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Short chain diamines are the physiological substrates of PACE family efflux pumps

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Abstract

Acinetobacter baumannii has rapidly emerged as a major cause of Gram-negative hospital infections worldwide. A. baumannii encodes for the transport protein AceI, which confers resistance to chlorhexidine, a widely-used antiseptic. AceI is also the prototype for the recently discovered Proteobacterial Antimicrobial Compound Efflux (PACE) family of transport proteins that confer resistance to a range of antibiotics and antiseptics in many Gram-negative bacteria, including pathogens. The gene encoding AceI is conserved in the core genome of A. baumannii, suggesting it has an important primordial function. This was incongruous with the only characterised substrate of AceI, chlorhexidine, an entirely synthetic biocide produced only during the last century. Here we investigated a potential primordial function of AceI and other members of the PACE family in the transport of naturally occurring polyamines. Polyamines are abundant in living cells, where they have physiologically important functions and play multifaceted roles in bacterial infection. Gene expression studies revealed that the aceI gene is induced in A. baumannii by the short chain diamines cadaverine and putrescine. Membrane transport experiments conducted in whole cells of A. baumannii and Escherichia coli, and in proteoliposomes showed that AceI mediated the efflux of these short chain diamines when energised by an electrochemical gradient. Assays conducted using eight additional diverse PACE family proteins identified three that also catalysed cadaverine transport. All together these results demonstrate that short chain diamines are common substrates for the PACE family of transport proteins, adding to their broad significance as a novel family of efflux pumps.

Significance

Multidrug efflux pumps are highly promiscuous determinants of antimicrobial resistance in bacterial pathogens. Since efflux pumps evolved long before the widespread use of antimicrobials, drug transport is likely to be a side reaction in many pumps, fortuitously beneficial to bacteria in hospitals. The AceI efflux protein from Acinetobacter baumannii is the prototype for the PACE family. AceI was only known to transport the synthetic biocide chlorhexidine, which was incongruous with its ancient origin. Here we demonstrate that short chain diamines are the physiological substrates of AceI and other PACE members, and that transport is energised by an electrochemical gradient of protons. These observations are important because diamines play vital roles in bacterial physiology and virulence, and have significant commercial uses.

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Introduction

Multidrug efflux pumps are encoded in all bacterial genomes sequenced to date (1, 2). These proteins are most studied for their functions in drug resistance. However, multidrug efflux pumps participate in many additional processes, such as cell adherence, invasion, biofilm formation, virulence, natural product secretion, and resistance to host encoded factors (3). This diverse array of functions reflects the promiscuous substrate recognition profiles of multidrug efflux pumps, which can extend well beyond antibiotics and biocides. Indeed, for many multidrug efflux pumps the natural physiological substrates are likely to be endogenously produced small molecules, and the recognition of antimicrobial compounds is a fortuitous consequence of flexible substrate recognition pockets that can accommodate a diverse array of chemical structures.

A major goal of this study was to elucidate the physiological function of the AceI efflux pump, the prototype for the most recently discovered family of multidrug efflux pumps, and also of its homologues known as the Proteobacterial Antimicrobial Compound Efflux (PACE) family (4, 5). AceI is encoded by a gene that is highly conserved in the core genome Acinetobacter baumannii. Prior to this study the only recognised substrate for the AceI efflux pump was the biocide chlorhexidine. Chlorhexidine is a bisbiguanide compound consisting of terminal proguanil groups separated by a 1,6-diaminohexane moiety. Chlorhexidine is of massive importance in pathogen control, it is listed as an “Essential Medicine” by the World Health Organisation and is used globally in a broad range of antiseptic and disinfectant preparations. Despite its current importance, chlorhexidine was first synthesised only last century, so would not have been present in the environment across the evolution of the AceI pump, and could not have imposed selective pressure for aceI gene maintenance during this time.

Some naturally occurring polyamines share structural similarities with chlorhexidine, since their molecules are comprised of aliphatic carbon chains with interspersed and/or terminal amino groups that are typically charged at physiological pH (6). Therefore, polyamines were viewed as candidates for potential physiological substrates of AceI and other PACE pumps. Polyamines such as cadaverine, putrescine, spermidine and spermine are common in prokaryotic and eukaryotic cells, where they may exist at high concentrations (mM) and have varied vital functions in protein and nucleic acid stability, metabolism, nitrogen storage, acid tolerance, cell to cell communication, motility, transcriptional regulation and protein expression (7, 8). These biologically abundant polyamines also function in host immune responses and bacterial virulence (8).

