to
69 the periplasmic protease, LapG. Here, we demonstrate that LapA is not secreted in a

70 canonical one-step T1SS fashion, but rather tethers to the cell surface through its T1SS
71 apparatus. A cleavable retention module at the N-terminus of LapA prohibits complete
72 secretion until cleaved by the LapG protease. We also provide evidence that this previously
73 unappreciated retention strategy is broadly conserved in Proteobacteria and represents a
74 third, distinct subclass of T1S systems.

75 **Results**

76 **Bioinformatic Identification of LapG Substrates.** Despite the importance of LapA and
77 related proteins as key biofilm adhesins (15), it is difficult to identify LapG substrates due
78 to their relatively low sequence similarity. Additionally, ORF analysis programs often
79 overlook or misannotate these large and complex adhesins (5, 16, 17). To overcome the
80 first limitation, we took advantage of the observation that the two proteins that control
81 LapA localization, the c-di-GMP-receptor LapD and the LapD-regulated protease LapG,
82 show high sequence similarity and functional conservation between microbes (3, 12, 18)
83 and can be identified by their respective domain architectures (LapG, pfam06035; LapD,
84 pfam16448). We utilized the NCBI conserved domain database (CDD) and genome
85 database to identify bacterial species encoding *lapDG* homologs; ~1300 such *lapGD*-
86 encoding species spanning 120 genera were identified. Each annotated genome was
87 investigated for proteins containing hallmarks of LapA: an N-terminal LapG cleavage site
88 and C-terminal RTX motifs. To accomplish this task, we developed an algorithm to
89 recognize large proteins (>1000 aa) with RTX-motifs (Dx[L/I]x(4)GxDx[L/I]xGGx(3)D) and
90 a canonical N-terminal dialanine LapG cleavage motif ([T/A/P]AA[G/V]).
91 Although our approach is constrained to properly annotated LapA-like ORFs, we still
92 identified over 500 putative LapG substrates in ~50 genera throughout Proteobacteria,

93 including *Legionella* and *Vibrio* species (Table S1). Importantly, characterized LapG
94 substrates LapA and BrtA (3, 19) were identified. Interestingly, some species likely encode
95 multiple LapG substrates, including *P. fluorescens* Pf0-1 (LapA and Pfl01_1463) and *V.*
96 *cholerae* O395 (FrhA and VC0395_0388).

97 **LapG Substrates Predicted *in silico* Are Processed *in vitro*.** LapG homologs cleave a
98 variety of LapA-like N-termini from unrelated species *in vitro* and *in vivo* (3, 18). To help
99 validate the utility of our algorithm and confirm predicted LapG substrates, we cloned and
100 expressed the N-terminal elements (~250-350 aa) of the putative LapG-proteolyzed
101 adhesins Pfl01_1463 from *P. fluorescens* and VC0395_0388 from *Vibrio cholerae* (Fig 1A).
102 Cell lysates of *E. coli* expressing C-terminally 6HIS-tagged, N-terminal fragments of
103 Pfl01_1463 (M1-240S, 117TAAG120) or VC0395_0388 (M1-363G, 127AAAG130) were
104 mixed with a lysate made from *E. coli* expressing *P. fluorescens* Pf0-1 LapG from a plasmid
105 or the empty vector control. The LapG-dependent cleavage product was tracked via
106 Western blot, with the N-terminus of LapA (M1-235V, 107TAAG110) and an uncleavable
107 variant (LapA^{TRRG}, AA108-109RR) serving as positive and negative controls, respectively
108 (9). *P. fluorescens* Pf0-1 LapG cleaves both predicted substrates (Fig 1 B & C). Unlike FrhA
109 (4), a role for VC0395_0388 in infection and/or biofilm formation is currently unknown;
110 however, these data implicate VC0395_0388 as a cell-surface associated, c-di-GMP-
111 regulated biofilm-promoting adhesin.

