OPEN ACCESS

Scholars International Journal of Biochemistry

Abbreviated Key Title: Sch Int J Biochem
ISSN 2616-8650 (Print) |ISSN 2617-3476 (Online)
Scholars Middle East Publishers, Dubai, United Arab Emirates
Journal homepage: https://saudijournals.com

Original Research Article

Proteobacterial Antimicrobial Compound Efflux (PACE) Family Transport Proteins from Gram-Negative Bacteria: A Strategy for Gene Cloning, Amplified Expression and Large-Scale Purification

Irshad Ahmad[1,2], Simon G. Patching[1]*

[1]School of Biomedical Sciences (Astbury Building), University of Leeds, Leeds LS2 9JT, UK
[2]Institute of Basic Medical Sciences, Khyber Medical University, Peshawar 25100, Pakistan

DOI: https://doi.org/10.36348/sijb.2026.v09i01.001 | **Received:** 26.12.2025 | **Accepted:** 28.02.2026 | **Published:** 02.03.2026

***Corresponding author:** Simon G. Patching
School of Biomedical Sciences (Astbury Building), University of Leeds, Leeds LS2 9JT, UK

Abstract

Proteobacterial Antimicrobial Compound Efflux (PACE) family proteins are one of seven types of multidrug efflux pumps in Gram-negative bacteria. PACE proteins can actively efflux synthetic biocides, including the antiseptic chlorhexidine, whilst their physiological substrates are polyamines. PACE proteins contain four putative transmembrane-spanning alpha-helices, and experimental evidence suggests that they mainly exist and function in a dimeric state, but the high-resolution structural organisation and molecular mechanism of PACE proteins are yet to be elucidated experimentally. As an essential first step to achieve this, we employed a strategy for gene cloning, expression screening and large-scale purification of representative PACE proteins. The genes of 24 proteins were successfully cloned into IPTG-inducible plasmid pTTQ18 directly upstream from a His_6-tag coding sequence and transformed into *E. coli* BL21(DE3) cells. Small-scale expression tests identified seven proteins amplified at a sufficient level for larger-scale cultures and purification. Based on results from 30-litre fermentor cultures and inner membrane preparations, four proteins (A1S_2063, Fbal_3166, STY_3166, Tmarg_opt) representing distinct phylogenetic groups of the PACE family, were progressed to detergent solubilisation and purification. These proteins had purities of 86, 84, 80 and 78%, and purification yields of 1.1, 1.1, 1.3 and 1.0 mg/litre cell culture, respectively. The detergent-solubilised purified proteins had far-UV circular dichroism spectra consistent with alpha-helical secondary structure, producing melting temperatures of 46.7, 34.2, 32.6 and 37.6 °C, respectively. A1S_2063 was most stable and might be best for structure elucidation. Secondary structure in all purified proteins appeared reasonably stable for performing biochemical and biophysical experiments up to 25 °C.

Keywords: Antibiotic Resistance, Gene Cloning, Gram-Negative Bacteria, Hospital-Acquired Infection, Multidrug Efflux, Protein Expression, Protein Purification, Structural Biology Pipeline.

1. INTRODUCTION

A principal mechanism that bacteria have evolved for resistance to antimicrobial agents is active multidrug efflux, whereby harmful compounds are expelled from the bacterial cell membrane or cytoplasm to the external environment by transport proteins driven by an ion gradient. Such proteins, also known as efflux pumps, may be highly specific for one compound or may be highly promiscuous, transporting a broad range of structurally dissimilar substrates. This mechanism is present in all types of bacteria, including those that are pathogenic to humans, animals and plants (Levy, 1992; Nikaido, 1994; Bolhuis *et al*., 1997; Nikaido, 2003; Webber & Piddock, 2003; Li & Nikaido, 2004; Kaatz, 2005; Kumar & Varela, 2012; Sun *et al*., 2014; Spengler *et al*., 2017; Yılmaz & Özcengiz, 2017; Ahmad *et al*., 2018a; Huang *et al*., 2022; Siasat & Blair, 2023; Ahmad *et al*., 2025; Elshobary *et al*., 2025). As a result of their cell structure and the activity of multidrug efflux proteins, Gram-negative bacteria (e.g. *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*) are intrinsically more resistant to antibiotics and biocides than Gram-positive bacteria (Zgurskaya & Nikaido, 2000; Zgurskaya *et al*., 2003; Pagès *et al*., 2010; Nikaido & Pagès, 2012; Blair *et al*., 2014; Li *et al*., 2015; Schindler & Kaatz, 2016). They are responsible for the majority of highly or extremely drug-resistant bacterial infections (Tacconelli

et al., 2018; Gurvic & Zachariae, 2024), including 30% to 70% of hospital acquired infections (Kunz & Brook, 2010; Peleg & Hooper, 2010; Chelazzi *et al*., 2015; Mehrad *et al*., 2015; Ruppé *et al*., 2015; Parajuli *et al*., 2017; Badger-Emeka *et al*., 2023; Alkhowaiter *et al*., 2024).

Based on amino acid sequence similarity, substrate specificity and the energy source used to export their substrates, seven distinct families of bacterial multidrug efflux transport proteins have been identified (Kourtesi *et al*., 2013; Du *et al*., 2015; Chitsaz & Brown, 2017; Shaheen *et al*., 2017; Ahmad *et al*., 2018a; Huang *et al*., 2022; Siasat & Blair, 2023; Ahmad *et al*., 2025; Elshobary *et al*., 2025): adenosine triphosphate (ATP)-binding cassette (ABC) superfamily (Chang, 2003; Lubelski *et al*., 2007), resistance-nodulation-division (RND) family (Tseng *et al*., 1999; Routh *et al*., 2011), major facilitator superfamily (MFS) (Saidijam *et al*., 2006; Ranaweera *et al*., 2015), small multidrug resistance (SMR) family (Paulsen *et al*., 1996; Bay & Turner, 2009), multidrug and toxic compound extrusion (MATE) family (Omote *et al*., 2006; Kuroda and Tsuchiya, 2009), proteobacterial antimicrobial compound efflux (PACE) family (Hassan *et al*., 2013; Hassan *et al*., 2015a), p-aminobenzoyl-glutamate transporter (AbgT) family (Su *et al*., 2015; Delmar & Yu, 2016). Of these, the PACE family is least characterised and the only one without experimentally derived high-resolution structure information.

The PACE family originated by exploiting the adaptive resistance response, whereby a novel protein called AceI (Acinetobacter chlorhexidine efflux protein I) from *A. baumanii* (gene locus A1S_2063) was identified to confer resistance to the bisbiguanide antiseptic chlorhexidine (1,6-bis(4-chloro-phenyl-biguanido)hexane) using an active efflux mechanism driven by the electrochemical proton gradient (Hassan *et al*., 2013; Hassan *et al*., 2015a). Because close homologues of AceI are especially prevalent in *Proteobacteria*, this type of bacterial multidrug efflux protein was designated as the Proteobacterial Antimicrobial Compound Efflux (PACE) family (Hassan *et al*., 2015a; Hassan *et al*., 2015b). AceI and many representative PACE family proteins not only confer resistance to chlorhexidine but also to other biocides including benzalkonium, dequalinium, proflavine and acriflavine (Hassan *et al*., 2015b). These biocides are all synthetic compounds only available for the past 50-100 years, which is long after the evolutionary development of the proteins, so they are not the original substrates of the proteins. This is supported by the observation that the genes encoding PACE family proteins are conserved in the core genomes of bacteria instead of on recently acquired mobile genetic elements, so they are likely to confer important core functions as well as biocide resistance (Hassan *et al*., 2018). Based on experiments with AceI, the physiological substrates of PACE family proteins were identified as polyamines (e.g. spermidine, spermine, putrescine and cadaverine), first by using near-UV synchrotron radiation circular dichroism spectroscopy (experiments conducted in 2013) (Patching, 2022) and then by membrane transport experiments conducted in whole bacterial cells and in proteoliposomes (Hassan *et al*., 2019).

Representative members of the PACE family, selected to encompass the full spectrum of currently sampled phylogenetic diversity, have a length of 135-180 residues (average 151 residues) and contain two putative tandem bacterial transmembrane pair (BTP) domains. The Pfam database (http://pfam.xfam.org/, version 31.0) (Finn *et al*., 2014) currently lists 914 proteins containing BTP (Pfam accession number PF05232) domains from 400 different bacterial species. Close homologues of PACE family/BTP-containing proteins have not been identified in any archaeal or eukaryotic organisms. In all PACE family proteins, a highly conserved glutamic acid residue (Glu50 in full-length AceI) is found in the middle of putative transmembrane helix 1, for which mutation to glutamine renders the protein unable to mediate chlorhexidine resistance and transport, but still able to bind chlorhexidine (Hassan *et al*., 2013). Native mass spectrometry experiments demonstrated that the functional form of AceI is a dimer, and that assembly of the dimer is mediated by binding of chlorhexidine and promoted by high pH (7 to 9). Mutation of the conserved glutamic acid residue to glutamine prevented dimerisation of the protein with increasing pH, confirming its important role in transporter function (Bolla *et al*., 2020). A phylogenetic and functional study demonstrated that PACE transporter PA2880 from *P. aeruginosa* exists mainly as a dimer in solution. Dimer formation was independent of pH and promoted by chlorhexidine, and the dimeric state was essential for proper function of PA2880. Like AceI, PA2880 transported chlorhexidine/H^+ via an antiport electrogenic mechanism (Zhao *et al*., 2022).

The structural organisation and molecular mechanism of PACE family proteins have yet to be elucidated by determination of high-resolution structure and further experimental investigation. As an essential first step to achieve this, we have performed expression screening in *E. coli* of twenty-four representative PACE family proteins, followed by scaling up to larger cultures, inner membrane preparation and purification of the best expressed proteins.