A number of different transport systems that promote the uptake of polyamines into bacteria have been characterised (9). These uptake systems may help to support the high cellular requirements for polyamines and facilitate catabolism of exogenous polyamines [9]. Despite their high abundance and broad physiological significance, polyamines can inhibit cell growth when they are in excess, requiring cells to have detoxification mechanisms. Efflux pumps that transport polyamines out of the cell have also been identified (10, 11). Active polyamine efflux systems could help to maintain cellular concentrations of polyamines at sub-toxic levels, and may also be required to export polyamines involved in cellular communication or bacterial motility. Bacterial multidrug efflux pumps from the small multidrug resistance (SMR; e.g., MdtIJ in Escherichia coli) family and the major facilitator superfamily (MFS; e.g., Blt from Bacillus subtilis) have previously been shown to mediate polyamine efflux (10, 11). In this study, we examined polyamines as potential physiological substrates of the prototypical PACE family member, AceI from Acinetobacter baumannii (4), and some of its homologues.

Active efflux pumps need to capture the energy required to move their primary substrate against its concentration gradient out of the cell. In previously characterised families of drug efflux proteins, the energy is provided by a secondary substrate such as ATP, which provides chemical energy during hydrolysis to ADP, or a transmembrane electrochemical gradient of monovalent cations (typically H^+ or Na^+, and occasionally K^+) that are exchanged for primary substrates in antiport reactions (12). AceI and other PACE family proteins do not include nucleotide binding domains that are typically associated with ATP-driven pumps and are not encoded in proximity to, or coordinately regulated with, genes encoding these domains in bacterial genomes. Therefore, it was likely that PACE proteins energised the transport of their primary substrates using an electrochemical gradient, which we now show is the proton-motive-force. In addition, we determine the specificities of induction and transport for polyamines using both intact cells and liposomes containing isolated AceI.

Results and Discussion

Expression of the aceI efflux pump gene in A. baumannii is induced by polyamines

We conducted qRT-PCR to determine whether the addition of exogenous polyamines would elicit a transcriptional response in aceI gene expression in Acinetobacter baumannii. The cells were treated with four polyamines including the short chain diamines cadaverine and putrescine, the tri-amine spermidine, and the tetra-amine spermine. These polyamines were chosen for analysis since they represent the major groups of polyamines found in most organisms (8). We found that both of the diamines caused strong induction of aceI gene expression, around 20-fold higher than in untreated cells (Figure 1A). In contrast, aceI expression was only moderately induced by spermidine (5.2-fold induction) and weakly induced by spermine (2.8-fold induction). The initial discovery of the AceI pump was based on upregulation of aceI by chlorhexidine (4). In these investigations chlorhexidine caused an approximately ten-fold increase in aceI expression in A. baumannii ATCC 17978 (4).

aceI is required for A. baumannii to tolerate exogenous diamines

The induction of aceI by the addition of cadaverine or putrescine, and to a lesser extent spermidine and spermine, suggested that these compounds may be substrates of the AceI efflux pump. Since these polyamines have some level of toxicity towards many species of bacteria, we tested this possibility by examining the requirement of the aceI gene for polyamine tolerance in A. baumannii. Minimum inhibitory concentration analyses were conducted to test tolerance to putrescine, cadaverine, spermidine and spermine, in wild-type A. baumannii AB5075-UW, and in an isogenic aceI-inactivated mutant strain (13). The parental strain tolerated high concentrations of all four polyamines; the minimum inhibitory concentrations were 40 μg/ml in the case of putrescine, cadaverine and spermidine, and 10 μg/ml for spermine (SI Appendix, Fig. S1). Inactivation of aceI in AB5075-UW resulted in at

least eight-fold reductions in tolerance to cadaverine and putrescine, but no change in tolerance to spermidine or spermine (SI Appendix, Fig. S1). This suggested that AceI may recognise diamines as substrates, but that it does not recognise tri- or tetra-amines, or does so only weakly. The minimum inhibitory concentration of chlorhexidine for A. baumannii is more than 1000-fold lower than that of cadaverine or putrescine, but inactivation of aceI in A. baumannii causes only a two-fold reduction in chlorhexidine tolerance (14). The high fold-reductions in tolerance after aceI inactivation observed for cadaverine and putrescine suggest efflux of short chain diamines may be a primary role of AceI.