112 Conversely, *P. fluorescens* Pf0-1 LapG is unable to cleave the N-terminus of PA1874(M1-
113 251T, 137AAAIG141) (Fig 1D), a large 238 kDa putative outer membrane adhesin encoded
114 by *Pseudomonas aeruginosa* PAO1. Although PA1874 resembles LapA, it was not detected
115 by our algorithm because it contains a degenerate LapG cleavage motif (137AAAIG141).

116 Instead, we manually identified PA1874 by its proximity to *lapEBC* homologs (20).

117 Together, these data support the predictive power of our algorithm for detecting LapA-like
118 LapG substrates *in silico*, and demonstrate the breadth of this c-di-GMP-regulated biofilm
119 strategy in Proteobacteria.

120 ***P. fluorescens* LapA Homologs Contain a Conserved N-terminal Domain.** Previous work
121 from our lab implicated the N-terminus of LapA in retaining the giant adhesin at the cell
122 surface (13, 19). To gain insight into LapA's retention mechanism, we aligned putative
123 LapA-like proteins from closely related *P. fluorescens* strains detected *in silico* and color-
124 filled residues according to the default Clustal X color parameters in Jalview to highlight
125 highly similar regions (Fig S1A, Table S2). Our analysis revealed these adhesins contain
126 two highly similar regions: a C-terminal region that corresponds to LapA's T1S signal
127 required for secretion (Fig S1A, blue box) and a N-terminal region that extends ~20
128 residues beyond the LapG dialanine cleavage motif (Fig S1A, red box and Fig 2A). Given the
129 C- to N-terminal secretion directionality of T1S substrates (21), we speculated that the N-
130 terminus may be involved in retaining these giant adhesins at the cell surface. Consistent
131 with this idea, analysis of BrtA and RtxA homologues from *B. bronchiseptica* and *L.*
132 *pneumophila* strains, respectively, revealed similar patterns of high identity N- and C-
133 termini (Fig S1B,C).

134 **The N-terminus of LapA is Required for Surface Retention.** Closer examination of the N-
135 terminus of *P. fluorescens* LapA homologs indicates their sequence similarity breaks down
136 shortly after a poly-glycine region (Figure 2A, Poly-G). This poly-glycine region is present
137 in many of the LapA-like proteins predicted by our algorithm (Fig S2A). Because poly-
138 glycine regions often serve as unstructured domain linkers, we hypothesized the N-

139 terminal region of LapA encompassing up to this linker may function as a retention module
140 (M1-125S).

141 To test this idea, we made targeted deletions in this putative N-terminal domain.
142 Analysis of the primary sequences and predicted secondary structures suggests the N-
143 termini of the LapA-like proteins identified by our algorithm share little sequence identity,
144 but may adopt similar secondary structures (Fig S2A-B). Given that glycine residues are
145 known to punctuate secondary structures, we used a “gly-to-gly” targeted truncation
146 strategy to disrupt secondary structures within the N-terminus of LapA, using the
147 alignment from Fig S2A as guide.

148 LapA is typically undetectable in the supernatant and enriched at the cell surface of the
149 *lapG* mutant (Fig 2B and 2C, WT vs *lapG*). Therefore, RM mutants were engineered into the
150 hyper-biofilm forming *lapG* gene deletion strain using unmarked allelic replacement,
151 allowing us to decouple retention defects from LapG-mediated proteolysis. Biofilm
152 formation and LapA localization were assayed by comparing each retention mutant to the
153 parental *lapG* mutant, and Western blotting for LapA in the whole cell (WC), supernatant
154 (S) and cell surface (CS) fractions.

155 Consistent with our hypothesis, a LapA truncation mutant lacking residues D31-95A
156 (Δ D31-95A) is unable to form a biofilm (Fig 2D), and this LapA variant is found in the
157 supernatant (Fig 2B) but not retained at the cell surface (Fig 2C). While the Δ V23-95A and
158 Δ D31-95A phenotypes were indistinguishable, most mutants engineered for this study
159 showed only a slightly reduced biofilm phenotype (Fig S2B-C). For those mutants with a
160 biofilm defect (i.e., Δ S02-107A), Western analysis showed that the mutant protein was
161 unstable. Together, these data indicate the N-terminus of LapA functions as a retention

162 module, contributing to the localization of LapA to the cell surface, and also suggests that
163 this region plays a critical, but unclear, role in LapA stability.