2. MATERIALS AND METHODS

2.1. General

All media, buffers and other solutions were prepared using either deionised water or MilliQ™ water. All media were sterilised by autoclaving or for thermally sensitive solutions by passage through 0.2 µM Minisart® high-flow sterile syringe-driven filters (Sartorius AG, Germany) or using vacuum-driven 0.2 µM Stericup® filters (Merck Millipore, USA). Protein determinations

were conducted using the method of Schaffner & Weissmann (1973) or a bicinchoninic acid (BCA) assay using Pierce® BCA protein assay reagent (Thermo Fisher Scientific, UK).

2.2. Gene Cloning and Transformation of *E. coli*

Gene cloning and amplification of protein expression in *E. coli* was achieved using a strategy that we and others have found successful with a wide range of bacterial and archaeal membrane proteins (Ward *et al*., 1999; Saidijam *et al*., 2003; Xie *et al*., 2004; Saidijam *et al*., 2005; Clough *et al*., 2006; Suzuki & Henderson, 2006; Szakonyi *et al*., 2007; Gordon *et al*., 2008; Ma *et al*., 2008; Bettaney *et al*., 2013; Ma *et al*., 2013; Ma *et al*., 2016; Ahmad *et al*., 2018b; Ahmad *et al*., 2020; Ahmad *et al*., 2021; Ahmad *et al*., 2022; Patching, 2024; Ali *et al*., 2025). PCR primers were designed to extract and amplify the specific gene from genomic DNA, introducing in-frame *Eco*RI (GAATTC) and *Pst*I (CTGCAG) restriction sites at the 5' and 3' ends, respectively. This was to allow ligation into the multi-cloning site of plasmid pTTQ18 (Stark, 1987) downstream from the isopropyl-β-D-1-thiogalactopyranoside (IPTG)-inducible *tac* promoter and immediately upstream from an RGS(His_6)-coding sequence that we had already engineered into the plasmid (Ward *et al*., 1999; Ward *et al*., 2000). Sequences of the genes of interest were obtained from the Transporter Protein Analysis Database (http://www.membranetransport.org/) (Paulsen *et al*., 1998; Ren *et al*., 2006) or the UniProt KnowledgeBase (http://www.uniprot.org/). Restriction sites in the genes of interest were mapped using Webcutter 2, which allowed checking for the presence of any *Eco*RI or *Pst*I restriction sites used for cloning. Only genes that did not contain internal *Eco*RI or *Pst*I sites were kept for cloning. Primers were designed with the following ideal properties where possible: a length of between 25 and 45 bases, a melting temperature ≥70 °C, a minimum GC content of 40%, termination with a G or C base, absence of primer dimers or other secondary structures. Primer properties and quality were predicted using OligoAnalyzer 3.1 software. Primers were prepared in sterilised water (10 pmol/µl) and stored at -20 °C.

PCR amplification was performed using a PTC-200 Peltier Thermal Cycler (MJ Research, UK). The restriction-digested PCR product was ligated with the restriction-digested pTTQ18. The ligation product was transformed into competent *E. coli* Omnimax cells (Agilent Technologies, USA) in the presence of carbenicillin (100 µg/ml) followed by PCR screening of colonies, extraction of plasmid DNA from positive clones and restriction digestion analysis using *Eco*R1 and *Pst*1 enzymes (New England Biolabs, USA). Plasmid DNA was isolated from bacterial overnight cultures and purified using a QIAprep Spin Miniprep Kit (QIAGEN Ltd, UK). Isolated plasmid DNA was stored at -20 °C. Plasmid DNA was sequenced by Beckman Coulter Genomics (BCG, UK) using minimum volumes of 15 µl at 100 ng/µl concentration. Plasmid DNA from successful ligations was transformed into *E. coli* BL21(DE3) cells (Novagen™, Merck Millipore, USA) followed by a test for inducible expression of the His_6-tagged protein by SDS-PAGE and Western blot analysis of membranes prepared by the water lysis method (Witholt *et al*., 1976; Ward *et al*., 2000) from small-scale (50 ml) cell cultures that were uninduced or induced with IPTG (Melford Laboratories Ltd, UK) (0.2 mM) (see further below). Clones of cells that showed successful amplified expression of the proteins were transferred into a freezing mixture (12.6 g/L K_2HPO_4, 0.9 g/L sodium citrate, 0.18 g/L $MgSO_4$, 1.8 g/L $(NH_4)_2SO_4$, 3.6 g/L KH_2PO_4, 96 g/L glycerol), frozen in liquid nitrogen and stored at -80 °C.

2.3. Preparation and Transformation of Competent Cells

E. coli competent cells were prepared using the method of Inoue *et al*. (1990). LB-agar plates containing carbenicillin (100 µg/ml) were incubated overnight and ten colonies were used to inoculate SOB medium (250 ml) in a 1-litre baffled flask. This was incubated at 18 °C with shaking at 220 rpm until the A_{600} reached ~0.6. Cells were placed on ice for 10 minutes before sedimentation (2500 x g, 10 minutes, 4 °C). Cells were resuspended in ice-cold transformation buffer (10 mM PIPES, 55 mM $MnCl_2$, 15 mM $CaCl_2.H_2O$, 250 mM KCl) (80 ml) using pre-cooled pipette tips and left on ice for 10 minutes. The cells were sedimented again (2500 x g, 10 minutes, 4 °C) and then gently resuspended in ice-cold transformation buffer (20 ml). DMSO was added to a final concentration of 7% (v/v) and the cells were left on ice for 10 minutes before dispensing in 50 µl aliquots, freezing in liquid nitrogen and storage at -80 °C.

Competent cells (50 µl) were thawed on ice prior to the addition of 5 µl (10-15 ng) of ligation mixture and incubated on ice for 30 minutes. The cells were heat-shocked at 42 °C for 45 seconds, followed by cooling on ice for 2 minutes. LB medium (450 µl) was added and the cells were incubated at 37 °C with shaking at 220 rpm for 1 hour to allow expression of the antibiotic resistance gene. The cells were sedimented by centrifugation (3000 x g, 2 minutes) and resuspended in LB medium (200 µl). An aliquot of the resuspended cells (100 µl) was streaked on to an LB-agar plate containing carbenicillin (100 µg/ml) and incubated at 37 °C overnight.

2.4. Small-Scale Expression Screening

Expression screening was performed on 50 ml cultures of *E. coli* BL21(DE3) cells harbouring the appropriate pTTQ18-based construct. Two batches of cells were grown to A_{600} = 0.8 in LB medium supplemented with glycerol (20 mM) and the antibiotic carbenicillin (Melford Laboratories Ltd, UK) (100 µg/ml). One batch was induced with IPTG (Melford Laboratories Ltd, UK) (0.2 mM) while the other was left uninduced and incubation was continued

for a further for 2 hours. Cells were harvested by centrifugation (4000 x g, 10 minutes, 4 °C) and the cell pellets were stored at -20 °C. At a later time, total/mixed (inner plus outer) membranes were prepared by the water lysis method (Witholt *et al*., 1976; Ward *et al*., 2000). The cells were resuspended in 10-ml Tris-HCl buffer (0.2 M, pH 8.0) and shaken for 20 minutes. At time zero, 4.85 ml of 1 M sucrose, 0.2 M Tris-HCl pH 8.0, 1 mM EDTA was added followed by the addition of 65 µl of 10 mg/ml lysozyme solution after 1.5 minutes. After 2 minutes the reaction was terminated by addition of 9.6 ml MilliQ water and left stirring for 20 minutes. The resultant spheroplasts were sedimented (45000 x g, 20 minutes, 4 °C) then resuspended and disrupted in 15 ml MilliQ water using a homogeniser. A further sedimentation step (45000 x g, 20 minutes, 4 °C) followed for fractionation of the cytoplasm (supernatant), and the generation of mixed cell membranes (pellet). Membrane preparations were washed three-times with 30 ml NaPi buffer (0.1 M pH 7.2) with 1 mM β-mercaptoethanol. The final membrane pellet was resuspended in 300 or 500 µl of NaPi buffer (0.1 M pH 7.2) with 1 mM β-mercaptoethanol and stored at -20 °C. Membrane preparations were analysed by SDS-PAGE and Western blotting using an antibody to the His_6 epitope for detection of amplified protein bands.

2.5. Large-Scale Culture and Inner Membrane Preparation

Culture volumes were scaled up to 30 litres in an ADI 1010 fermentor vessel (Applikon Biotechnology, Netherlands). Cells were grown to an A_{600} = 0.8 then pTTQ18-directed gene expression was induced with IPTG (0.2 mM) for 2 hours. Cells were harvested and wet cell pellets in buffer (20 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 10% v/v glycerol) were stored at -80 °C. At a later time, cells were thawed gradually at 4 °C with gentle mixing and resuspended in disruption buffer (20 mM Tris-HCl pH 7.5 containing 0.5 mM EDTA). Usually, 3 or 4 ml of disruption buffer was used for 1 g (wet weight) of bacterial cells. The suspension was stirred for 1 minute using an Ultra Turrax homogeniser (Cole-Parmer, UK) and thawed cells, which typically had a yoghurt-like consistency, were passed through a needle and syringe to ensure that the cells were fully defrosted and completely homogenised. Cells were disrupted at 4 °C by passing twice through a cell disrupter (Constant Systems Ltd, UK) at 30 kpsi according to the manufacturer's instructions. Undisrupted cells and cell debris were removed by centrifugation (12000 x g, 45 minutes, 4 °C). The supernatant containing total (inner plus outer) membranes was collected and retained.

Inner/outer membranes were separated by sucrose gradient ultracentrifugation using a six-step sucrose gradient prepared with 55, 50, 45, 40, 35 and 30% w/v sucrose solutions. Membranes were resuspended in an appropriate volume, typically 5 ml, of 25% w/v sucrose dissolved in Tris-HCl buffer (20 mM, pH 7.5) with EDTA (0.5 mM) and loaded on to the sucrose gradient. Following ultracentrifugation (131,000 x g, 16 hours, 4 °C) the dense golden inner membrane fraction was drawn off at the 35-40% interface and resuspended in Tris-HCl buffer (20 mM, pH 7.5). Isolated membranes were washed three-times using Tris-HCl buffer (20 mM, pH 7.5) with sedimentation by ultracentrifugation (131,000 x g, 16 hours, 4 °C), resuspended in Tris-HCl buffer (20 mM, pH 7.5) and stored as aliquots at -80 °C.

