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1 **Increasing the PACE of characterising novel transporters by functional genomics**

2

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18

19 **Abstract**

20 Since the late 1990's the genome sequences for thousands of species of bacteria have been
21 released into public databases. The release of each new genome sequence typically revealed
22 the presence of tens to hundreds of uncharacterised genes encoding putative membrane proteins
23 and more recently, microbial metagenomics has revealed countless more of these
24 uncharacterised genes. Given the importance of small molecule efflux in bacteria, it is likely
25 that a significant proportion of these genes encode for novel efflux proteins, but the elucidation
26 of these functions is challenging. We used transcriptomics to predict that the function of a gene
27 encoding a hypothetical membrane protein is in efflux-mediated antimicrobial resistance. We
28 subsequently confirmed this function and the likely native substrates of the pump by using
29 detailed biochemical and biophysical analyses. Functional studies of homologs of the protein
30 from other bacterial species determined that the protein is a prototype for a family of multidrug
31 efflux pumps – the Proteobacterial Antimicrobial Compound Efflux (PACE) family. The
32 general functional genomics approach used here, and its expansion to functional
33 metagenomics, will very likely reveal the identities of more efflux pumps and other transport
34 proteins of scientific, clinical and commercial interest in the future.

35

36 **Introduction**

37 Small molecule efflux pumps are important mediators of bacterial resistance to antimicrobial
38 drugs. These pumps also contribute to a variety of basic physiological functions, including
39 virulence related phenotypes, and are thus central to the pathogenic lifestyles of many bacteria.
40 In the late decades of the last century five families of transport proteins that include small
41 molecule drug efflux pumps were discovered – the ATP-Binding Cassette (ABC) Superfamily,
42 the Major Facilitator Superfamily (MFS), the Resistance/Nodulation/Division (RND)
43 Superfamily, the Multidrug/Oligosaccharidyl-lipid/Polysaccharide (MOP) Superfamily, and
44 the Drug/Metabolite Transporter (DMT) Superfamily. Some individual protein members of
45 these widespread families have since been studied extensively using microbiological,
46 genetical, biochemical and structural approaches [1]. This large body of research has
47 demonstrated that members of these families can be grouped on the basis of their primary
48 sequences and tertiary structures, and that the molecular mechanisms of transport used by
49 members of different families can be fundamentally different. Altogether, these findings show
50 that the functions of several large transport protein families have separately converged on small
51 molecule efflux, highlighting the importance of this process in bacteria [2].

52 The 21st century has seen an explosion in genome and metagenome sequence information for
53 organisms that reside in diverse environmental niches, including on and in human hosts. These
54 sequence data have highlighted that we have a basic understanding of function for only a very
55 small fraction of genes encoded by bacteria. Indeed, in even the best studied bacterial species,
56 such as *Escherichia coli*, we lack functional information for approximately 25–35 % of the
57 predicted gene content [3,4]. In light of the tremendous diversity of bacteria and the importance
58 of small molecule efflux for their physiology and niche adaptation, members of our team have
59 long speculated that many uncharacterised bacterial genes are likely to encode novel small
60 molecule efflux pumps [5]. These proteins may have arisen during the adaptation of bacteria

61 to diverse biological niches, and could well be exploited in microbial biotechnology, but their
62 identification and characterisation remains a challenge. In this article we illustrate current
63 thinking on how to address this challenge, exemplified by our recent experiences in
64 characterising a new group of efflux proteins, the Proteobacterial Antimicrobial Compound
65 Efflux, or PACE, family.