164 **Cell Surface Associated LapA Engages the LapEBC T1SS Machinery Through its N-**

165 **Terminal Domain.** Next, we investigated how the RM tethers LapA to the cell surface.

166 LapA is found in the outer membrane fraction (24); however, LapA's RM lacks elements

167 previously shown to be involved in forming outer membrane pores and translocation

168 structures in target cell membranes (15, 22, 23). Thus, we considered the possibility that

169 LapA is anchored to the cell surface by remaining in the T1SS apparatus as a secretion

170 intermediate, using its N-terminal RM to prevent complete secretion of the adhesion.

171 Although unheard of for T1S proteins, we previously demonstrated the LapG substrate

172 from *Pseudomonas aeruginosa*, CdrA, a two partner secreted protein, uses a "cysteine-hook"

173 formed by an intramolecular disulfide bond to anchor itself to the cell surface through its

174 outer membrane pore, CdrB (12, 25). Additionally, recent structural analysis and modeling

175 performed on MpIBP, a large LapA-like adhesion of *M. primoryensis*, suggests the RM of

176 MpIBP, which shares secondary structure features with LapA, may form a plug that

177 prohibits complete secretion of the adhesin through its outer membrane T1S-associated

178 TolC-like protein (26). However, to date, there has been no experimental data supporting

179 the retention model we explore here.

180 To determine if LapA is secreted via the classical one-step T1SS model or retained

181 within its translocation machinery, we conducted a secretion competition experiment to

182 compare secretion of the C-terminal secretion domain of LapA tagged with a HA-epitope

183 tag for Western blotting (HA-C235; Fig 3A, far right). This tool allows us to discern if cell

184 surface-associated LapA impacts the availability of LapEBC T1SS to secrete the HA-C235

185 peptide. We examined secretion of the HA-C235 protein in strains where LapA is locked at
186 the cell surface (a $\Delta lapG$ mutant), or alternatively, in strains where LapA is continuously
187 secreted into the supernatant ($\Delta lapD$ or $lapG lapA\Delta D31-95A$ mutants). In the $\Delta lapD$ mutant,
188 constitutive LapG activity removes LapA from the cell surface, while the $lapG lapA\Delta D31-$
189 $95A$ strain expresses a variant of LapA that lacks the complete retention signal (see Fig 2).

190 Western blot analysis of the supernatant fraction indicates like LapA, HA-C235 peptide
191 secretion is LapEBC-dependent. Deletion of $lapE$ results in loss of biofilm formation, as
192 reported previously (6) and eliminates HA-C235 secretion (Fig 3B). HA-C235 secretion is
193 restored when HA-C235 and $lapE$ are introduced into the $\Delta lapE$ mutant and expressed as a
194 transcriptional fusion from a plasmid (Fig 3B, far right).

195 In the $\Delta lapA\Delta lapG$ mutant, HA-C235 is constitutively secreted because it lacks the LapA
196 RM (Fig 3C). HA-C235 supernatant levels in the $\Delta lapA\Delta lapG$ and $\Delta lapD$ mutants are
197 identical, indicating that lack of LapA or constitutive LapA secretion, respectively, does not
198 impinge on HA-C235 secretion (Fig 3C, left). However, when LapA is locked at the cell
199 surface in the $\Delta lapG$ mutant strain, HA-C235 secretion is nearly abolished (Fig 3C, center).
200 HA-C235 secretion is restored in a $\Delta lapG$ mutant when LapA does not associate with the
201 cell surface (compare $\Delta lapG$ mutant to $\Delta lapG lapA\Delta D31-95A$; Fig 3C). Together, these data
202 indicate retention of LapA, but not its secretion, limits LapEBC availability for secreting
203 other peptides, and is consistent with the model that the RM of LapA is retained in the
204 LapEBC secretion apparatus.