2.6. Protein Solubilisation and Purification

Inner membrane preparations were resuspended in solubilisation buffer (20 mM Tris-HCl pH 8.0, 20 mM imidazole pH 8.0, 300 mM NaCl, 20% v/v glycerol, 1% w/v DDM [Melford Laboratories Ltd, UK]) and mixed for 2-3 hours at 4 °C. The membranes were then sedimented (131,000 x g, 1 hour, 4 °C) to remove the insoluble fraction. For purification by immobilised-metal affinity chromatography (IMAC), the supernatant was incubated with Ni-NTA resin (QIAGEN Ltd, UK) for 16 hours at 4 °C with mixing and then transferred to a BioRad column and the supernatant was run through the column to elute unbound components. The resin was washed with 150-200 ml of wash buffer 1 (10 mM Tris-HCl pH 8.0, 40 mM imidazole pH 8.0, 10% v/v glycerol, 0.05% w/v DDM) to remove any remaining unbound material. The His_6-tagged protein was removed by addition of elution buffer (10 mM Tris-HCl pH 8.0, 200 mM imidazole pH 8.0, 2.5% v/v glycerol, 0.05% w/v DDM). Volumes of eluted samples were reduced to 3 ml using Vivaspin 20 tube concentrators (4,000 x g) with molecular weight cut off (MWCO) 30 kDa (Sartorius AG, Germany). To remove the high concentration of imidazole, the 3 ml sample was then applied to a BioRad Econo-pac 10 DG desalting column (BioRad Laboratories, UK). Wash buffer 2 (10 mM Tris-HCl pH 8.0, 2.5% v/v glycerol, 0.05% w/v DDM) (5 ml) was applied to the column and the eluted fraction was collected in a Vivaspin 6 tube MWCO 30 kDa and spun at 4500 x g. The purified eluted fraction that had been concentrated to 4-20 mg/ml was dispensed into aliquots, frozen in liquid nitrogen and stored at -80 °C.

2.7. SDS-PAGE and Western Blotting

SDS-PAGE was performed by the method of Laemmli (1970), refined for membrane proteins as described by Henderson & Macpherson (1986), using 4% stacking gels and 15% resolving gels prepared from acrylamide (40%) and bisacrylamide (2%) solutions (BioRad Laboratories, UK) in a Mini PROTEAN 3 apparatus (BioRad Laboratories, UK). Estimation of protein molecular weights was achieved by reference to SDS-7 protein molecular weight markers (Sigma-Aldrich Co., USA). For gel analysis, samples containing 16 µg of protein were solubilised in 4x sample loading buffer (60 mM Tris-HCl pH 7.2, 10% v/v glycerol, 2% w/v SDS, 0.005% bromophenol blue, 3% β-

mercaptoethanol). Proteins were visualised by staining the gel in EZBlue™ stain and densitometric analysis was performed using the program ImageJ.

For Western blotting, samples containing 4 µg protein were separated on a gel followed by semi-dry transfer to a Fluorotrans™ membrane (Pall BioSupport, UK) using a BioRad TRANS-BLOT® SD apparatus (BioRad Laboratories, UK) operating at 18 volts for 35 minutes. Four pieces of filter paper were pre-soaked in 0.5 x SDS-PAGE running buffer. Two pieces of filter paper followed by the membrane, the polyacrylamide gel and two further pieces of filter paper were layered on to one another. The membrane was incubated with BSA (3%) in TBST (20 mM Tris-HCl pH 7.6, 0.05% v/v Tween-20, 0.5M NaCl) for 3 hours at 4 °C to block non-specific binding sites. The membrane was washed twice with TBST (20 ml) at room temperature for 10 minutes. The membrane was then incubated for 1 hour with HisProbe-HRP antibody (Thermo Fisher Scientific, UK) (10 ml) diluted to 1:5000 with TBST followed by three washes with TBST (20 ml) for 10 minutes each. A 6 ml SuperSignal West Pico chemiluminescent solution (Thermo Fisher Scientific, UK) was prepared by mixing West Pico luminol/enhancer solution (3 ml) and West Pico stable peroxide solution (3 ml) and the membrane was incubated for 3 minutes before wrapping in acetate paper for exposure using a Syngene G:BOX EF Gel Imaging Doc W Trans-Illuminator. Estimates of molecular weights of detected protein bands was achieved by reference to 12-225 kDa Rainbow markers (GE Healthcare Ltd, UK).

2.8. Circular Dichroism Spectroscopy

The secondary structure content of purified proteins was measured by far-UV circular dichroism (CD) spectroscopy using a Chirascan Plus instrument (Applied Photophysics, UK) at 20 °C with constant nitrogen flushing. Protein samples (0.15 mg/ml) in 10 mM NaPi (pH 7.5) buffer plus 0.05% DDM were analysed in a Hellma quartz cuvette with a 1.0 mm pathlength. Measurements in the wavelength range 180-260 nm used a scan rate of 1 nm/second. A spectrum of buffer alone was subtracted from all CD data, although the signal produced by the buffer was almost negligible. Thermal stability was analysed by ramping the temperature from 5-90 °C and finally back to 5 °C, where each increment was held for 60 seconds before a measurement was made. Changes in secondary structure were monitored at 209 nm. Melting temperatures were estimated using Global Analysis CD software-3. CD ellipticity values were converted to mean residue ellipticity (MRE, deg.cm^{-2}.dmol^{-1}).

3. RESULTS AND DISCUSSION

3.1. Details of PACE Family Proteins for Expression Screening

The twenty-four selected PACE family proteins for expression screening (Table 1) are from a range of bacterial species and were chosen to encompass the full spectrum of currently sampled phylogenetic diversity. They vary in length from 134 to 179 residues and their sequence identity with AceI ranges from 24.9% (for Mlut_15630 from *Micrococcus luteus*) to 62.6% (for ACIAD1978 from *Acinetobacter baylyi* and Arad_01702 from *Acinetobacter radioresistens*) (Table 1). Analysis of the protein sequences by online tools TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) (Krogh *et al*., 2001) and TOPCONS (http://topcons.cbr.su.se/) (Bernsel *et al*., 2009; Tsirigos *et al*., 2015) predicted four transmembrane spanning helices with both N- and C-terminal ends at the cytoplasmic side of the membrane helices in all cases (Table 1). The TOPCONS tool, which has been demonstrated as one of the best performing (Tsirigos *et al*., 2012; Saidijam *et al*., 2018), predicts exceptionally high conservation for locations of the four transmembrane helices, differing at most by only one or two residues at both cytoplasmic and periplasmic sides of each helix (Figure 1). All proteins conformed to the 'positive-inside' rule (von Heijne, 1992), whereby there is a predominance of positively charged residues in cytoplasmic regions. There are high concentrations of positively charged residues in the regions preceding TM1, loop TM2-TM3 and following TM4 (Figure 1). The predicted transmembrane regions do not contain any positively charged residues at all, which may only be a consequence of the methods used by the prediction tools (Saidijam *et al*., 2017). Negatively charged residues are slightly less abundant and predominantly found in the regions preceding TM1, loop TM1-TM2, loop TM2-TM3 and following TM4. Eight residues are identically conserved in all proteins, of which seven are in transmembrane regions (Figure 1). These include the glutamate in the middle of TM1 that is involved in chlorhexidine transport by AceI (Hassan *et al*., 2013).

Table 1: Details of 24 PACE family proteins from Gram-negative bacteria selected for gene cloning and expression screening.

	Protein/gene locus[a]	Organism of origin	UniProt[b]	Length[c]	MW (Da)[d]	Homology with AceI[e]	TM helices[f]	
1	AceI (A1S_2063)	*Acinetobacter baumannii*	A0A0E8PCH0	179	20,958	100% / 100%	4	4
2	A1S_1503	*Acinetobacter baumannii*	A0A062FCY8	145	16,529	34.5% / 59.3%	4	4
3	ACIAD1978	*Acinetobacter baylyi* ADP1	Q6FAW0	134	15,525	62.6% / 78.3%	4	4

	Protein/gene locus[a]	Organism of origin	UniProt[b]	Length[c]	MW (Da)[d]	Homology with AceI[e]	TM helices[f]	
4	PA14_26850	*Pseudomonas aeruginosa* PA14	A0A1T2VHI1	171	19,233	31.0% / 55.0%	4	4
5	PFL_4585	*Pseudomonas fluorescens* Pf-5	Q4K7W4	143	16,046	31.0% / 54.9%	4	4
6	PFL_4558	*Pseudomonas fluorescens* Pf-5	Q4K7Z1	144	16,641	34.0% / 62.5%	4	4
7	Vpar_0264	*Veillonella parvula* DSM 2008	D1BQY5	134	15,519	32.4% / 55.6%	4	4
8	VP1155	*Vibrio parahaemolyticus*	Q87QJ4	140	15,913	30.3% / 55.6%	4	4
9	Fbal_3166	*Ferrimonas balearica* DSM 9799	E1SV65	146	16,172	26.5% / 54.8%	4	4
10	P20429_2969	*Pseudoalteromonas sp.* BSi20429	G7F6R6	145	16,287	32.9% / 61.4%	4	4
11	MHA_0890_opt	*Mannheimia haemolytica* PHL213	A0A1S1ZIS3	137	16,072	33.3% / 59.0%	4	4
12	ROS217_23162	*Roseovarius sp.* 217	A3VYE7	145	16,270	30.2% / 58.6%	4	4
13	Mlut_15630	*Micrococcus luteus* NCTC 2665	C5CBD2	179	19,002	24.9% / 46.4%	4	4
14	Arad_01702 (HMPREF0018_01702)	*Acinetobacter radioresistens* SH164	C6RMX1	144	16,644	62.6% / 80/.6%	4	4
15	Entcl_2273	*Enterobacter cloacae* SCF1	E3G7A3	152	17,319	31.6% / 58.6%	4	4
16	Yreg_01962 (HMPREF0880_01962)	*Yokenella regensburgei*	G9Z395	160	18,339	27.5% / 55.0%	4	4
17	KPK_0842	*Klebsiella pneumoniae* 342	B5XUL5	153	17,626	29.4% / 58.2%	4	4
18	Ec_3891 (ECTW07793_0407)	*Escherichia coli* TW07793		152	17,736	28.9% / 54.6%	4	4
19	STY3166	*Salmonella typhi* CT18	Q8XG43	160	18,421	27.5% / 55.0%	4	4
20	PP_3512	*Pseudomonas putida* KT2440	Q88H53	146	16,568	28.3% / 62.3%	4	4
21	PSPTO_3587	*Pseudomonas syringae pv. tomato str.* DC3000	Q87Z44	169	19,505	29.6% / 55.0%	4	4
22	Bcen2424_2356	*Burkholderia cenocepacia* HI2424	A0A1U9MS91	148	16,582	30.4% / 59.5%	4	4
23	RP_mp1531 (RPS107_mp1531)	*Ralstonia solanacearum* PSI07	D8N2I5	145	16,171	35.1% / 64.1 %	4	4
24	Tmarg_opt	*Tepidiphilus margaritifer*		159	17,742	29.5% / 56.0%	4	4