66 **The discovery of AceI**

67 In the early 2010's, while investigating the molecular mechanisms that the human pathogen,
68 *Acinetobacter baumannii*, uses to tolerate the biocide chlorhexidine, transcriptomic analyses
69 drew our attention to a hypothetical gene that was predicted on the basis of hydropathy analysis
70 to encode a membrane protein. The gene, denoted by the locus tag A1S_2063, was more highly
71 expressed after a 30-minute exposure to a near inhibitory concentration of chlorhexidine than
72 in control cells that were not exposed to chlorhexidine [6]. The only other genes to show similar
73 changes in expression following chlorhexidine exposure encoded components of the AdeABC
74 multidrug efflux pump, classified as a member of the RND superfamily, which had previously
75 been found to increase the resistance of *A. baumannii* cells to chlorhexidine [7]. To determine
76 whether A1S_2063 similarly provided resistance to chlorhexidine this gene was isolated,
77 cloned and expressed in *E. coli* cells. These cells were 8–16-fold more tolerant to chlorhexidine
78 when A1S_2063 was expressed compared to parental cells, demonstrating the ability of
79 A1S_2063 to function as a chlorhexidine resistance determinant in *E. coli* [6]. We conducted
80 a broad phenotypic resistance screen (> 200 antimicrobials) of *E. coli* cells expressing
81 A1S_2063 using the Biolog Phenotype Microarray system [8], to determine if A1S_2063 could
82 mediate resistance to multiple antimicrobials, similar to *adeABC*. This analysis suggested that
83 resistance mediated by A1S_2063 was specific to chlorhexidine [6]. Tucker *et al.* subsequently
84 made a deletion mutant of A1S_2063 in *A. baumannii* ATCC 17978, which had a 50 %
85 reduction in chlorhexidine tolerance compared to the parental strain, confirming a

86 chlorhexidine resistance function in the native *A. baumannii* ATCC 17978 host [9]. A similar
87 loss of resistance was also seen in *A. baumannii* AB5075-UW and *Acinetobacter baylyi* ADP1,
88 strains in which the orthologous gene had been inactivated or deleted [6,10].

89 Hydropathy analysis of the amino acid sequence of the protein product of A1S_2063 predicted
90 that it was comprised of four transmembrane alpha-helices (TMH), with both N- and C-termini
91 in the cytoplasm [6,11]. Consistent with this, A1S_2063 protein expressed in *E. coli* was
92 present in the *E. coli* inner-membrane fraction [6]. Since it was embedded in the membrane,
93 we suspected that the protein might be a novel chlorhexidine efflux system. Indeed, [¹⁴C]-
94 chlorhexidine accumulation into *E. coli* cells that expressed A1S_2063 was reduced compared
95 to parental cells provided that the cells were energised [6]. Additionally, these cells were able
96 to export pre-loaded [¹⁴C]-chlorhexidine into the medium more rapidly than otherwise isogenic
97 controls lacking the *A. baumannii* gene [6]. This was consistent with the A1S_2063 product
98 functioning in the active efflux of chlorhexidine. Chlorhexidine binding to the purified
99 A1S_2063 protein product was then demonstrated using fluorescent and circular dichroic
100 spectroscopic methods [6], and subsequently by protein mass spectrometry [12], consistent
101 with the protein functioning in chlorhexidine transport directly, rather than via cooperation
102 with another transport system. On the basis of these results the protein was named the
103 *Acinetobacter* chlorhexidine efflux I (AceI) protein.

104 **Regulation of expression of the *aceI* gene**

105 Since the expression of *aceI* was induced by the presence of chlorhexidine, it was reasonable
106 to expect that it was controlled by a chlorhexidine responsive transcription factor. The most
107 likely candidate was a LysR family regulator encoded adjacent and divergent to *aceI* in *A.*
108 *baumannii*. A related regulator was also observed adjacent to *aceI* homologs in the genomes
109 of other *Acinetobacter* species. Inactivation or deletion of the LysR family regulator gene in
110 both *A. baumannii* AB5075-UW and *A. baylyi* ADP1 abolished chlorhexidine-mediated

111 induction of *aceI*, and caused a reduction in chlorhexidine tolerance equivalent to that seen
112 upon inactivation of *aceI* [10,13]. These results suggested that the LysR family protein is likely
113 to be an activator of AceI expression. Electrophoretic gel mobility shift assays and DNase I
114 footprinting assays demonstrated that the LysR family regulator protein was indeed able to
115 bind upstream of the *aceI* gene in the presence of chlorhexidine at sites adjacent to the putative
116 promoter site [10]. Direct binding of chlorhexidine to the LysR family regulator was also
117 demonstrated using surface plasmon resonance and tryptophan fluorescence quenching assays
118 [10], and subsequently by protein mass spectrometry [12]. Altogether, these results indicated
119 that the LysR family regulator was a direct chlorhexidine-responsive activator of *aceI* gene
120 expression, and it was named AceR [10].