205 **Chemical Inhibition of LapG Locks LapA at Cell Surface, Occluding the LapEBC T1SS**
206 **Machinery.** LapG is a calcium-dependent cysteine protease that can be chemically
207 inhibited *in vivo* and *in vitro* with micromolar levels of EGTA (19). Addition of EGTA to the

208 *ΔlapD* mutant, which usually exhibits constitutive LapG activity, restores LapA cell-surface
209 localization by inhibiting LapG (19). To determine if chemical inhibition of LapG in the
210 *ΔlapD* background can block HA-C235 secretion by enhancing surface associated LapA, we
211 compared biofilm formation and HA-C235 secretion in the *ΔlapD* mutant grown in the
212 absence or presence of 500 μM EGTA (-EGTA or +EGTA). Each strain was grown for 5 hr,
213 then washed and resuspended for 20 min in the indicated medium (Fig 3D, growth/wash).
214 The supernatants were then collected and HA-C235 protein was probed via Western blot
215 analysis.

216 EGTA treatment restored biofilm formation and inhibited HA-C235 secretion in the
217 *ΔlapD* mutant (Fig 3D). Additionally, washing EGTA grown cells in medium lacking EGTA
218 restored C235 secretion (Fig 3D, right), indicating that removal of EGTA reactivated LapG
219 activity, allowing this protease to cleave LapA from the cell surface and thus allow
220 secretion of HA-C235. These data support our model that LapA localizes to the cell surface
221 via anchoring in its T1S machinery.

222 **LapE Overexpression Rescues HA-C235 Secretion in a *lapG* Mutant Background.** The
223 LapEBC proteins likely form a tripartite complex (LapE-LapBC) to secrete LapA. Given that
224 secretion of HA-C235 is blocked when LapA is locked at the cell surface (i.e., a *lapG* mutant
225 or addition of EGTA), we reasoned that a T1SS component(s) participating in cell surface
226 localization of LapA is likely limiting. Therefore, overexpression of the limiting secretion
227 component should allow additional secretion, and thus should rescue HA-C235 secretion in
228 a *ΔlapG* mutant. Previous work from our lab comparing *lapA*, *lapE*, *lapB*, and *lapC* gene
229 expression in the presence and absence of the biofilm promoting nutrient phosphate
230 indicates only *lapE* is down regulated under phosphate-limiting conditions that inhibit

231 biofilm formation (27). Thus, we suspected that LapE might be the limiting component of
232 the secretion machinery.

233 To determine if *lapE* overexpression can rescue HA-C235 secretion in a *lapG* mutant, we
234 assayed for HA-C235 in the supernatant of the $\Delta lapG$ mutant carrying a pC235-*lapE*
235 transcriptional fusion construct. This construct allows simultaneous expression of HA-
236 C235 and LapE protein from the same promoter. Western analysis of the supernatant
237 fraction indicates co-expression of HA-C235 and LapE, but not HA-C235 alone, can rescue
238 HA-C235 secretion in a *lapG* mutant background (Fig 3E, right). HA-C235 secretion in the
239 $\Delta lapA\Delta lapG$ mutant was used as a baseline secretion control. Together, these data are
240 consistent with the model that LapA is retained on the cell surface by LapE, LapE levels are
241 limiting, and retained LapA occupies the secretion pathway thus blocking secretion of any
242 other substrate.