[a]Name of the protein or gene locus according to NCBI (https://www.ncbi.nlm.nih.gov/)
[b]UniProt KnowledgeBase (http://www.uniprot.org/) entry identifier
[c]Length of the protein: number of amino acid residues
[d]Molecular weight of the protein in Da
[e]Percentage sequence homology with AceI (identical residues / identical + highly similar residues) determined from sequence alignment using Clustal Omega 1.1.0 (http://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers *et al*., 2011)
[f]Predicted number of transmembrane helices in the protein given by TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) (Krogh *et al*., 2001) (*left*) and TOPCONS (http://topcons.cbr.su.se/) (Bernsel *et al*., 2009; Tsirigos *et al*., 2015) (*right*).

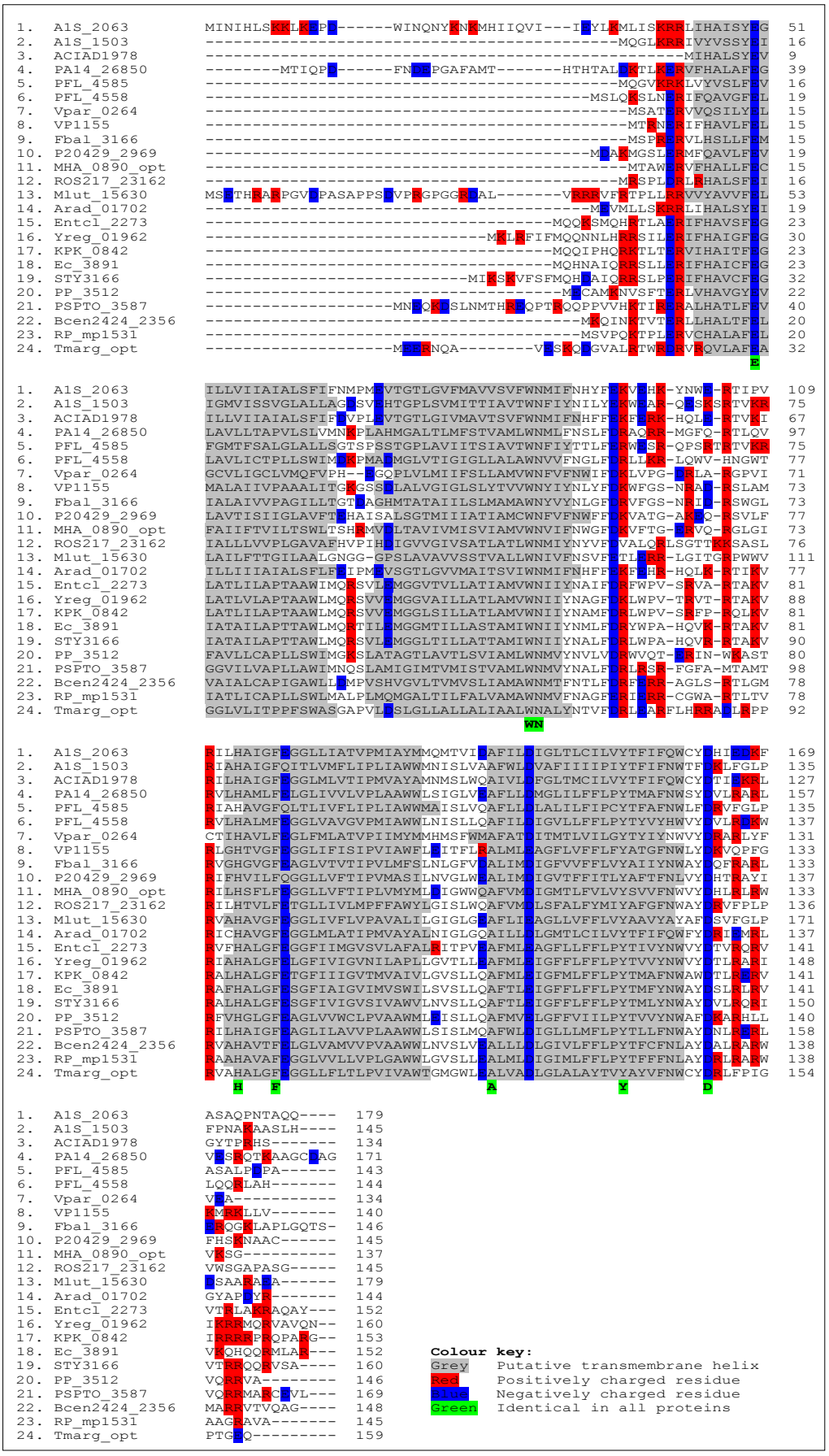

Figure 1: Putative transmembrane regions in selected PACE family proteins

Sequence alignment for PACE family proteins listed in Table 1, produced using Clustal Omega 1.1.0 (http://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers *et al*., 2011) and coloured to show the putative locations of transmembrane spanning helices as predicted by TOPCONS (http://topcons.cbr.su.se/) (Bernsel *et al*., 2009; Tsirigos *et al*., 2015) (*grey*). Also highlighted are positively charged residues (Arg + Lys) (*red*), negatively charged residues (Asp + Glu) (*blue*) and residues that are identically conserved in all the proteins (*green*).

3.2. Expression Screening of PACE Family Proteins

Expression screening was the initial step carried out to test for a sufficient amount of appropriate quality protein, which is one of the main bottlenecks in the structural biology pipeline. To accelerate the purification of proteins, small-scale expression screening was carried out to identify quickly those that would allow sufficient quantities of protein to be produced from larger-scale cultures. Genes for expressing the selected twenty-four PACE family proteins were successfully amplified from genomic DNA and ligated into plasmid pTTQ18 immediately upstream from a His_6-tag coding sequence, as determined by restriction digestion analysis and DNA sequencing. Plasmid constructs were transformed into *E. coli* BL21(DE3) cells, which are commonly used for recombinant protein expression (Ahmad *et al*., 2018b), and the results of small-scale expression tests are shown in Figure 2. Usually, the proteins that are identified by Western blot analysis but fail to visualise in SDS-PAGE, are not considered the best candidates to pursue for purification due to low yields and high levels of contaminants. On this basis, seven of the proteins (A1S_2063, PSPTO_3587, A1S_1503, PFL_4558, Fbal_3166, Tmarg_opt and STY_3166) were considered to be expressed at a level sufficient for producing larger-scale cultures, purification, and for structural and functional characterisation. These proteins showed clear and intense bands on both gels and Western blots (Figure 2). Detection of a signal on the Western blot confirmed that the recombinant expressed protein had retained its His_6-tag. Focusing on a smaller number of proteins that show the most potential at this stage was important to make the best use of time and materials. This type of strategy is commonly used in pipelines for structural biology of membrane proteins (Ward *et al*., 2000; Gordon *et al*., 2008; Ma *et al*., 2008; Bettaney *et al*., 2013; Ma *et al*., 2013; Moraes *et al*,. 2014; Alegre & Law, 2015).

3.3. Scale-Up and Purification of PACE Family Proteins

Out of the seven proteins that were progressed to larger scale cultures, four proteins (A1S_2063, Fbal_3166, STY_3166 and Tmarg_opt) representing four phylogenetic groups of PACE proteins (Hassan *et al*., 2015b) were chosen for purification. These proteins produced good quantities of cells and of inner membrane preparations from 30-litre fermentor cultures (Table 2). The amplified His_6-tagged proteins were solubilised from inner membranes using 1% DDM and then purified using a Ni-NTA column that was washed with 40 mM imidazole before elution with 200 mM imidazole. The various fractions were analysed on SDS-PAGE gels and on Western blots (Figure 3). Comparison of inner membranes and the supernatant following solubilisation showed that a large majority of the protein of interest had been solubilised in all cases (Figure 3). Absence of a significant band on the Western blots for the unbound fraction showed that a large majority of the protein of interest had bound to the column in all cases. Under conditions of the SDS-PAGE separation, the A1S_2063 purified protein was largely present in its monomeric form at a position of around 16 kDa, with some dimeric form present at around 25 kDa. Fbal_3166 was approximately equally distributed between monomeric and dimeric forms at positions of around 18 kDa and 30 kDa, respectively. STY_3166 was predominantly present as a dimer at a position of around 32 kDa and also present as higher oligomeric states at positions of around 45 kDa and 50 kDa. Tmarg_opt was distributed between three main bands at positions of around 20, 30 and 40 kDa (Figure 3). Based on densitometric analysis of the SDS-PAGE gels, A1S_2063, Fbal_3166, STY_3166 and Tmarg_opt had protein purities of 86, 84, 80 and 78%, respectively. The purified yields of these proteins were 1.1, 1.1, 1.3 and 1.0 mg per litre of cell culture (Table 2). These results make the proteins tractable to structure determination and to analysis by a range of biochemical and biophysical techniques.