121 **The physiological substrates of AceI**

122 A number of questions arose from the discovery of the AceI chlorhexidine transport protein.
123 Foremost amongst these was the native role of AceI in the physiology of *A. baumannii*. A
124 clustering analysis indicated that AceI is encoded by genes in the core genomes of all *A.*
125 *baumannii* strains for which complete genomes were publicly available [13]. Furthermore, an
126 orthologous protein is encoded in 69 of 70 *Acinetobacter* species with genome sequences
127 presented in the RefSeq genomes database (Figure 1), and an adjacent *aceR* ortholog is found
128 in >90 % of cases (data not shown). A phylogenetic analysis of *aceI* orthologs harboured in the
129 representative genome sequences of *Acinetobacter* species was constructed and compared to a
130 core housekeeping gene tree for the same species (Figure 1). The membership of most clades
131 within the *aceI* phylogenetic tree followed that of the core housekeeping gene tree (Figure 1).
132 Taken together, this suggests that *aceI/aceR* have been present in the core *Acinetobacter*
133 genome for millennia – at least since the divergence of this genus from related bacterial genera,
134 whereas, chlorhexidine is an artificial biocide produced only during the last century. Therefore,
135 it is not possible that AceI evolved to confer resistance specifically to this antimicrobial.

136 Rather, the native function of AceI seemed likely to relate to an ancient physiological need of
137 the *Acinetobacter* species or possibly the family Moraxellaceae.

138 Polyamines were viewed as potential native substrates for AceI because they consist of
139 aliphatic carbon chains with charged termini at physiological pH, similar to the general
140 properties of chlorhexidine (Figure 2). Furthermore, these compounds are widely found in the
141 environment, including in almost all living cells at high concentrations (usually high μM -low
142 mM) [14,15]. Polyamines commonly found in biology were screened for their potential to be
143 recognised by the AceI transport protein. These compounds displayed some toxicity towards
144 *A. baumannii*, so the tolerance of an *aceI*-inactivated mutant was initially compared to that of
145 the parental strain. This experiment showed that tolerance to the diamines, putrescine (1,4-
146 diaminobutane) and cadaverine (1,5-diaminopentane), was 8–16-fold lower in the *aceI* mutant
147 than in the parental strain, whereas tolerance to spermidine and spermine did not change
148 between the mutant and parental strain [16]. These results fit with the hypothesis that some
149 diamines could be recognised as substrates of AceI, and this hypothesis was then tested using
150 transport assays.

151 The accumulation of [^{14}C]-cadaverine into an *A. baumannii* AB5075-UW *aceI* mutant was
152 compared to accumulation into the parental strain. The loss of *aceI* resulted in a significantly
153 higher accumulation of [^{14}C]-cadaverine over time [16]. Similarly, the expression of active
154 *aceI* in *E. coli* cells, which do not normally express this gene or any homolog, significantly
155 reduced the accumulation of [^{14}C]-cadaverine [16]. Expression of an *aceI* mutant (E15Q),
156 which was unable to effect resistance to chlorhexidine [6], was also unable to diminish the rate
157 of cadaverine accumulation, consistent with active AceI functioning in the efflux of cadaverine
158 as it entered the cells [16].

