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Increasing the PACE of characterising novel transporters by functional genomics

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Abstract

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

Introduction

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

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

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

The discovery of AceI

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

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

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

Regulation of expression of the *aceI* gene

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

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

The physiological substrates of AceI

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

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

Characterisation of the extensive family of PACE proteins

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

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

The generic structure and molecular mechanism of PACE family pumps

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

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

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

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

Future perspectives

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

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

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

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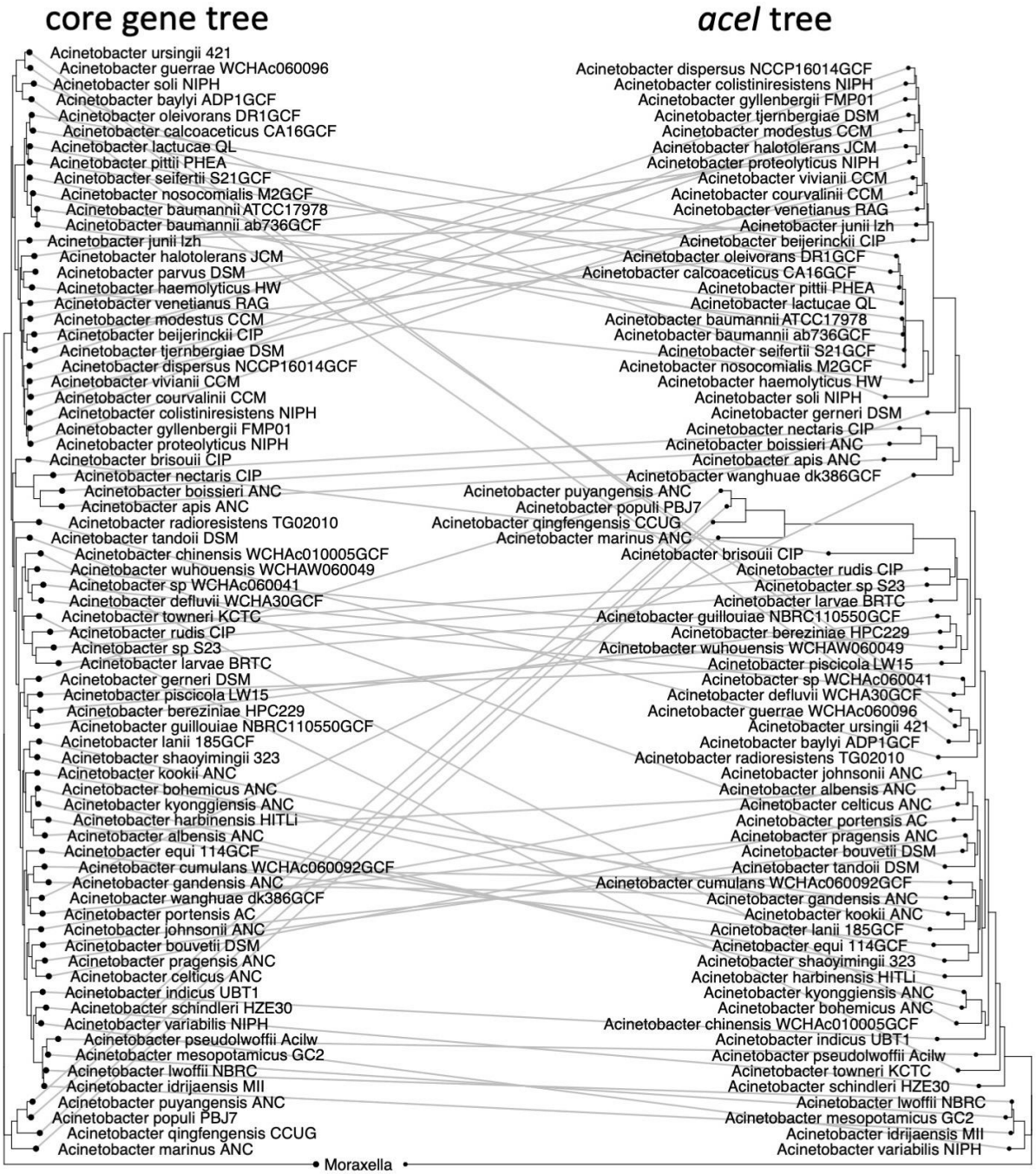
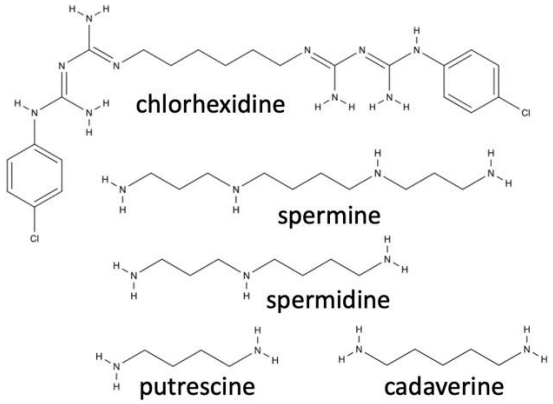