243 **Evidence of a Conserved Retention Strategy.** PHYRE analysis (28) predicts the N-termini
244 of putative LapG substrates may adopt similar secondary structures, suggesting these
245 adhesins could tether through the outer membrane like LapA. To test this idea, we replaced
246 the RM of LapA (M1-125S) with low-identity putative RMs from *V. cholerae* and *P.*
247 *aeruginosa* proteins described earlier (Fig 3F,G, also Fig 1C,D). Figure 3A shows the LapG
248 cleavage site in LapA and VC0395_0338 (boxed in green), the degenerate LapG cleavage
249 site in PA1874 (boxed in yellow), and the putative polyglycine linker common to all three
250 N-termini (boxed in red). Based on our *in vitro* LapG cleavage experiments, the putative
251 RMs may represent separate classes of LapA-like adhesins. VC0395_0338 has a conserved
252 LapG cleavage motif (AAAG) and is processed by LapG *in vitro* (Fig 1C). In contrast, while
253 the N-terminus of the *P. aeruginosa* protein PA1874 has features similar to LapA, it appears

254 to contain a degenerate LapG processing motif (AAA/G) and is not processed *in vitro* (Fig.
255 1D). Thus, we predicted PA1874 anchors to the cell surface but is not subject to LapG-
256 mediated proteolysis and release. Using allelic exchange, we replaced the DNA encoding
257 the N-terminus of LapA with DNA corresponding to the amino acids detailed in the
258 alignment in Fig 3A in both the wild type *P. fluorescens* and the $\Delta lapG$ mutant background.
259 These chimeras are called VC-Swap and PA-Swap. Biofilm formation and chimera
260 localization were compared for the wild type and the $\Delta lapG$ mutant strains expressing the
261 chimeras to determine if these proteins were retained on the surface, could support biofilm
262 formation in *P. fluorescens* and/or were subject to LapG proteolysis to release the chimeric
263 adhesins from the cell surface.

264 Western blot analysis of the whole-cell fraction indicates both VC-Swap and PA-Swap
265 chimeras are stable in the wild type (light grey) and *lapG* (dark grey) mutant backgrounds
266 (Fig 3F, WC). The biofilm phenotype and chimera localization of the VC-Swap and PA-Swap
267 retention chimeras are consistent with their differential susceptibility to LapG cleavage *in*
268 *vitro* (Fig 1 C and D). Western blot analysis of the VC-Swap chimera in the supernatant (Fig
269 3F, S) and cell surface fractions (Fig 3G, CS) indicates deletion of the *lapG* gene decreases
270 levels of the chimera in the supernatant fraction (Fig 3F), and more importantly, enhances
271 chimera levels at the cell surface (Fig 3F, G; VC-Swap, light grey vs. dark grey) and
272 increased biofilm formation (Fig 3H, VC-Swap; light grey vs. red bar). This trend mimics
273 wild type LapA in the parental wild type and *lapG* backgrounds (Fig 3H WT; light grey vs.
274 red bar). These data indicate the RM from VC0395_0338 (Fig 3A, bottom) can complement
275 LapA localization and LapG-dependent release from the cell surface, suggesting LapA and
276 VC0395_0338 localize to the cell surface by a similar mechanism. Conversely, PA-Swap

277 chimera localization and corresponding biofilm formation were not impacted by LapG
278 activity. Consistent with our *in vitro* LapG cleavage analysis, these data indicate that the
279 PA1874 RM can complement LapA's RM for cell surface retention and biofilm formation,
280 but not for cleavage by LapG due the presence of a degenerate cleavage motif (Fig 3F-H).
281 **ABC Transporters of LapA-like Adhesins Form a Distinct T1SS Subgroup.** ABC
282 transporters are found throughout all three domains of life, but can often be functionally
283 grouped based on common residues that are critical for secreting their substrate(s) (29–
284 31). Studies of the T1SS ATPase HlyB of *E. coli*, which transports the RTX toxin HlyA,
285 demonstrated HlyB contains a N-terminal domain that is critical for binding HlyA's C-
286 terminal RTX motifs (31). The N-terminal domain of HlyB resembles the C39 peptidase
287 domain (C39) typically found at the N-terminus of the ATPase component of bacteriocin
288 ABC transporters, but HlyB lacks the catalytic cysteine residue required to cleave and
289 activate immature bacteriocins during secretion (32). Instead, the C39-like domain (CLD)
290 of HlyB contains a tryptophan involved in binding HlyA that is conserved among many ABC
291 transporters involved in secreting RTX toxins, including the ABC transporter secreting
292 CyaB of *B. bronchiseptica*. Thus, the C39 and CLD of bacteriocin and RTX toxin transporters
293 can be distinguished by their amino acid sequence (31).