Expression of the AceI protein in Acinetobacter baumannii or Escherichia coli reduces the accumulation of cadaverine

To test whether the reduced tolerance to diamines in the aceI mutant A. baumannii strain was related directly to the efflux of these compounds, we examined accumulation of [^{14}C]-labelled cadaverine into mutant cells compared to the parental strain. The parental AB5075-UW strain accumulated minimal amounts of [^{14}C]-cadaverine when it was added to the media (Fig. 1B). In contrast, the aceI inactivated mutant readily accumulated [^{14}C]-cadaverine, suggesting that normal levels of AceI induced by cadaverine in Acinetobacter baumannii prevent high accumulation of the diamine into the cells, consistent with cadaverine efflux activity.

Laboratory E. coli strains are excellent hosts for studies examining the function of PACE family proteins because they do not carry genes encoding endogenous PACE family proteins that could interfere with the activity of heterologously expressed proteins; indeed PACE family genes have been found in less than 0.2 % of sequenced E. coli genomes and never in laboratory strains (4, 15). To confirm that AceI isolated from A. baumannii does mediate the efflux of short chain diamines, we examined the level of [^{14}C]-cadaverine accumulation in E. coli cells expressing AceI compared to cells expressing an inactive AceI mutant harbouring an E15Q substitution (AceI-E15Q) and to control cells carrying the empty vector expression

plasmid (Fig. 1C). Importantly, immunoblot analyses demonstrated that both AceI and AceI-E15Q were expressed at similar levels in the induced cells (SI Appendix, Fig. S2). [^{14}C]-Cadaverine was readily accumulated in both the empty vector control cells and the cells expressing the AceI-E15Q mutant protein. In contrast, the concentration of [^{14}C]-cadaverine in E. coli cells expressing the parental AceI protein did not significantly increase over the time course of the experiment (Fig. 1C), suggesting that AceI can transport cadaverine out of the cell at a rate equal to or higher than its rate of accumulation. The AceI-E15Q protein was previously shown to be incapable of mediating chlorhexidine resistance or transport (4). Since cells expressing this mutant accumulated [^{14}C]-cadaverine to a similar level as the negative control (Fig. 1C) it appears that an acidic residue at position 15 is necessary for all transport activity of AceI.

The AceI protein promotes the transport of [^{14}C]-cadaverine in a reconstituted system

Previous attempts to obtain measurements of AceI mediated transport of chlorhexidine in a reconstituted system were not successful (4). At that time, chlorhexidine was the only recognised substrate for the protein, and chlorhexidine is very poorly suited to experiments using proteoliposomes. Specifically, chlorhexidine is poorly soluble, it adsorbs non-specifically to most filter membranes and to biological membranes, and it is a membrane active biocide, so even low concentrations tend to disrupt naked proteoliposomes. The identification of cadaverine and putrescine as potential substrates of the AceI transport system in this study presented a new opportunity to examine the activities of AceI in a reconstituted membrane bilayer system.

Both the wild-type AceI protein and the inactive AceI-E15Q mutant protein were purified and reconstituted into preformed liposomes composed of E. coli polar lipids (SI Appendix, Methods). For comparison empty liposomes were also generated using the same approach. An experimental system was established to generate an electrochemical gradient across the

(proteo)liposome membranes, consisting of both a chemical proton gradient (ΔpH; inside acidic) and an electrical potential ($\Delta\Psi$; inside positive) (Fig. 2A). The lumen of the (proteo)liposomes contained Na^+ at pH 7.0. The (proteo)liposomes were diluted into a buffer containing isosmolar K^+ and a low concentration of the potassium ionophore valinomycin at pH 8.0. The difference in pH of the buffers generated ΔpH, and the valinomycin promoted the downhill movement of K^+ into the (proteo)liposomes, generating $\Delta\Psi$ inside positive (Fig. 2A) (16, 17). The polarity of the pH and charge differential (inside positive and acidic) across the membrane could energise the uptake of externally applied substrates in exchange for a cation, such as a proton, by any active antiport system (16), in this case AceI (Fig. 2A).