159 **Energisation of polyamine transport**

160 In order to observe active extrusion of chlorhexidine or cadaverine by the expressed AceI
161 protein, it was necessary that energy metabolism in the cells was intact, usually accomplished
162 by addition of glucose to the cells; if an energy uncoupling agent such as the protonophore
163 carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was added to the cells, extrusion of the
164 substrate by AceI was prevented [6]. To test directly whether AceI itself was responsible for
165 the energised extrusion phenotype, the protein was purified to near homogeneity and
166 reconstituted into proteoliposomes containing the pH sensitive dye pyranine. Upon the
167 establishment of an electrochemical gradient of protons (inside positive and acidic, opposite to
168 that in intact cells), AceI promoted the uptake of [¹⁴C]-cadaverine (as measured by liquid
169 scintillation) and the alkalinisation of the proteoliposome lumen (as measured by pyranine
170 fluorescence), consistent with the export of protons in exchange for cadaverine [16].
171 Proteoliposomes containing the inactive AceI mutant E15Q or empty liposomes accumulated
172 [¹⁴C]-cadaverine at a comparatively very slow rate. Notably, the glutamate residue
173 corresponding to E15 in AceI is very highly conserved in the aligned AceI proteins from
174 *Acinetobacter* (Figure 3) and more broadly, in AceI homologs from other genera [17]. On this
175 basis we speculate that it may be involved in a coupling mechanism, and its conversion to
176 glutamine would, of course, prevent its participation in proton-coupled transport. Putrescine
177 was nearly as effective as cadaverine in promoting a pH change, but spermidine and spermine
178 were ineffective [16]. Taken together, these results indicated that AceI is likely to recognise
179 the diamines cadaverine and putrescine as substrates, and that in intact cells their efflux is
180 driven against a concentration gradient by the trans-membrane electrochemical gradient of
181 protons [16]. Consistently, the expression of *aceI* in *A. baumannii* was induced by the presence
182 of the diamines putrescine and cadaverine to a higher level than by chlorhexidine, and to a
183 lesser extent by spermidine, strongly reinforcing the suggestion that diamines are physiological
184 substrates of AceI [2,16].

185 **Characterisation of the extensive family of PACE proteins**

186 Another important question arising from the discovery of AceI, was whether similar proteins
187 existed outside the *Acinetobacter* genus and whether these had the potential to contribute to
188 chlorhexidine tolerance, and/or diamine transport, similarly to AceI. AceI is comprised of two
189 Bacterial Transmembrane Pair (BTP) conserved Pfam domains, each consisting of a pair of
190 TMH [17-19] (Figure 3). The Pfam database includes almost 800 protein sequences that
191 include a BTP domain, almost all of which have the same dual BTP domain structure as AceI
192 [18]. These proteins are homologous to AceI and are found in more than 650 representative
193 bacterial species, predominantly Proteobacteria, but some Actinobacteria [18].

194 Our research groups have cloned more than 40 diverse AceI homologs (most from synthetic
195 DNA), to assess their functions by heterologous expression in *E. coli*. Approximately 85 % of
196 the cloned homologs were readily expressed in a pTTQ18 expression system in *E. coli* [20,21].
197 Minimum inhibitory concentration analyses demonstrated that approximately half of the
198 expressed AceI homologs confer resistance to chlorhexidine, including all homologs from the
199 *Acinetobacter* genus [19]. Furthermore, many of the proteins from other genera were able to
200 confer resistance to a broader set of antimicrobials, including other biocides and the DNA
201 intercalating fluorescent dye acriflavine [17,19]. This allowed the transport activity of these
202 homologs to be confirmed using fluorescent transport assays [17,19]. Diamine transport has
203 been assessed in a subset of clones and similarly was seen to be mediated by approximately
204 half of the proteins tested [16].

205 **The generic structure and molecular mechanism of PACE family pumps**

206 Current thinking suggests that PACE proteins: 1) have four TMH with two sets of repeating
207 motifs (Figure 3); 2) are likely to be energised by a substrate/cation antiport mechanism; 3) are
208 likely to have polyamines as their physiological substrates; and 4) are likely to bind and

209 possibly transport chlorhexidine and other biocides. Primarily, information is now needed
210 about the tertiary and quaternary structures of the PACE proteins.