294 Interestingly, the T1SS ATPase for LapA, called LapB, contains a N-terminal domain that
295 lacks both the conserved cysteine and tryptophan residues of C39 and CLD involved in
296 bacteriocin-processing and RTX-binding, respectively. Given LapA's unusual retention
297 strategy and the observation that LapEBC is often encoded nearby LapDG homologs (11,
298 33), we were curious if LapB-like transporters may represent a distinct subgroup of ABC
299 transporters involved in secreting adhesins with the newly defined N-terminal RM. To test

300 this idea, we assessed the phylogenetic relationship between C39 and CLD sequences from
301 the ATPases of bacteriocin and RTX toxin transporters, and the N-terminal domain of the
302 ATPase from transporters involved in adhesin retention. The phylogenetic relationships of
303 N-terminal domains from the ATPase component several characterized RTX toxin and
304 bacteriocin transporters were identified using Interpro (<http://www.ebi.ac.uk/interpro/>),
305 and compared with N-terminal domain of the putative LapB-like ATPase component.
306 LapB-like ABC transporters were defined as those encoded near LapDG homologs. We also
307 included LssB of *L. pneumophila* and PA1876 of *P. aeruginosa* in the analysis. Recent studies
308 indicate LssB is an ABC transporter involved with secreting an RTX adhesin, RtxA, which
309 was predicted to be a LapG substrate by our algorithm (Fig S1B) (34). PA1876 is likely
310 involved with transporting PA1874, the LapA-like protein with a degenerate cleavage motif
311 described here (Fig 1D and Fig 3F-H) (20).

312 Our phylogenic analysis suggests the ATPase component of the ABC transporters of
313 LapA-like adhesins form a distinct group that lack the functional residues critical for RTX
314 motif-binding and bacteriocin-processing (Fig 4A, see WebLogo). These differences at the
315 amino acid level likely reflect functional differences, rather than phylogenetic diversity of
316 the organisms analyzed, because characterized RTX toxin and LapA-like adhesin ATPases
317 encoded within the same genome cluster with their predicted substrate type. For example,
318 the RTX toxin transporter CyaB (Fig 4A; orange circle) and LapA-like adhesin transporter
319 BB1189 (red circle) encoded by *B. bronchiseptica* map to different clusters in this analysis.
320 These data support the idea that adhesins with an N-terminal RM are secreted by a distinct
321 group of T1SS ABC transporters.

322 **Discussion.** Here, we describe a novel N-terminal element in LapA - called the retention
323 module - that is responsible for localizing this giant adhesin to the cell surface. We propose
324 structural features within LapA's RM play a role in tethering LapA to the cell surface and
325 show that disrupting these predicted secondary structures can lead to uncontrolled release
326 of the adhesin from the cell surface, as was observed for the LapA Δ D31-95A mutant.
327 Consistent with these observations, low sequence identity RMs from putative LapA-like
328 proteins share similar predicted secondary structures and can complement LapA function
329 and localization, suggesting LapA's unusual retention strategy is broadly conserved among
330 this group of adhesins. The bioinformatic analysis presented here and previously (33, 35)
331 suggest LapA-like proteins are secreted by an unappreciated subgroup of T1SS ABC
332 transporters that engage TolC-like pores dedicated to the secretion and retention of these
333 large adhesins.