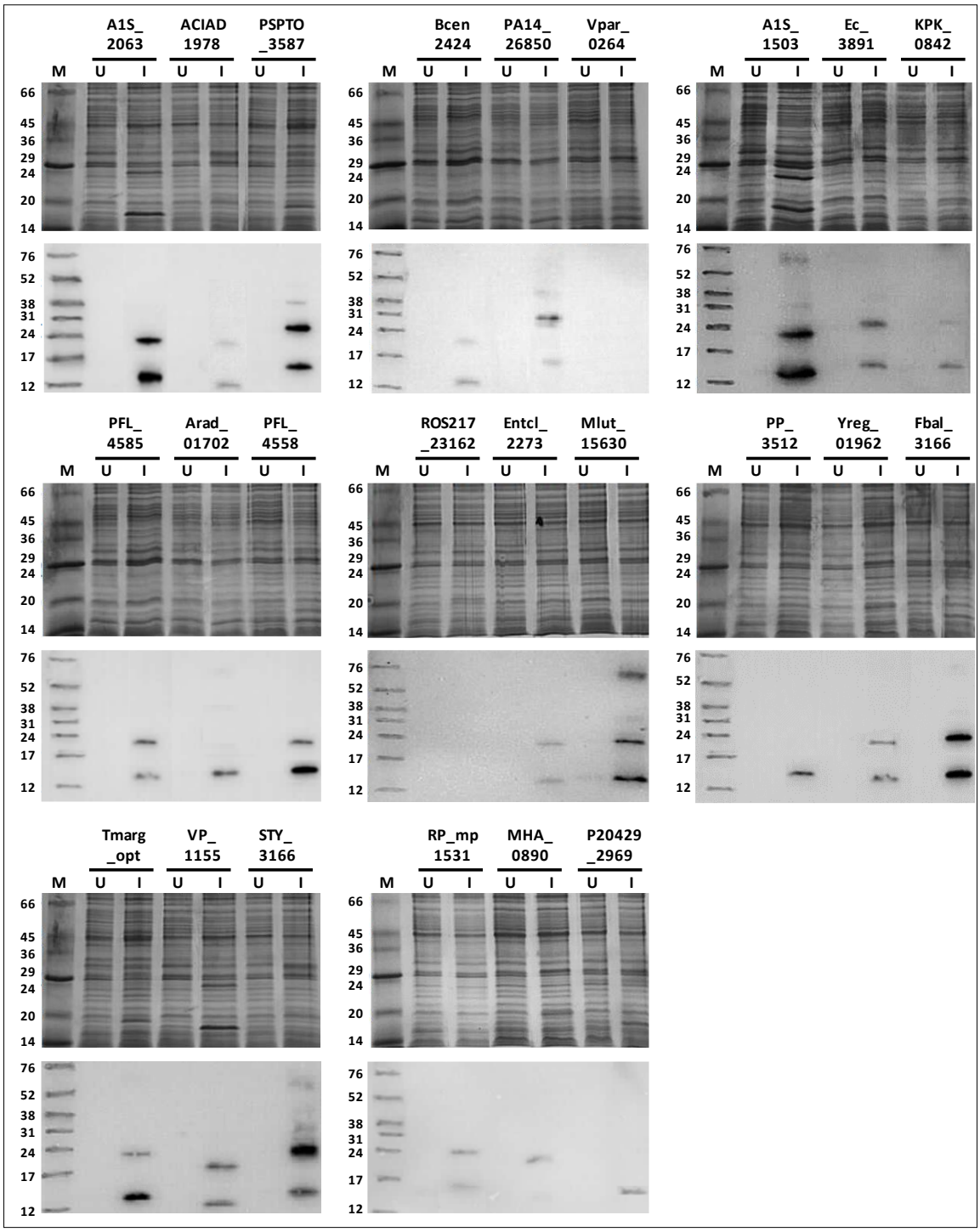

Figure 2: Expression screening of PACE family proteins

SDS-PAGE (*top panel*) and Western blot (*lower panel*) analysis of membrane preparations from small-scale cultures of *E. coli* BL21(DE3) cells harbouring plasmids for expressing the recombinant PACE family proteins listed in Table 1. Cells were cultured in LB medium containing glycerol (20 mM) and carbenicillin (100 µg/ml) at 37 °C with shaking at 220 rpm. Cells were left uninduced (U) or were induced (I) at A_{600} = 0.8 with IPTG (0.2 mM). Cells were harvested 2 hours post-induction and mixed membranes were prepared using the water lysis method. M = molecular weight markers (kDa).

This table gives results for production of four PACE family proteins (A1S_2063, Fbal_3166, STY_3166, Tmarg_opt) from 30-litre fermentor cultures, followed by inner membrane preparation, purification (Figure 3) and CD analysis of thermal stability (Figure 4).

Table 2: Results for large-scale production and purification of PACE family proteins

Protein	Mass of wet cell pellet from 30 litres (fermentor)	Volume of inner membranes	Purification yield (per litre cell culture)	Protein purity	Protein melting temperature (CD)
A1S_2063	129.4 g	14.3 ml	1.1 mg/litre	86%	46.7 ºC
Fbal_3166	112.7 g	12.9 ml	1.1 mg/litre	84%	34.2 ºC
STY_3166	116.9 g	16.5 ml	1.3 mg/litre	80%	32.6 ºC
Tmarg_opt	120.2 g	14.1 ml	1.0 mg/litre	78%	37.6 ºC

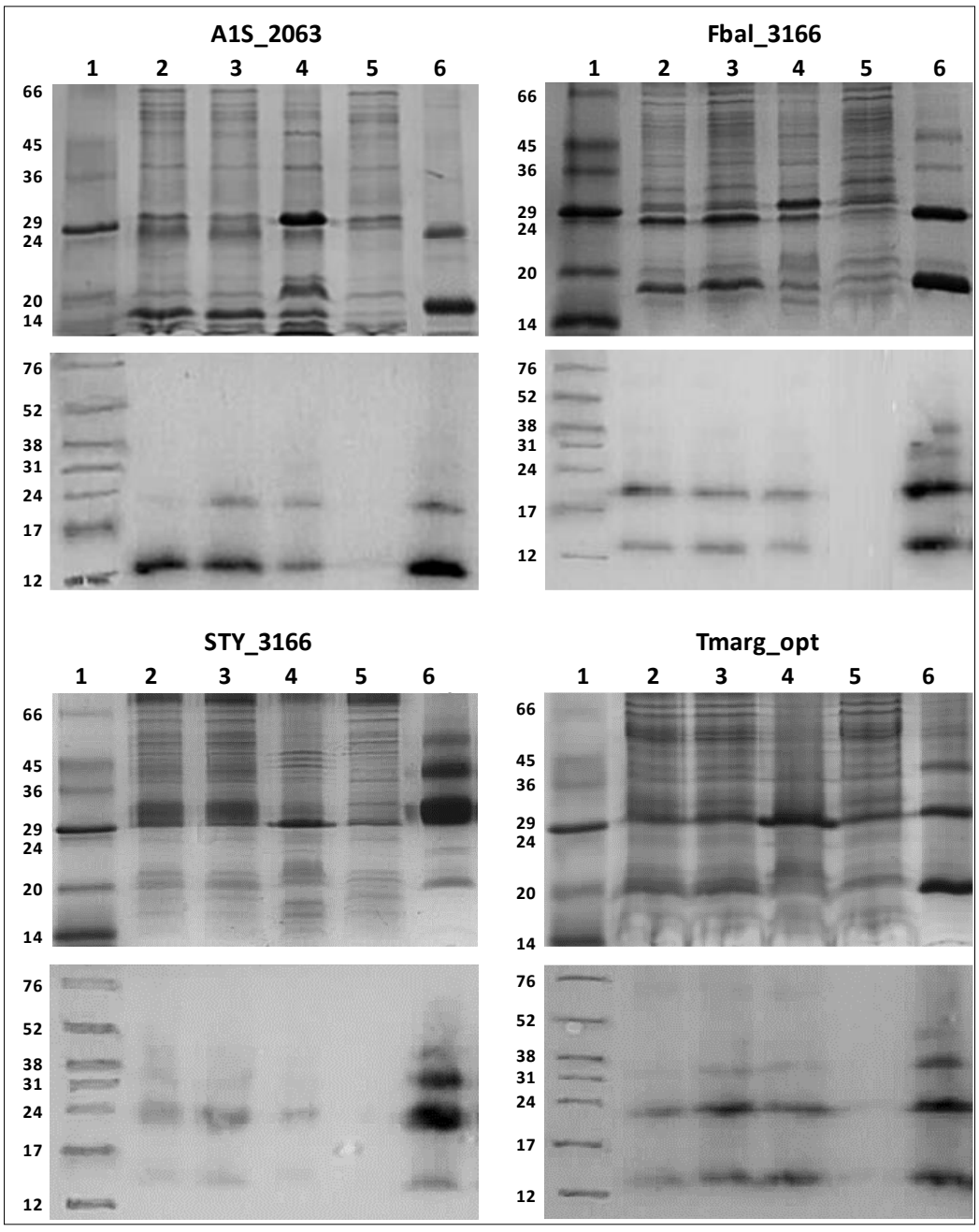

Figure 3: Purification of PACE family proteins from inner membranes

SDS-PAGE (*top panel*) and Western blot (*lower panel*) analyses of fractions from the detergent solubilisation and purification of four PACE family proteins (A1S_2063, Fbal_3166, STY_3166, Tmarg_opt) from inner membrane preparations of *E. coli* BL21(DE3) cells grown in a 30-litre fermentor. Samples were loaded on the gel (16 µg protein) and the blot (4 µg protein) as follows: (1) molecular weight markers (kDa); (2) inner membranes; (3) supernatant (following solubilisation); (4) membrane pellet (following solubilisation); (5) unbound flow through (from column); (6) purified and concentrated protein.