211 Given their small size, PACE proteins are likely to function as oligomers. Preliminary
212 investigations into the oligomeric state of PACE pumps have been conducted using native
213 PAGE. AceI protein that had been solubilised and purified in the non-denaturing detergent n-
214 dodecyl- β -D-maltoside (DDM) was examined using blue-native PAGE (Figure 4A). This
215 analysis suggested a very high order oligomeric state, possibly a dodecamer or 24-mer based
216 on the band size relative to comigrated soluble proteins of known molecular mass. To
217 demonstrate a large oligomer, the denaturing detergent sodium dodecyl sulphate (SDS) was
218 added to equivalent samples at increasing concentrations (Figure 4A). Upon addition of SDS,
219 the high molecular weight AceI complex broke down into smaller complexes, possibly
220 representing dimers, trimers and/or tetramers. Since blue native PAGE relies on membrane
221 proteins first being purified in detergent, it may produce artefactual results due to protein
222 aggregation. The recently developed styrene maleic acid copolymer (SMA) PAGE approach
223 does not require proteins to be removed from the membrane prior to electrophoresis, since they
224 are solubilised directly in styrene maleic acid copolymers to form lipid nanodiscs [22].
225 Therefore, this approach may demonstrate the oligomeric state of proteins in the native
226 membrane environment and has been used to predict the oligomeric state of a diverse set of
227 membrane proteins with good accuracy [23]. SMA PAGE was used to examine the quaternary
228 organisation of the *Vibrio parahaemolyticus* PACE family protein VP1155. This protein is an
229 important model for the PACE family. It is a multidrug transporter that recognises a broad
230 range of antimicrobial substrates, such as chlorhexidine, acriflavine and benzalkonium [19].
231 Two bands of VP1155 protein were observed in the SMA-solubilised sample, of which the
232 lower molecular weight band was dominant (Figure 4B). By comparison to soluble molecular
233 weight markers these bands had approximate molecular weights of 87 and 321 kDa (Figure

234 4B). Given that VP1155 has a predicted molecular weight of ~18 kDa and allowing for
235 associated lipids, this analysis suggested that VP1155 may predominantly exist as a tetramer.
236 Recent evidence from mass spectrometry has also supported the oligomerisation of AceI –
237 upon binding to chlorhexidine, mass spectrometry was used to suggest that the detergent
238 solubilised protein is likely to form a dimer [12]. Efforts to obtain high resolution structural
239 information for PACE proteins are underway, including in our laboratories. These data will be
240 of significant interest for understanding the similarities and differences of function and of the
241 molecular mechanism for transport by these proteins.

242 **Future perspectives**

243 A common goal for projects in microbial biotechnology is the biological production of high
244 value small molecules from cheap feedstocks like glucose and ammonia. With molecular and
245 synthetic biology, it is possible to assemble biosynthetic pathways in engineered
246 microorganisms that allow efficient production of target molecules. A key step in this process
247 is the export of the biosynthetically produced compounds to facilitate simpler extraction from
248 culture and reduced end-product inhibition [24]. Novel efflux systems for a diverse range of
249 valuable biosynthetic products are needed to expand the range of compounds that could be
250 microbially produced in a commercially viable manner. The PACE family includes several
251 members that mediate the active transport of the diamines cadaverine and putrescine. These
252 compounds can be used as precursors in the production of nylons that have advantageous
253 physical properties over the common nylon-6 [25,26]. Thus, there is a large potential market
254 for cadaverine and putrescine, but they are not as easily derived from petrochemical sources as
255 the nylon-6 precursor 1,6-diaminohexane. Many bacterial species naturally produce cadaverine
256 and/or putrescine and could serve as a renewable source of these compounds in the production
257 of bio-based nylons [27]. However, the compound yield must be improved before this
258 production is a viable alternative to petroleum derived precursors. The expression of a PACE

259 family protein that recognises cadaverine and/or putrescine in the producing organism could
260 enhance their production.