There was some uptake of cadaverine into liposomes without incorporated protein (Fig. 2B), which was probably the result of uncharged cadaverine diffusing across the membrane and accumulating in the lumen by protonation. The proteoliposomes containing wild-type AceI and energised by an electrical and pH gradient positive and acidic inside accumulated [^{14}C]-cadaverine (Fig. 2B) much more rapidly. After subtraction of the small amount of accumulation in liposomes the initial rate of cadaverine uptake between the first and second assay time points (30 and 120 sec) was 16.7±5.9 nmol/mg protein/min (SI Appendix, Fig. S3). In contrast, the rate of [^{14}C]-cadaverine uptake into proteoliposomes containing the inactive AceI-E15Q mutant was indistinguishable from the rate into liposomes without any incorporated protein, calculated to be -0.2 ±0.7 nmol/mg protein/min between the first and second time points (Fig. 2B and S3). AceI proteoliposomes that were not treated with valinomycin, and thus had ΔpH (inside acidic) but no $\Delta\Psi$, accumulated [^{14}C]-cadaverine at a much lower rate of 3.9±3.7 nmol/mg protein/min between the first and second assay time points (Fig. 2B and S3, SI Appendix, Table S1). AceI proteoliposomes were also diluted into the same buffer without valinomycin, but with the protonophore CCCP that discharges both electrical and pH gradients. In this case, the proteoliposomes still took up [^{14}C]-cadaverine,

214 but at a much slower rate of 3.1 ±2.0 nmol/mg protein/min between the first and second assay
215 time points (Fig. 2B and S3, SI Appendix, Table S1) a reduction of about 80% in activity
216 from the energised rate. Based on these results and those described below, energised AceI-
217 mediated cadaverine transport appears to be driven by proton exchange. The intermediate
218 levels of [^{14}C]-cadaverine accumulation seen in the absence of valinomycin or presence of
219 CCCP, which were above that seen in the liposome negative control, may be driven by the
220 unenergized downhill movement of [^{14}C]-cadaverine into the proteoliposomes facilitated by
221 AceI, i.e. a relatively low level of 'uniport' (facilitated diffusion) activity, which might even
222 reflect a variable stoichiometry of cadaverine:H^+, as seen in other secondary transporters (18).
223 Also, low level transport may be driven by an alternative coupling ion, such as Na^+, which is
224 present in the lumen of the proteoliposomes.

225 Overall, the rapid non-linear accumulation of [^{14}C]-cadaverine into the reconstituted
226 proteoliposomes fully energised by both $\Delta\Psi$ and ΔpH, markedly above the levels seen in the
227 control and uncoupled experiments, demonstrated that AceI can promote the active transport
228 of cadaverine in exchange for a cation.

229 [^{14}C]-cadaverine uptake into AceI proteoliposomes is inhibited by putrescine

230 With a reconstituted AceI transport assay system in place for [^{14}C]-cadaverine it was possible
231 to assess whether AceI can recognise alternative substrates by examining competitive
232 inhibition of [^{14}C]-cadaverine transport. Since putrescine and spermidine induced measurable
233 increases in expression of the aceI gene (Fig. 1A), we tested whether unlabelled putrescine or
234 spermidine reduced [^{14}C]-cadaverine uptake into AceI proteoliposomes at 50-fold (1 mM) and
235 500-fold (10 mM) molar excess over [^{14}C]-cadaverine. Putrescine caused significant
236 inhibition of [^{14}C]-cadaverine uptake into AceI proteoliposomes, 43.9 % inhibition of uptake
237 by 1 mM putrescine and 85.9 % inhibition by 10 mM putrescine, equivalent to the addition of
238 excess unlabelled cadaverine (Fig. 2C). In contrast, spermidine caused only a marginal level

of inhibition of [^{14}C]-cadaverine uptake; 1 mM spermidine caused 15.7 % inhibition and 10 mM spermidine caused 31.0 % inhibition (Fig. 2C). These results suggest that putrescine can be transported by AceI at an equivalent rate to cadaverine, but that spermidine is poorly recognised by AceI, consistent with the results of the polyamine tolerance experiments (SI Appendix, Fig. S1), where inactivation of aceI in A. baumannii caused a reduction in tolerance to both putrescine and cadaverine, but not to spermidine. Therefore, of the polyamines tested, AceI appears to recognise specifically the short chain diamines, cadaverine and putrescine as substrates.

AceI-mediated cadaverine and putrescine transport into proteoliposomes occurs in parallel with internal pH changes

The results described above provided good evidence for AceI being a secondary active transport protein for cadaverine and putrescine, i.e., the reconstituted transport experiments showed that the generation of an inside positive electrical potential significantly increased the rate of uptake of externally applied [^{14}C]-cadaverine into AceI proteoliposomes (Fig. 2). This suggested that AceI may catalyse a diamine:cation antiport reaction. To help confirm whether the cation was a proton we examined pH changes inside the lumen of AceI proteoliposomes during transport of diamine achieved by using a membrane impermeable pH sensitive fluorescent dye, 8-hydroxypyrene-1,3,6-trisulfonic acid (pyranine, see SI Appendix, Methods).