Figure 1. Twin tree figure comparing phylogenetic relationships of core housekeeping genes and putative *aceI* orthologs encoded in *Acinetobacter* species. The "reference" genome sequences for 70 *Acinetobacter* species, and the genome sequence for *A. baumannii* ATCC 17978, were obtained from the NCBI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

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

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

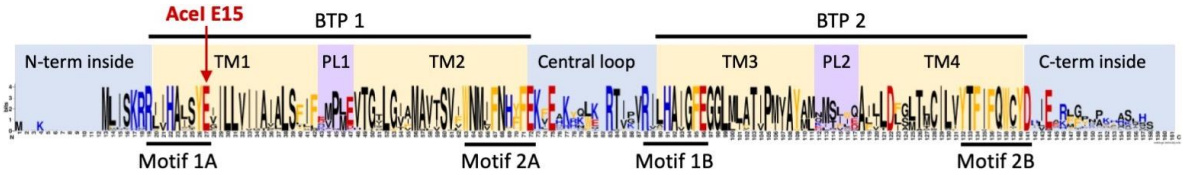


Figure 2. Sequence conservation within putative AceI orthologs across the *Acinetobacter* genus. The "reference" genome sequences for 70 *Acinetobacter* species, and the sequence for *A. baumannii* ATCC 17978, were obtained from the NCBI RefSeq database (15 Feb 2021). Putative orthologs were identified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

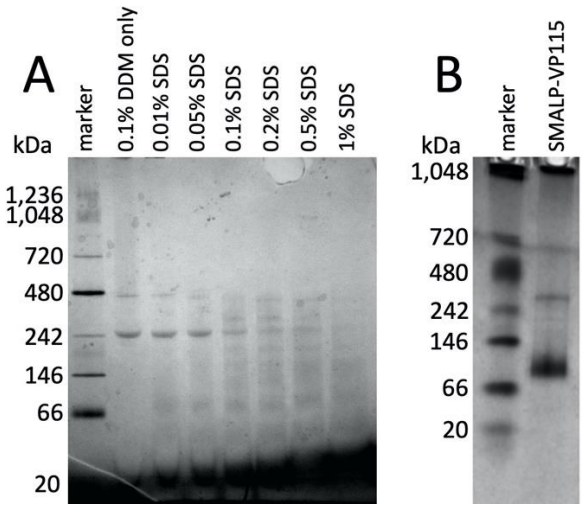


Figure 4. Native PAGE analyses on PACE family proteins. A) Blue-native PAGE of AceI. AceI protein fused to the C-terminal tag RGSH6 was overproduced from the expression vector pTTQ18 in *E. coli* DH5α (plasmid construct described in [6]). The cells were mechanically lysed and total membranes isolated by ultracentrifugation. The membranes were solubilised using in DDM and AceI purified by Ni-chelation chromatography. AceI protein was eluted in buffer containing 0.1 % DDM. The purified protein was electrophoresed on a precast Thermofisher NativePAGE Bis-Tris gel with blue-native PAGE buffers. The gel was subsequently stained using Coomassie blue. To disrupt the oligomeric state of the protein, increasing concentrations of SDS were added to equivalent samples of the DDM solubilised protein prior to electrophoresis. B) SMA-PAGE of VP1155 solubilised in styrene maleic acid copolymer lipid nanoparticles (SMALPs). VP1155 was overproduced from pTTQ18-VP1155 in *E. coli* DH5α. The cells were mechanically lysed and total membranes isolated by ultracentrifugation. The membranes were solubilised using SMA and VP1155 purified by Ni-chelation chromatography. Native PAGE was performed using the purified VP1155 protein. The gel was subsequently silver stained. (N=2). Lane 1: Native protein marker (Thermo Scientific). Lane 2: SMA-isolated VP1155. The Rf values of each protein band were used to estimate the molecular weight (MW) of each of the bands observed in the sample.

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* special interest

** outstanding interest

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