3.4. Secondary Structure Integrity and Thermal Stability of Purified PACE Family Proteins

Far-UV CD spectroscopy was used to assess the secondary structure content and thermal stability of the four purified and DDM-solubilised PACE family proteins (Figure 4). The resultant spectra confirmed that the proteins were predominantly alpha-helical, with a characteristic positive peak at ~192 nm and negative peaks at 209 nm and 222 nm (Wallace *et al*., 2003; Kelly *et al*., 2005; Miles & Wallace, 2016). A small variability in the CD signal intensities was probably due to unintentional differences in protein concentration between the samples. These measurements suggest that all four purified proteins were correctly folded and had retained their secondary structure architecture after passing through various steps of the purification. Thermal stability measurements on the proteins monitored at a wavelength of 209 nm suggest that A1S_2063 and Tmarg_opt are more slowly denatured than Fbal_3166 and STY_3166 (Figure 4). The melting temperatures obtained for A1S_2063, Fbal_3166, STY_3166 and Tmarg_opt were 46.7, 34.2, 32.6 and 37.6 °C, respectively (Table 2). These results suggest that A1S_2063 is the most stable protein compared with the others and might be best for structure determination. The melting temperatures also suggest that performing experiments on the purified proteins up to a temperature of 25 °C should have no significant effects on their secondary structure stability.

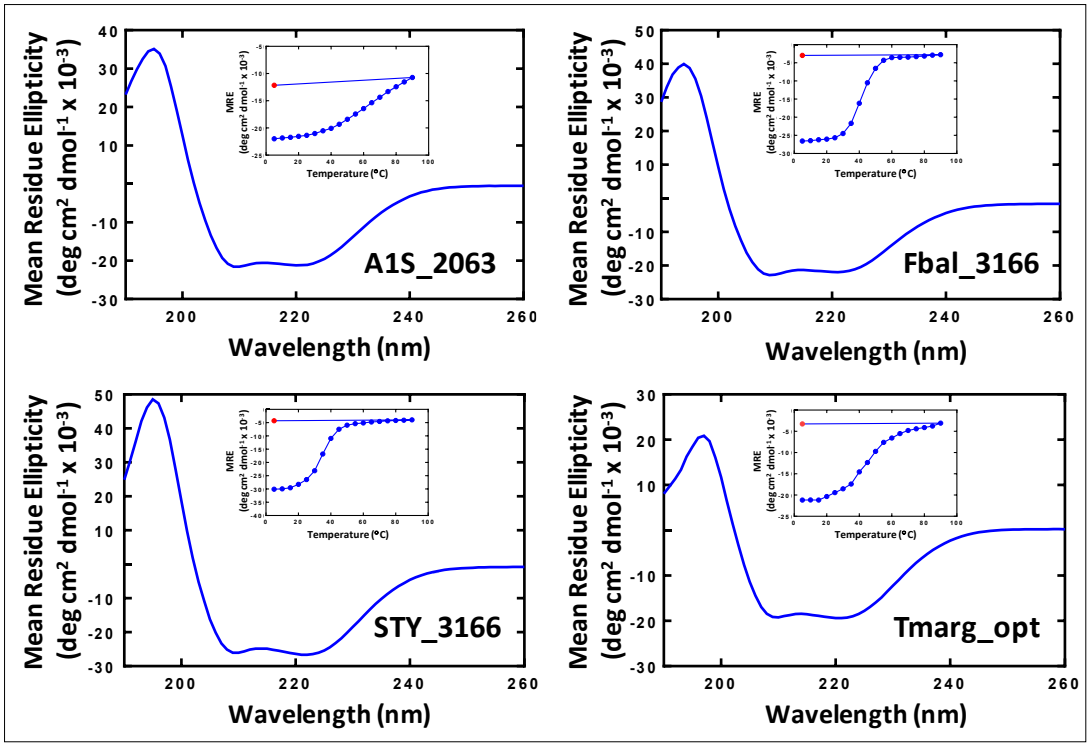

Figure 4: Secondary structure integrity and thermal stability of purified PACE family proteins

Far-UV (180-260 nm) CD spectra of four purified and detergent solubilised PACE family proteins (A1S_2063, Fbal_3166, STY_3166, Tmarg_opt) (Figure 3). Measurements were made using a Chirascan Plus instrument (Applied Photophysics, UK) at 20 ºC with constant liquid nitrogen flushing. Samples were prepared in a Hellma quartz cuvette of 1.0 mm pathlength at a final protein concentration of 0.15 mg/ml in NaPi buffer (10 mM, pH 7.5) containing DDM (0.05%). Inset are thermal unfolding curves obtained by ramping the temperature in 5 °C increments from 5 °C to 90 °C, followed by cooling back to 5 °C (*red*), from monitoring the CD signal at 209 nm.

4. CONCLUSIONS

We have demonstrated a robust strategy for the expression screening and purification of PACE family transport proteins preliminary to understanding structural biology. Starting with twenty-four proteins, small-scale expression screening identified seven proteins (A1S_2063, PSPTO_3587, A1S_1503, PFL_4558, Fbal_3166, Tmarg_opt and STY_3166) that were suitable for scaling up to a larger culture volume, inner membrane preparation and purification. Four of these proteins (A1S_2063, Fbal_3166, STY_3166 and Tmarg_opt) produced good quantities of cells and inner membranes from 30-litre fermentor cultures and all four proteins were purified in yields of ≥ 1 mg per litre of cell culture and had protein purities of 78-86%. The purified and detergent-solubilised proteins retained secondary structure integrity that was predominantly alpha-helical and showed thermal stability properties making them tractable to structure determination and to analysis by biochemical and biophysical techniques. Of the four

proteins, A1S_2063 was the most stable to thermal unfolding and may be the best candidate for structure determination. All four purified proteins should be reasonably stable to experiments performed up to a temperature of 25 °C. These results are very important because the high-resolution structural organisation and molecular mechanism of PACE family proteins are yet to be elucidated. Future studies also need to investigate the roles of conserved and other potentially key residues in PACE family proteins. Such information will enhance our understanding about the function of secondary active transport proteins, especially with respect to the ongoing worldwide problem of antimicrobial resistance.

Acknowledgements: The authors thank Mr David Sharples (University of Leeds, UK) for culturing cells in a fermentor.

Conflicts of Interest: The authors do not have any conflicts of interest to declare.

REFERENCES

- Ahmad, I., Ali, M., Ali, R., Nawaz, N., & Patching, S. G. (2025). Multidrug efflux protein families in bacteria: ABC, RND, MFS, SMR, MATE, PACE, AbgT (an update). *Advances in Life Sciences*, 3, 1-44. DOI: 10.62778/int.book.596
- Ahmad, I., Hassan, K. A., Henderson, P. J. F., & Patching, S. G. (2022). Cloning, amplified expression, functional characterisation and purification of a *Pseudomonas putida* NCS1 family transport protein. *Int J Adv Multidiscip Res*, 9(12), 127-156.
- Ahmad, I., Lee, Y., Nawaz, N., Elahi, R., Khan, I. A., Mustafa, M. Z., & Patching, S. G. (2021). Cloning, amplified expression and bioinformatics analysis of a putative Nucleobase Cation Symporter-1 (NCS-1) protein from *Rhodococcus erythropolis*. *BioScientific Review*, 3(4). DOI: 10.32350//BSR.0304.05
- Ahmad, I., Ma, P., Nawaz, N., Sharples, D. J., Henderson, P. J. F., & Patching, S. G. (2020). Cloning, amplified expression, functional characterisation and purification of *Vibrio parahaemolyticus* NCS1 cytosine transporter VPA1242. Chapter 8. In: Patching, S. G. (Ed.), A Closer Look at Membrane Proteins. Independent Publishing Network, UK, pp. 241-267. ISBN: 978-1-83853-535-3.
- Ahmad, I., Nawaz, N., Darwesh, N. M., Ur Rahman, S., Mustafa, M. Z., Khan, S. B., & Patching, S. G. (2018b). Overcoming challenges for amplified expression of recombinant proteins using *Escherichia coli*. *Protein Expr Purif*, 144, 12-18.
- Ahmad, I., Nawaz, N., Dermani, F. K., Kohlan, A. K., Saidijam, M., & Patching, S. G. (2018a). Bacterial multidrug efflux proteins: A major mechanism of antimicrobial resistance. *Curr Drug Targets*, 19, 1-13. DOI: 10.2174/1389450119666180426103300.
- Alegre, K. O., & Law, C. J. (2015). Purification of a multidrug resistance transporter for crystallization studies. *Antibiotics (Basel)*, 4(1), 113-135.
- Ali, M., Ali, R., Khan, I. A., Ahmad, I., Nawaz, N., & Patching, S. G. (2025). The *Escherichia coli* zinc exporter ZitB of the cation diffusion facilitator (CDF) protein family: Properties, cloning, amplified expression, purification. *Sch Int J Biochem* 8(2), 50-65.
- Alkhowaiter, H. S., AL-Jaddawi, A., Abu-Zeid, M., & Abo-Aba, S. E. M. (2024). A review: Hospital acquired infection in some Gram negative pathogenic bacterial strains. *J Contemp Med Sci*, 10(1). DOI: 10.22317/jcms.v10i1.1475
- Badger-Emeka, L., Al Rashed, A. S., Aljindan, R. Y., Emeka, P. M., Quadri, S. A., & Almutairi, H. H. (2023). Incidence of drug-resistant hospital-associated Gram-negative bacterial infections, the accompanying risk factors, and clinical outcomes with treatment. *Antibiotics*, 12(9), 1425.
- Bay, D. C., & Turner, R. J. (2009). Diversity and evolution of the small multidrug resistance protein family. *BMC Evol Biol*, 9, 140.
- Bernsel, A., Viklund, H., Hennerdal, A., & Elofsson, A. (2009). TOPCONS: Consensus prediction of membrane protein topology. *Nucleic Acids Res*, 37(Web Server issue), W465-W468.
- Bettaney, K. E., Sukumar, P., Hussain, R., Siligardi, G., Henderson, P. J., & Patching, S. G. (2013). A systematic approach to the amplified expression, functional characterization and purification of inositol transporters from *Bacillus subtilis*. *Mol Membr Biol*, 30(1), 3-14.
- Blair, J. M., Richmond, G. E., & Piddock, L. J. (2014). Multidrug efflux pumps in Gram-negative bacteria and their role in antibiotic resistance. *Future Microbiol*, 9(10), 1165-1177.
- Bolhuis, H., van Veen, H. W., Poolman, B., Driessen, A. J., & Konings, W. N. (1997). Mechanisms of multidrug transporters. *FEMS Microbiol Rev*, 21(1), 55-84.
- Bolla, J. R., Howes, A. C., Fiorentino, F., & Robinson, C. V. (2020). Assembly and regulation of the chlorhexidine-specific efflux pump AceI. *Proc Natl Acad Sci U S A*, 117(29), 17011-17018.
- Chang, G. (2003). Multidrug resistance ABC transporters. *FEBS Lett*, 555(1), 102-105.
- Chelazzi, C., Pettini, E., Villa, G., & De Gaudio, A. R. (2015). Epidemiology, associated factors and outcomes of ICU-acquired infections caused by Gram-negative bacteria in critically ill patients: An observational, retrospective study. *BMC Anesthesiol*, 15, 125.
- Chitsaz, M., & Brown, M. H. (2017). The role played by drug efflux pumps in bacterial multidrug resistance. *Essays Biochem*, 61(1), 127-139.