Experimental conditions were established to generate a proton electrochemical gradient (inside acidic and positive), described above using [^{14}C]-cadaverine for the assay of AceI transport activity in the reconstituted system. Proteoliposomes containing AceI or the AceI-E15Q mutant, and empty control liposomes were formed in Na^+ buffer at pH 7.0. To establish a pH gradient in the assay the (proteo)liposomes were diluted into buffer at higher pH (pH 8.0). An electrical gradient, $\Delta\Psi$, was generated by the inclusion of isosmolar K^+ in the

external assay buffer and the subsequent addition of 5 nM valinomycin to catalyse the downhill movement of K^+ into the lumen of the proteoliposomes (Fig. 3A). The addition of valinomycin caused a slow increase in the F_{509} (ex. 450 nm)/ F_{509} (ex. 400 nm) fluorescence ratio of the AceI proteoliposomes and empty liposomes, consistent with a gradual increase in the luminal pH likely due to the leakage of protons down their electrical gradient out of the (proteo)liposomes (Fig. 3A). The addition of 1 mM cadaverine to the energised AceI proteoliposomes resulted in a far more rapid alkalisation of the proteoliposome lumen, observed through a rapid increase in the F_{509} (ex. 450 nm)/ F_{509} (ex. 400 nm) fluorescence ratio of pyranine (Fig. 3A). In contrast, there was no significant change in the internal pH in empty liposomes or proteoliposomes containing AceI-E15Q after cadaverine addition (Fig. 3A). This result suggested that AceI catalyses the exchange of protons for cadaverine, i.e., the luminal pH increases as protons are exported in exchange for cadaverine. The level of fluorescence change induced by different concentrations of cadaverine (0-10 mM) in this assay system was examined to determine whether the rate of pH change could be saturated. The results indicated that the rate of fluorescence change was saturable (Fig. 3B) consistent with the operation of a protein-catalysed active transport activity specific for cadaverine. A Michaelis-Menten curve fit suggested a Km of 2.65±0.52 mM (Fig. 3B).

We next determined the effect of excluding valinomycin from the assay until after cadaverine addition to determine whether ΔpH alone could promote AceI-mediated cadaverine transport. Proteoliposomes containing AceI or the AceI-E15Q mutant, and empty control liposomes were formed in buffer containing Na^+ at pH 7.0, then diluted into isosmolar K^+ buffer at pH 8.0. Valinomycin was initially omitted and 1 mM cadaverine was added; this induced a pH change in the lumen of AceI proteoliposomes that was above the background level seen in the liposome control or in proteoliposomes containing AceI-E15Q (Fig. 3C). However, the fluorescence change was well below that seen when cadaverine was added to AceI

proteoliposomes after the generation of an electrical potential by valinomycin (Fig. 3A). Subsequent addition of valinomycin to the AceI proteoliposomes, thus generating an inside positive electrical potential, again resulted in a very rapid increase in the internal pH, well above that seen in the liposome control or in proteoliposomes containing AceI-E15Q (Fig. 3C). Similar to the reconstituted transport experiments using [^{14}C]-cadaverine, these results suggest that an electrical gradient, $\Delta\Psi$, can promote rapid AceI-mediated cadaverine transport.

To examine the effect of Δ_Ψ in the absence of ΔpH on AceI activity in this system, proteoliposomes containing AceI or the AceI-E15Q mutant, and empty control liposomes, were formed in buffer containing Na^+ at pH 7.0, then diluted into isosmolar K^+ buffer at pH 7.0. Valinomycin was added to generate an electrical potential, followed by cadaverine. In this system 1 mM cadaverine did not induce a significant change in pH in the interior of AceI proteoliposomes or in control liposomes that was reliably detectable using the pyranine indicator. However, increasing the concentration of cadaverine to 100 mM resulted in a large pH change inside the AceI proteoliposomes that was similar in amplitude to that seen after 1 mM cadaverine addition at pH 8.0 with valinomycin energisation (Fig. 3A and 3D). No significant change in pH was observed in the control liposomes or AceI-E15Q proteoliposomes after addition of 100 mM cadaverine under these conditions, showing that the effect was mediated by AceI (Fig. 3D) and not by the change in osmolarity across the liposome membrane. We also examined the effect of putrescine and spermidine addition to AceI proteoliposomes in this experimental system. The addition of 100 mM putrescine induced a significant pH change in AceI proteoliposomes, but spermidine did not (SI Appendix, Fig. S4). However, we did observe some precipitation of spermidine or buffer components upon its addition. These results are consistent with our observations that aceI promoted putrescine but not spermidine tolerance in A. baumannii (SI Appendix, Fig. S1), and that putrescine but not spermidine was able significantly to inhibit AceI-mediated [^{14}C]-

cadaverine transport into proteoliposomes (Fig. 2C). The results provide additional support that AceI can transport putrescine and cadaverine well, but spermidine poorly, at best.