334 How does the RM contribute to LapA's cell surface localization? We propose the RM
335 stalls the final steps of LapA translocation, leaving LapA threaded through the TolC-like
336 outer membrane pore, LapE, with its RM localized in the periplasm, accessible to LapG, and
337 C-terminal adhesive repeats displayed at the cell surface (Fig 4B). Although this model
338 opposes the classical one-step paradigm detailed in over ~25 years, the model is consistent
339 with our artificial HA-C235 substrate competition experiments, EGTA-mediated inhibition
340 of LapG activity, and expression studies with the TolC-like LapE outer membrane protein
341 presented here. Given the abundance of LapA-like adhesins in pathogens and
342 environmental microbes, and the conserved role of c-di-GMP in regulating their cell surface
343 localization, this means of secreting and anchoring an adhesion appears to be a general
344 strategy used for cell-surface and perhaps cell-cell adherence.

345 Based on the recently reported *Marinomonas* MpIBP structural modeling (26), and the
346 data presented here and reported previously (13), we built a model of the LapA retention
347 module (Fig 4B). Our genetic analysis of the N-terminal RM is in good agreement with the
348 proposed N-terminal domain of MpIBP being localized to the periplasm. Interestingly, the
349 proposed model for the MpIBP adhesin of *Marinomonas* (26) indicates the LapG proteolysis
350 site may be obscured by the TolC pore, and our mutational analysis suggests regions not
351 included in the MpIBP NMR structure are also important for retention and LapG processing
352 (13). In contrast, we propose that the LapG processing site is accessible in the periplasm, a
353 conclusion consistent with our modeling (Fig. 4B) and our previous biochemical and
354 genetic studies (9, 12, 24).

355 **Materials and Methods.**

356 **Strains and media.** *P. fluorescens* and *E. coli* strains listed in Table S3 were grown on
357 lysogeny broth at 30°C and 37°C, respectively. Gentamycin was used when appropriate (10
358 µg/mL for *E. coli*, 30µg/mL for *P. fluorescens*). For biofilm and LapA localization analysis, *P.*
359 *fluorescens* strains subcultured in K10T-1 for 6 hr statically and with rotation, respectively
360 (27).

361 **Static Biofilm Assay.** The static biofilm assay was performed and quantified as described
362 previously (13).

363 **LapA Localization.** Whole cell, supernatant, and cell surface localization analysis was
364 performed as previously using a HA-tagged variant of LapA (13).

365 **HA-C235 Localization.** Each strain was subcultured for 5.5 hr, washed, and then
366 resuspended for 20 min in the indicated medium under non-inducing conditions. For
367 chemical inhibition assays, K10T-1 medium was supplemented with 500µM EGTA as noted.

368 Whole cell and supernatant fractions were prepared as for LapA localization. Western blot
369 analysis against the HA epitope was used to detect HA-C235.

370 ***In Vitro* LapG Cleavage Analysis.** *In vitro* cleavage analysis was performed as described
371 previously (13).

372 ***In Silico* Prediction of LapG Substrates.** LapG and LapD homologs were defined as ORFs
373 encoding proteins with the pfam06035 and pfam16448 domains, respectively. The NCBI
374 CDD was utilized to generate a list of LapG- and LapD-encoding bacteria, and the
375 programming language R was used to determine the intersecting LapD/LapG-encoding
376 bacteria. The protein annotation of these genomes were downloaded from the NCBI
377 genome database and each annotated locus was interrogated for the presence of a LapG
378 cleavage site within amino acids 80-150 ([T/A/P]AA[G/V]) and at least one RTX motif
379 (Dx[L/I]x(4)GxDx[L/I]xGGx(3)D).

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471

472 **Figures**

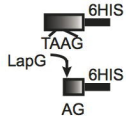
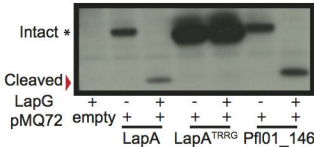
473 **Figure 1. *P. fluorescens* Pf0-1 LapG *in vitro* cleavage analysis.** (A) Overview of *in vitro*
474 LapG cleavage assay. (B-D). Intact (*) and cleaved (red arrow) N-termini were visualized
475 by Western blot. Equal protein concentrations of the substrate and extracts from LapG-
476 expressing strains were used.