- Clough, J., Saidijam, M., Bettaney, K. E., Szakonyi, G., Patching, S. G., Mueller, J., Shibayama, K., Bacon, M., Barksby, E., Groves, M., Herbert, R. B., Phillips-Jones, M., Ward, A., Gunn-Moore, F., O'Reilly, J., Rutherford, N. G., Bill, R., & Henderson, P. J. F. (2006). Prokaryotic membrane transport proteins: amplified expression and purification. In: Lundstrom, K. H. (Ed.), Structural genomics on membrane proteins. Taylor and Francis, pp. 21-42.
- Delmar, J. A., & Yu, E. W. (2016). The AbgT family: A novel class of antimetabolite transporters. *Protein Sci*, 25(2), 322-337.
- Du, D., van Veen, H. W., Murakami, S., Pos, K. M., & Luisi, B. F. (2015). Structure, mechanism and cooperation of bacterial multidrug transporters. *Curr Opin Struct Biol*, 33, 76-91.
- Elshobary, M. E., Badawy, N. K., Ashraf, Y., Zatioun, A. A., Masriya, H. H., Ammar, M. M., Mohamed, N. A., Mourad, S., & Assy, A. M. (2025). Combating antibiotic resistance: Mechanisms, multidrug-resistant pathogens, and novel therapeutic approaches: An updated review. *Pharmaceuticals (Basel)*, 18(3), 402.
- Finn, R. D., Bateman, A., Clements, J., Coggill, P., Eberhardt, R. Y., Eddy, S. R., Heger, A., Hetherington, K., Holm, L., Mistry, J., Sonnhammer, E. L., Tate, J., & Punta, M. (2014). Pfam: The protein families database. *Nucleic Acids Res*, 42(Database issue), D222-D230.
- Gordon, E., Horsefield, R., Swarts, H. G., de Pont, J. J., Neutze, R., & Snijder, A. (2008). Effective high-throughput overproduction of membrane proteins in *Escherichia coli*. *Protein Expr Purif*, 62(1), 1-8.
- Gurvic, D., & Zachariae, U. (2024). Multidrug efflux in Gram-negative bacteria: Structural modifications in active compounds leading to efflux pump avoidance. *NPJ Antimicrob Resist*, 2(1), 6.
- Hassan, K. A., Elbourne, L. D., Li, L., Gamage, H. K., Liu, Q., Jackson, S. M., Sharples, D., Kolstø, A. B., Henderson, P. J., & Paulsen, I. T. (2015a). An ace up their sleeve: A transcriptomic approach exposes the AceI efflux protein of *Acinetobacter baumannii* and reveals the drug efflux potential hidden in many microbial pathogens. *Front Microbiol*, 6, 333.
- Hassan, K. A., Jackson, S. M., Penesyan, A., Patching, S. G., Tetu, S. G., Eijkelkamp, B. A., Brown, M. H., Henderson, P. J., & Paulsen, I. T. (2013). Transcriptomic and biochemical analyses identify a family of chlorhexidine efflux proteins. *Proc Natl Acad Sci U S A*, 110(50), 20254-20259.
- Hassan, K. A., Liu, Q., Elbourne, L. D. H., Ahmad, I., Sharples, D., Naidu, V., Chan, C. L., Li, L., Harborne, S. P. D., Pokhrel, A., Postis, V. L. G., Goldman, A., Henderson, P. J. F., & Paulsen, I. T. (2018). Pacing across the membrane: The novel PACE family of efflux pumps is widespread in Gram-negative pathogens. *Res Microbiol*, 169(7-8), 450-454.
- Hassan, K. A., Liu, Q., Henderson, P. J., & Paulsen, I. T. (2015b). Homologs of the *Acinetobacter baumannii* AceI transporter represent a new family of bacterial multidrug efflux systems. *mBio*, 6(1), e01982-14.
- Hassan, K. A., Naidu, V., Edgerton, J. R., Mettrick, K. A., Liu, Q., Fahmy, L., Li, L., Jackson, S. M., Ahmad, I., Sharples, D., Patching, S. G., Henderson, P. J. F., & Paulsen, I. T. (2019). Short-chain diamines are the physiological substrates of PACE family efflux pumps. *Proc Natl Acad Sci U S A*, 116(36), 18015-18020.
- Henderson, P. J., & Macpherson, A. J. (1986). Assay, genetics, proteins, and reconstitution of proton-linked galactose, arabinose, and xylose transport systems of *Escherichia coli*. *Methods Enzymol*, 125, 387-429.
- Huang, L., Wu, C., Gao, H., Xu, C., Dai, M., Huang, L., Hao, H., Wang, X., & Cheng, G. (2022). Bacterial multidrug efflux pumps at the frontline of antimicrobial resistance: An overview. *Antibiotics (Basel)*, 11(4), 520.
- Inoue, H., Nojima, H., & Okayama, H. (1990). High efficiency transformation of *Escherichia coli* with plasmids. *Gene*, 96(1), 23-28.
- Kaatz, G. W. (2005). Bacterial efflux pump inhibition. *Curr Opin Investig Drugs*, 6(2), 191-198.
- Kelly, S. M., Jess, T. J., & Price, N. C. (2005). How to study proteins by circular dichroism. *Biochim Biophys Acta*, 1751(2), 119-139.
- Kourtesi, C., Ball, A. R., Huang, Y. Y., Jachak, S. M., Vera, D. M., Khondkar, P., Gibbons, S., Hamblin, M. R., & Tegos, G. P. (2013). Microbial efflux systems and inhibitors: Approaches to drug discovery and the challenge of clinical implementation. *Open Microbiol J*, 7, 34-52.
- Krogh, A., Larsson, B., von Heijne, G., & Sonnhammer, E. L. (2001). Predicting transmembrane protein topology with a hidden Markov model: Application to complete genomes. *J Mol Biol*, 305(3), 567-580.
- Kumar, S., & Varela, M. F. (2012). Biochemistry of bacterial multidrug efflux pumps. *Int J Mol Sci*, 13(4), 4484-4495.
- Kunz, A. N., & Brook, I. (2010). Emerging resistant Gram-negative aerobic bacilli in hospital-acquired infections. *Chemotherapy*, 56(6), 492-500.
- Kuroda, T., & Tsuchiya, T. (2009). Multidrug efflux transporters in the MATE family. *Biochim Biophys Acta*, 1794(5), 763-768.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227(5259), 680-685.
- Levy, S. B. (1992). Active efflux mechanisms for antimicrobial resistance. *Antimicrob Agents Chemother*, 36(4), 695-703.