The reason that a higher concentration of cadaverine was needed to induce a strong pH change in the $\Delta\psi$ assay system at pH 7.0 may be the result of the chemical properties of cadaverine and the nature of electrochemically driven transport reactions. Cadaverine has two amines with pKas of 10.25 and 9.13 (6). Consequently, the majority of the cadaverine added at neutral pH will be protonated and thus charged at both amines. Transport reactions driven by an electrical gradient alone must be electrogenic themselves (17). Therefore, if AceI mediates the exchange of cadaverine for protons driven by the electrical potential, the number of protons exchanged must increase with the charge state of cadaverine, i.e., one or more protons exchanged for the neutral form of cadaverine, two or more for a singly charged form and three or more for cadaverine charged at both amines. This may favour the transport of neutrally charged cadaverine. Since cadaverine has two ionisable groups with high pKas, at pH 7.0 the concentration of neutral compound would be approximately 100 times lower than at pH 8.0, since for each amine group there will be an approximately ten-fold increase in the non-protonated form for each 1 pH unit increase. This may be why a 100-fold increase in cadaverine concentration was required at pH 7.0 (Fig. 3D) to promote a pH change similar to that seen at pH 8.0 (Fig. 3A). If transport of fully neutral or monocationic forms of cadaverine is favoured by AceI under these experimental conditions, the alkalisation of the proteoliposome lumen may be at least partly related to the proton accepting potential of the deprotonated amines, i.e., protons may be accepted by the cadaverine accumulating within the proteoliposome, so increasing the internal pH. Therefore, it is not possible to conclude with complete certainty that protons, rather than an alternative cation, such as Na^+ ions are exchanged for substrates by AceI.

<u>pH changes induced in AceI proteoliposomes by chlorhexidine</u>

Chlorhexidine was the first substrate identified for AceI and is one of the most important biocides used in healthcare worldwide. As stated above, chlorhexidine is a membrane active biocide and our previous attempts to examine AceI-mediated transport in a reconstituted system were not successful, due to non-specific adsorption to filters and biological membranes, and potential lysis of proteoliposomes by chlorhexidine. The nature of the assays using pyranine-containing proteoliposomes alleviate the problems of adsorption to filters and biological membranes, since they do not require the proteoliposomes to be filtered, nor the total substrate associated with the proteoliposomes to be measured. Therefore, considering our success in developing this assay system to demonstrate AceI-mediated transport of cadaverine and putrescine, we attempted assays using chlorhexidine. An experimental system was employed to generate a pH gradient; (proteo)liposomes were formed in K^+ buffer at pH 7.0 and diluted into K^+ buffer at pH 8.0. The addition of 100 μM chlorhexidine to AceI proteoliposomes caused alkalisation of the AceI proteoliposome lumen, observed as an increase in pyranine fluorescence (450 nm excitation/509 nm emission; SI Appendix, Fig. S5). Addition of the same concentration of chlorhexidine to empty control liposomes caused a lower level of alkalisation [Note that the addition of an equivalent amount of DMSO, the solvent for chlorhexidine, to AceI proteoliposomes did not cause a change in the internal pH (SI Appendix, Fig. S5)]. These observations are consistent with AceI-mediated chlorhexidine:H^+ exchange, but must be viewed with caution, since the chlorhexidine may have damaged the naked (proteo)liposomes.