477 **Figure 2. LapA's N-terminus Serves as a Retention Module.** (A) The first ~175 aa of the
478 *P. fluorescens* LapA-like N-termini (Fig. S1A, red box) with LapG cleavage motif (red arrow)
479 and putative polyglycine linker (Poly-G, black bar). (B) Western blot analysis of
480 supernatant and whole cell fractions for LapA. (C) Dot blot analysis of surface-associated
481 LapA. (D) Biofilm analysis of LapA N-terminal mutants (n=8, +/-SEM).

482 **Figure 3. LapA Cell Surface Localization Impacts LapEBC Activity.** (A) Top: Scaled
483 representation of LapA and HA-C235. Black bar indicates 3XHA tag in LapA and HA-C235.
484 Bottom: MUSCLE alignment of putative RMs from *P. fluorescens*, *P. aeruginosa*, and *V.*
485 *cholerae* adhesins with residues colored according to the default Clustal X coloring scheme
486 in Jalview. The putative polyglycine linker is boxed in red while the canonical and
487 degenerate LapG cleavage sites are boxed in green and yellow, respectively. (B-C) Biofilm
488 formation (top) and Western blot analysis of HA-C235 in the supernatant (S) and whole cell
489 (WC) fractions (bottom). In these experiments, LapE and HA-C235 are co-expressed as a
490 transcriptional fusion from a plasmid. (D) Biofilm analysis (top) and HA-C235 secretion
491 (bottom) in $\Delta lapD$ mutants with and without EGTA treatment. (E) Biofilm analysis (top)
492 and HA-C235 secretion (bottom) in $\Delta lapG$ mutants. (F) Western blot analysis of LapA RM
493 chimeras VC0395_0388 (VC-Swap; also panel A, bottom.) and PA1874 (PA-Swap; also panel
494 A, bottom) in the supernatant (S) and whole cell (WC) fractions in the indicated genetic
495 backgrounds. (G) Western blot analysis of cell surface associated LapA RM chimeras (top)
496 and quantification (bottom). Strains in panel G correspond to strains in panel F. (H) Biofilm
497 formation of LapA RM chimeras from strains in panel G. For biofilm and dot blot
498 quantification (n=8, +/-SEM).

499 **Figure 4. Phylogenetic Analysis of ABC Transporter Subfamilies.** (A) Phylogenetic
500 analysis of C39 peptidase domain, C39-like peptidase domain (CLD), and N-terminal ~125
501 aa of LapB-like ATPases (Table S4) using the online phylogenetic analysis program
502 phylogeny.fr under default settings (<http://www.phylogeny.fr>). WebLogos were generated
503 by MUSCLE aligning members from each branch and truncated to highlight functional
504 residues when applicable (red). Bootstrap values are indicated in red. (B) Left: Domain

505 architecture of LapA. Right: Surface retention model for LapA. The structural model of the
506 LapA retention module was generated using the Phyre2 protein fold recognition server and
507 PDB 5ix9 as the template (red domain). The sequence following the retention domain was
508 modeled as an extended peptide to reflect secondary structure predictions indicating this
509 region (residues 97-173) to be flexible. The unstructured sequence was threaded through
510 LapE, represented by the crystal structure of TolC (PDB: 1ek9). The LapG-targeting
511 dialanine motif (TAAG) and regions critical for LapG binding (Helix 1 and DPxxxLxx) are
512 shown, and proposed to be exposed in the periplasm. Two glycine-rich motifs in LapA,
513 which flank the sequences within the TolC pore, are indicated in yellow.

A**B****C****D**

anti-6HIS

A

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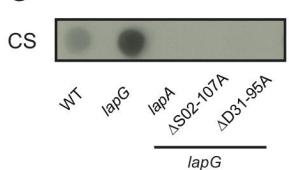
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LapG Poly-G

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B**C****D**