261 Bacterial genomes carry large numbers of genes of unknown function that are predicted to
262 encode integral membrane proteins. In some bacteria, more than 15 % of open reading frames
263 have been recognised to encode a transport protein [28]. Therefore, it seems likely that a
264 considerable proportion of membrane proteins of unknown function are involved in small
265 molecule transport. However, assigning function to uncharacterised membrane proteins is not
266 trivial. In this regard, rapid throughput genome scale analyses, such as transcriptomics,
267 proteomics and random transposon sequencing approaches have proven exceptionally useful.
268 With the identification of *aceI* and subsequently the PACE family, we have shed light on a tiny
269 fraction of the dark matter in microbial genomes. Future illumination of PACE depends
270 currently on elucidation of their subunit composition, their three-dimensional structures and
271 the roles of highly conserved residues in their molecular mechanism(s) of transport.

272

273

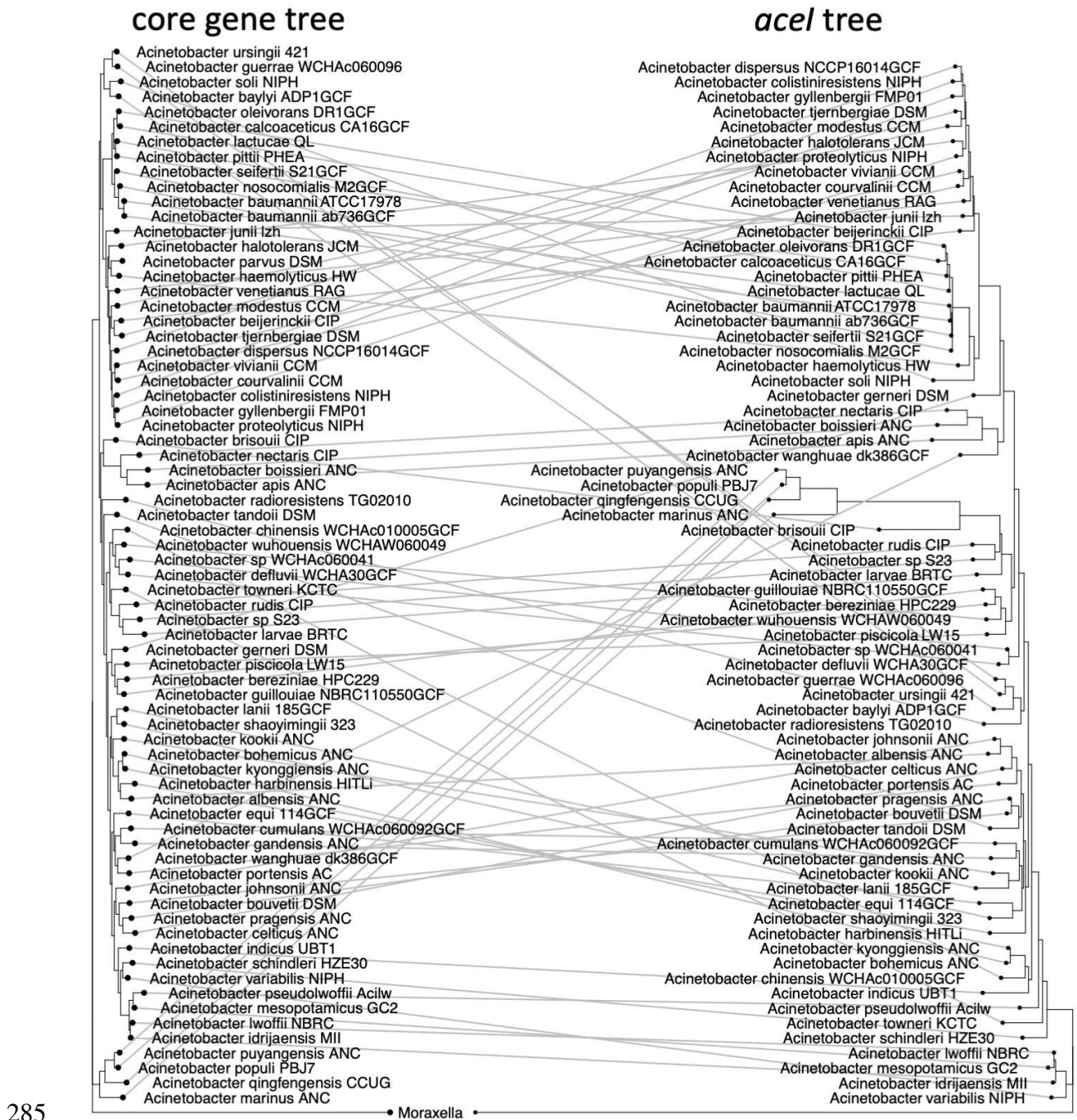
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283