Expression of several other PACE family proteins in Escherichia coli reduces cadaverine accumulation

The results described above focused only on the AceI transport protein as the prototypical representative of the PACE family. To examine whether other members of the PACE family can catalyse the transport of cadaverine, we examined the level of [^{14}C]-cadaverine

accumulation in E. coli cells expressing eight additional PACE family proteins, Fbal_3166 from Ferrimonas balearica, PFL_4558 from Pseudomonas protegens, Pf-5, 655492601 (GenBank protein ID) from Tepidiphilus margaritifer, A1S_1503 from Acinetobacter baumannii, Mlut_15630 from Micrococcus luteus, PSPTO_3587 from Pseudomonas syringae pv. tomato str., STY3166 from Salmonella enterica subsp. enterica serovar Typhi str. and VP1155 from Vibrio parahaemolyticus RIMD 2210633 (SI Appendix, Fig. S6). Each of these proteins was produced at readily detectable levels in E. coli BL21 cells upon IPTG induction (SI Appendix, Fig. S7). Of the additional proteins examined, three prevented the accumulation of [^{14}C]-cadaverine into E. coli, similar to AceI (SI Appendix, Fig. S6). These results indicate that cadaverine, and possibly other short chain diamines, are the likely physiological substrates of many PACE family proteins.

Conclusions

The PACE family is the most recently discovered family of multidrug efflux proteins (5). The genes encoding PACE proteins are conserved in a range of opportunistic Gram-negative pathogens and may contribute to serious outbreaks of drug resistant infections in hospitals. Owing to the recency of their identification, their physiological substrates and mechanisms of transport energisation were unknown. In this study, we made several major advances in understanding the function of the prototypical PACE family protein, AceI from A. baumannii. Firstly, polyamines, specifically the short chain primary diamines cadaverine and putrescine, are strong inducers of aceI in A. baumannii (Fig. 1A). Secondly, cadaverine and putrescine are substrates of AceI, whereas the longer polyamine, spermidine is only a weak substrate of AceI, even though it induced aceI expression, albeit at a lower level than the equivalent concentrations of cadaverine and putrescine (Fig. 1A). Thirdly, transport of cadaverine was effected by AceI protein reconstituted into liposomes. Finally, the trans-membrane electrical gradient of protons is the primary source of energy for AceI-mediated cadaverine transport, and the pH gradient may make a contribution.

Polyamines are produced in all cells and have a raft of functions in cellular regulation, maintaining stability of nucleic acids and proteins, motility and cell to cell signalling (3). Furthermore, polyamines have multifaceted roles in bacterial virulence and in host immune responses (8). Many of these effects are likely to be mediated by transport proteins, such as AceI, that catalyse the efflux or uptake of polyamines. Indeed, activating or deactivating expression of polyamine transport proteins in various bacteria attenuated virulence (8, 19). Therefore, compounds that modulate polyamine transport systems may have potential to be used as virulence attenuating drugs. The design of such drugs will now be facilitated by the knowledge that the physiological substrates are short chain polyamines. In addition to functions in human health, diamines including cadaverine and putrescine examined in this

401 study, have a range of industrial uses especially as precursors of polymers related to nylon
402 (20). The discovery that AceI is a novel secondary transport system for these compounds adds
403 potential for developing new biological platforms for their large scale biotechnological
404 production based around AceI-mediated efflux, which would provide much needed “green”
405 alternatives to petroleum-based precursors currently produced (20).
406

Methods

A detailed description of all experimental procedures is provided in the SI Appendix, Methods section. Briefly, for quantitative real-time PCR analyses of aceI gene expression we followed methods described previously (4, 21). For polyamine tolerance tests we used strains from the Manoil laboratory collection (13), and a broth microdilution method (22). [^{14}C]-cadaverine dihydrochloride was obtained from American Radiolabelled Chemicals. Whole cell [^{14}C]-cadaverine accumulation assays were performed following routine methods (4). The reconstitution method used for AceI and AceI-E15Q proteins was developed from that used by Ramos Aires and Nikaido (2005) (23). The approaches for generating proton electrochemical gradients across the proteoliposome membranes are described in the Results and Discussion, and in detail in the SI Appendix, Methods section.

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Author contributions

KAH, PJFH and ITP conceived the research. KAH, JRE, KAM LF, SMJ, IA and DS performed the biochemical experiments and protein purifications. KAH, VN, KAM QL and LL performed microbiological experiments. KAH, PJFH and ITP analysed the data and wrote the manuscript, with all authors contributing to the final version.

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498 Figure legends.

499 Figure 1. (A) Induction of aceI gene expression by polyamines. Polyamines were added to the
500 A. baumannii AB5075-UW media when the cells were in mid-exponential growth phase (SI
501 Appendix, Methods). The bars represent the change in aceI gene expression compared to an
502 untreated control after 30 mins growth in the presence of the polyamines. Error bars show the
503 standard deviations of at least two biological and four technical replicates. (B) Accumulation
504 of [^{14}C]-cadaverine into A. baumannii wild-type (AB5075-UW) or an aceI inactivated mutant
505 (ΔaceI) (13). The cells were exposed to a low concentration of cadaverine for 30 mins to
506 allow aceI expression, then washed and incubated with 50 μM [^{14}C]-cadaverine using 1 %
507 succinate provided as an energy source. Each sample included 100 μL of cells at OD_{600} = 1.0
508 (SI Appendix, Methods). (C) Accumulation of [^{14}C]-cadaverine into E. coli BL21 cells
509 overexpressing the wild-type AceI protein (AceI), the inactive E15Q AceI mutant protein
510 (E15Q) or no additional protein (Negative). In these E. coli cells, expression of aceI or its
511 E15Q variant was induced by IPTG and the harvested, washed cells were incubated with 50
512 μM [^{14}C]-cadaverine with 1% glucose provided as an energy source. Each sample included
513 100 μL of cells at OD_{600} = 1.0 (SI Appendix, Methods). Error bars show the standard
514 deviation of at least three independent replicate experiments.

515 Figure 2. Uptakes of [^{14}C]-cadaverine into (proteo)liposomes. (A) Schematic representation of
516 the strategy used for generating a proton electrochemical gradient ($\Delta\bar{\mu}_{H+}$) across the
517 proteoliposome membrane. (Proteo)liposomes were formed in Na^+-containing buffer at pH
518 7.0, then diluted into K^+ buffer at pH 8.0 containing the K^+ ionophore valinomycin. (B)
519 Liposomes and proteoliposomes containing AceI or AceI-E15Q were formed (SI Appendix,
520 Methods) and treated as shown in panel A. The results show [^{14}C]-cadaverine uptake per
521 fraction. Uptake of [^{14}C]-cadaverine was faster in energised AceI proteoliposomes (blue line)

compared to empty liposomes (green line) or proteoliposomes containing the inactive AceI-E15Q protein (tan line). [^{14}C]-cadaverine uptake into AceI proteoliposomes without valinomycin, or with the addition of CCCP occurred at an intermediate rate (purple and red lines, respectively). (C) Inhibition of AceI mediated [^{14}C]-cadaverine transport by alternative AceI substrates. Transport experiments were performed as for the blue trace in panel B, but in the presence of 1 or 10 mM unlabelled cadaverine, putrescine or spermidine. The values shown are the amount of [^{14}C]-cadaverine accumulated after ten minutes as a percentage of the amount accumulated with no inhibitor. Error bars show the standard deviations of at least three independent replicate experiments.

Figure 3. Cadaverine-induced pH changes inside proteoliposomes containing AceI. Proteoliposomes were formed in buffer containing the pH sensitive fluorescent dye pyranine (SI Appendix, Methods). The excitation maximum of pyranine shifts from 400 nm to 450 nm with increasing in pH, whereas the emission maximum is stable at approximately 509 nm. Plots A, C and D show the 450:400 nm fluorescence (509 nm) excitation ratio of pyranine in the lumen as follows: control liposomes without added protein (green); proteoliposomes containing the inactive AceI-E15Q mutant protein (tan); and proteoliposomes containing wild-type AceI (blue). An increase in this ratio is indicative of an increase in internal pH. All (proteo)liposomes were formed in Na^+ buffer at pH 7.0 (SI Appendix, Methods). (A) (Proteo)liposomes were diluted into K^+ buffer at pH 8.0, 5 nM valinomycin (val) was added at the first arrow and 1 mM cadaverine was added at the second arrow. (B) AceI proteoliposomes were diluted into K^+ buffer at pH 8.0 containing 5 nM valinomycin (val) and the indicated concentrations of [^{14}C]-cadaverine. The initial rates of fluorescence change are plotted relative to the concentration of cadaverine, corrected for the small level of fluorescence change observed in liposomes containing no protein. From a Michaelis-Menten curve fitted to the data points an apparent Km of 2.65±0.52 mM was derived. (C)

(Proteo)liposomes were diluted into K^+ buffer at pH 8.0, 1 mM cadaverine was added at the first arrow and 5 nM valinomycin was added at the second arrow. (D) (Proteo)liposomes were diluted into K^+ buffer at pH 7.0, 5 nM valinomycin was added at the first arrow and 100 mM cadaverine was added at the second arrow. The experiments were performed using at least three independent batches of (proteo)liposomes and error bars show the standard deviations.