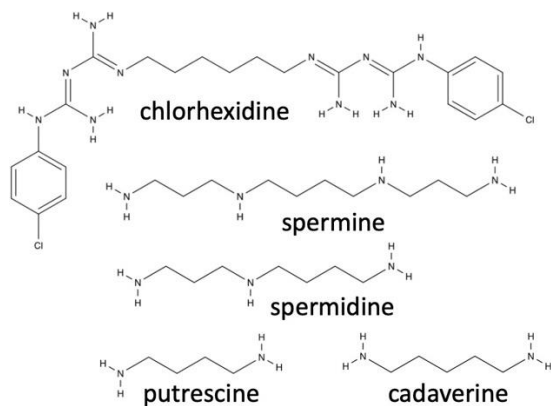
284



285

286 Figure 1. Twin tree figure comparing phylogenetic relationships of core housekeeping genes
 287 and putative *acelI* orthologs encoded in *Acinetobacter* species. The “reference” genome
 288 sequences for 70 *Acinetobacter* species, and the genome sequence for *A. baumannii* ATCC
 289 17978, were obtained from the NCBI RefSeq database (15 Feb 2021; listed in Supplemental
 290 file 1). Putative orthologs were identified using Proteinortho [29] (ortholog identity > 75 %).
 291 The tree on the left side was made using the concatenated sequences of core *Acinetobacter*

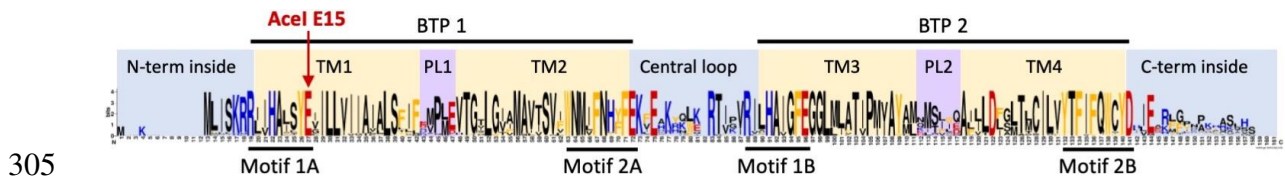
292 housekeeping genes (*gltA*, *gyrB*, *rpoD*, *fusA*, *rplB*) and the tree on the right side was made
293 using the sequences of all *aceI* orthologs. Alignments were made with MAFFT [30] using
294 defaults, ModelTest-NG [31] was used to validate the alignment and determine the best model
295 (GTR+I+G4) in both cases. RAxML-NG [32] was then used to infer the trees. Grey lines
296 connect the corresponding branch tips of each species represented in the trees. These lines
297 illustrate that the structure of individual subclades is conserved between the trees, suggesting
298 that *aceI* has been present within these genomes for significant evolutionary time.
299



300

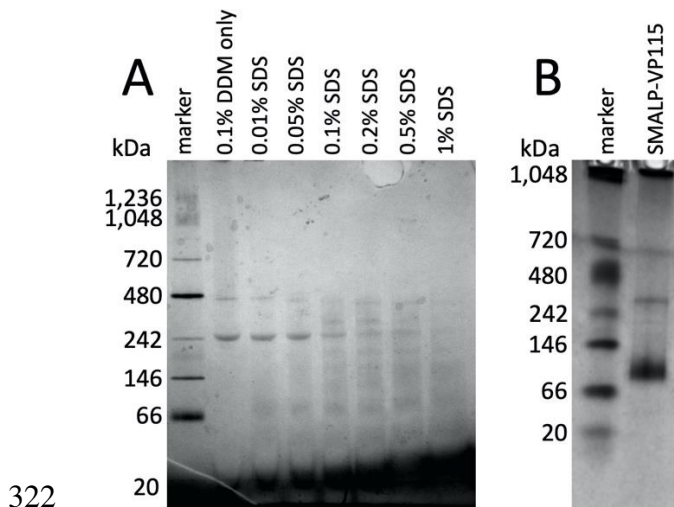
301 Figure 2. Chemical structures of chlorhexidine and representative polyamines found
 302 abundantly in biology: putrescine (1,4-diaminobutane); cadaverine (1,5-diaminopentane);
 303 spermidine; and spermine.

304



305
 306 Figure 2. Sequence conservation within putative AceI orthologs across the *Acinetobacter*
 307 genus. The “reference” genome sequences for 70 *Acinetobacter* species, and the sequence for
 308 *A. baumannii* ATCC 17978, were obtained from the NCBI RefSeq database (15 Feb 2021).
 309 Putative orthologs were identified using Proteinortho (ortholog identity > 75 %). Predicted
 310 orthologs of the *A. baumannii* ATCC 17978 AceI sequence were aligned using MAFFT [30]
 311 and the alignment used to create a sequence logo using weblogo [33], where the height of
 312 amino acid sequence characters is indicative of the level of amino acid conservation. The
 313 locations of conserved amino acid sequence motifs previously identified within PACE family
 314 proteins (1A, 2A, 1B and 2B; [17]) are underlined. The two Pfam [18] Bacterial
 315 Transmembrane Pair (BTP) domains are indicated. The amino acid residues encompassing
 316 transmembrane regions (TM1–TM4) are shown with yellow shading, those amino acids
 317 predicted to lie within periplasmic loops (PL1 and PL2) are shown with purple shading, and
 318 those within the cytoplasm (the N- and C- terminal regions and central loop) are shown with
 319 blue shading. The location of the conserved glutamate residue targeted for mutagenesis in AceI
 320 (E15) is indicated with an arrow.

321



322
 323 Figure 4. Native PAGE analyses on PACE family proteins. A) Blue-native PAGE of AceI.
 324 AceI protein fused to the C-terminal tag RGSH6 was overproduced from the expression vector
 325 pTTQ18 in *E. coli* DH5 α (plasmid construct described in [6]). The cells were mechanically
 326 lysed and total membranes isolated by ultracentrifugation. The membranes were solubilised
 327 using in DDM and AceI purified by Ni-chelation chromatography. AceI protein was eluted in
 328 buffer containing 0.1 % DDM. The purified protein was electrophoresed on a precast
 329 ThermoFisher NativePAGE Bis-Tris gel with blue-native PAGE buffers. The gel was
 330 subsequently stained using Coomassie blue. To disrupt the oligomeric state of the protein,
 331 increasing concentrations of SDS were added to equivalent samples of the DDM solubilised
 332 protein prior to electrophoresis. B) SMA-PAGE of VP1155 solubilised in styrene maleic acid
 333 copolymer lipid nanoparticles (SMALPs). VP1155 was overproduced from pTTQ18-VP1155
 334 in *E. coli* DH5 α . The cells were mechanically lysed and total membranes isolated by
 335 ultracentrifugation. The membranes were solubilised using SMA and VP1155 purified by Ni-
 336 chelation chromatography. Native PAGE was performed using the purified VP1155 protein.
 337 The gel was subsequently silver stained. (N=2). Lane 1: Native protein marker (Thermo
 338 Scientific). Lane 2: SMA-isolated VP1155. The Rf values of each protein band were used to
 339 estimate the molecular weight (MW) of each of the bands observed in the sample.

340

341 **References**

342 * special interest

343 ** outstanding interest

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