- Li, X. Z., & Nikaido, H. (2004). Efflux-mediated drug resistance in bacteria. *Drugs*, 64(2):159-204.
- Li, X. Z., Plésiat, P., & Nikaido, H. (2015). The challenge of efflux-mediated antibiotic resistance in Gram-negative bacteria. *Clin Microbiol Rev*, 28(2), 337-418.
- Lubelski, J., Konings, W. N., & Driessen, A. J. (2007). Distribution and physiology of ABC-type transporters contributing to multidrug resistance in bacteria. *Microbiol Mol Biol Rev*, 71(3), 463-476.
- Ma, P., Varela, F., Magoch, M., Silva, A. R., Rosário, A. L., Brito, J., Oliveira, T. F., Nogly, P., Pessanha, M., Stelter, M., Kletzin, A., Henderson, P. J., & Archer, M. (2013). An efficient strategy for small-scale screening and production of archaeal membrane transport proteins in *Escherichia coli*. *PLoS One*, 8(10), e76913.
- Ma, P., Yuille, H. M., Blessie, V., Göhring, N., Iglói, Z., Nishiguchi, K., Nakayama, J., Henderson, P. J., & Phillips-Jones, M. K. (2008). Expression, purification and activities of the entire family of intact membrane sensor kinases from *Enterococcus faecalis*. *Mol Membr Biol*, 25(6-7), 449-473.
- Ma, P.†, Patching, S. G.†, Ivanova, E., Baldwin, J. M., Sharples, D., Baldwin, S. A., & Henderson, P. J. (2016). Allantoin transport protein, PucI, from *Bacillus subtilis*: Evolutionary relationships, amplified expression, activity and specificity. *Microbiology*, 162(5), 823-836. †Equal contribution
- Mehrad, B., Clark, N. M., Zhanel, G. G., & Lynch, J. P. 3rd. (2015). Antimicrobial resistance in hospital-acquired gram-negative bacterial infections. *Chest*, 147(5), 1413-1421.
- Miles, A. J., & Wallace, B. A. (2016). Circular dichroism spectroscopy of membrane proteins. *Chem Soc Rev*, 45(18), 4859-4872.
- Moraes, I., Evans, G., Sanchez-Weatherby, J., Newstead, S., & Stewart, P. D. (2014). Membrane protein structure determination - the next generation. *Biochim Biophys Acta*, 1838(1 Pt A), 78-87.
- Nikaido, H. (1994). Prevention of drug access to bacterial targets: Permeability barriers and active efflux. *Science*, 264(5157), 382-388.
- Nikaido, H. (2003). Molecular basis of bacterial outer membrane permeability revisited. *Microbiol Mol Biol Rev*, 67(4), 593-656.
- Nikaido, H., & Pagès, J. M. (2012). Broad-specificity efflux pumps and their role in multidrug resistance of Gram-negative bacteria. *FEMS Microbiol Rev*, 36(2), 340-363.
- Omote, H., Hiasa, M., Matsumoto, T., Otsuka, M., & Moriyama, Y. (2006). The MATE proteins as fundamental transporters of metabolic and xenobiotic organic cations. *Trends Pharmacol Sci*, 27(11), 587-593.
- Pagès, J. M., Sandrine, A. F., Mahamoud, A., Bolla, J. M., Davin-Regli, A., Chevalier, J., & Garnotel, E. (2010). Efflux pumps of gram-negative bacteria, a new target for new molecules. *Curr Top Med Chem*, 10(18), 1848-1857.
- Parajuli, N. P., Acharya, S. P., Mishra, S. K., Parajuli, K., Rijal, B. P., & Pokhrel, B. M. (2017). High burden of antimicrobial resistance among gram negative bacteria causing healthcare associated infections in a critical care unit of Nepal. *Antimicrob Resist Infect Control*, 6, 67.
- Patching, S. G. (2022). Spermidine binding to the *Acetinobacter baumannii* efflux protein AceI observed by near-UV synchrotron radiation circular dichroism spectroscopy. *Radiation*, 2(2), 228-233.
- Patching, S. G. (2024). Structure and substrate recognition of the *Escherichia coli* transport protein NupG from the Nucleoside:H^+ Symporter (NHS) family. *Int J Curr Sci Res Rev*, 7(6), 3863-3868.
- Paulsen, I. T., Skurray, R. A., Tam, R., Saier, M. H. Jr, Turner, R. J., Weiner, J. H., Goldberg, E. B., & Grinius, L. L. (1996). The SMR family: A novel family of multidrug efflux proteins involved with the efflux of lipophilic drugs. *Mol Microbiol*, 19(6), 1167-1175.
- Paulsen, I. T., Sliwinski, M. K., & Saier, M. H. Jr. (1998). Microbial genome analyses: Global comparisons of transport capabilities based on phylogenies, bioenergetics and substrate specificities. *J Mol Biol*, 277(3), 573-592.
- Peleg, A. Y., & Hooper, D. C. (2010). Hospital-acquired infections due to gram-negative bacteria. *N Engl J Med*, 362(19), 1804-1813.
- Ranaweera, I., Shrestha, U., Ranjana, K. C., Kakarla, P., Willmon, T. M., Hernandez, A. J., Mukherjee, M. M., Barr, S. R., & Varela, M. F. (2015). Structural comparison of bacterial multidrug efflux pumps of the major facilitator superfamily. *Trends Cell Mol Biol*, 10, 131-140.
- Ren, Q., Chen, K., & Paulsen, I. T. (2007). TransportDB: A comprehensive database resource for cytoplasmic membrane transport systems and outer membrane channels. *Nucleic Acids Res*, 35(Database issue), D274-D279.
- Routh, M. D., Zalucki, Y., Su, C. C., Zhang, Q., Shafer, W. M., & Yu, E. W. (2011). Efflux pumps of the resistance-nodulation-division family: A perspective of their structure, function, and regulation in gram-negative bacteria. *Adv Enzymol Relat Areas Mol Biol*, 77, 109-146.
- Ruppé, É., Woerther, P. L., & Barbier, F. (2015). Mechanisms of antimicrobial resistance in Gram-negative bacilli. *Ann Intensive Care*, 5(1), 61.
- Saidijam, M., Azizpour, S., & Patching, S. G. (2017). Amino acid composition analysis of human secondary transport proteins and implications for reliable membrane topology prediction. *J Biomol Struct Dyn*, 35(5), 929-949.
- Saidijam, M., Azizpour, S., & Patching, S. G. (2018). Comprehensive analysis of the numbers, lengths and amino acid compositions of transmembrane helices in prokaryotic, eukaryotic

and viral integral membrane proteins of high-resolution structure. *J Biomol Struct Dyn*, 36(2), 443-464.

- Saidijam, M., Benedetti, G., Ren, Q., Xu, Z., Hoyle, C. J., Palmer, S. L., Ward, A., Bettaney, K. E., Szakonyi, G., Meuller, J., Morrison, S., Pos, M. K., Butaye, P., Walravens, K., Langton, K., Herbert, R. B., Skurray, R. A., Paulsen, I. T., O'Reilly, J., Rutherford, N. G., Brown, M. H., Bill, R. M., & Henderson, P. J. (2006). Microbial drug efflux proteins of the major facilitator superfamily. *Curr Drug Targets*, 7(7), 793-811.
- Saidijam, M., Bettaney, K. E., Szakonyi, G., Psakis, G., Shibayama, K., Suzuki, S., Clough, J. L., Blessie, V., Abu-Bakr, A., Baumberg, S., Meuller, J., Hoyle, C. K., Palmer, S. L., Butaye, P., Walravens, K, Patching, S. G., O'Reilly, J., Rutherford, N. G., Bill, R. M., Roper, D. I., Phillips-Jones, M. K., & Henderson, P. J. (2005). Active membrane transport and receptor proteins from bacteria. *Biochem Soc Trans*, 33(Pt 4), 867-872.
- Saidijam, M., Psakis, G., Clough, J. L., Meuller, J., Suzuki, S., Hoyle, C. J., Palmer, S. L., Morrison, S. M., Pos, M. K., Essenberg, R. C., Maiden, M. C., Abu-bakr, A., Baumberg, S. G., Neyfakh, A. A., Griffith, J. K., Stark, M. J., Ward, A., O'Reilly, J., Rutherford, N. G., Phillips-Jones, M. K., & Henderson, P. J. (2003). Collection and characterisation of bacterial membrane proteins. *FEBS Lett*, 555(1), 170-175.
- Schaffner, W., & Weissmann, C. (1973). A rapid, sensitive, and specific method for the determination of protein in dilute solution. *Anal Biochem*, 56(2), 502-514.
- Schindler, B. D., & Kaatz, G. W. (2016). Multidrug efflux pumps of Gram-positive bacteria. *Drug Resist Updat*, 27, 1-13.
- Shaheen, A., Iqbal, M., Mirza, O., & Rahman, M. (2017). Structural biology meets drug resistance: An overview on multidrug resistance transporters. *J Indian Institute Sci*, 97(2), 165-175.
- Siasat, P. A., & Blair, J. M. A. (2023). Microbial primer: Multidrug efflux pumps. *Microbiology (Reading)*, 169(10), 001370.
- Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Söding, J., Thompson, J. D., & Higgins, D. G. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol*, 7, 539.
- Spengler, G., Kincses, A., Gajdács, M., & Amaral, L. (2017). New roads leading to old destinations: Efflux pumps as targets to reverse multidrug resistance in bacteria. *Molecules*, 22(3), 468.
- Stark, M. J. (1987). Multicopy expression vectors carrying the lac repressor gene for regulated high-level expression of genes in *Escherichia coli*. *Gene*, 51(2-3), 255-267.
- Su, C. C., Bolla, J. R., Kumar, N., Radhakrishnan, A., Long, F., Delmar, J. A., Chou, T. H., Rajashankar, K. R., Shafer, W. M., & Yu, E. W. (2015). Structure and function of *Neisseria gonorrhoeae* MtrF illuminates a class of antimetabolite efflux pumps. *Cell Rep*, 11(1), 61-70.
- Sun, J., Deng, Z., & Yan, A. (2014). Bacterial multidrug efflux pumps: Mechanisms, physiology and pharmacological exploitations. *Biochem Biophys Res Commun*, 453(2), 254-267.
- Suzuki, S., & Henderson, P. J. (2006). The hydantoin transport protein from *Microbacterium liquefaciens*. *J Bacteriol*, 188(9), 3329-3336.
- Szakonyi, G., Leng, D., Ma, P., Bettaney, K. E., Saidijam, M., Ward, A., Zibaei, S., Gardiner, A. T., Cogdell, R. J., Butaye, P., Kolsto, A. B., O'Reilly, J., Hope, R. J., Rutherford, N. G., Hoyle, C. J., & Henderson, P. J. (2007). A genomic strategy for cloning, expressing and purifying efflux proteins of the major facilitator superfamily. *J Antimicrob Chemother*, 59(6), 1265-1270.
- Tacconelli, E., Carrara, E., Savoldi, A., Harbarth, S., Mendelson, M., Monnet, D. L., Pulcini, C., Kahlmeter, G., Kluytmans, J., Carmeli, Y., Ouellette, M., Outterson, K., Patel, J., Cavaleri, M., Cox, E. M., Houchens, C. R., Grayson, M. L., Hansen, P., Singh, N., Theuretzbacher, U., & Magrini, N.; WHO Pathogens Priority List Working Group. (2018). Discovery, research, and development of new antibiotics: The WHO priority list of antibiotic-resistant bacteria and tuberculosis. *Lancet Infect Dis*, 18(3), 318-327.
- Tseng, T. T., Gratwick, K. S., Kollman, J., Park, D., Nies, D. H., Goffeau, A., & Saier, M. H. Jr. (1999). The RND permease superfamily: An ancient, ubiquitous and diverse family that includes human disease and development proteins. *J Mol Microbiol Biotechnol*, 1(1), 107-125.
- Tsirigos, K. D., Hennerdal, A., Käll, L., Elofsson, A. (2012). A guideline to proteome-wide α-helical membrane protein topology predictions. *Proteomics*, 12(14), 2282-2294.
- Tsirigos, K. D., Peters, C., Shu, N., Käll, L., & Elofsson, A. (2015). The TOPCONS web server for consensus prediction of membrane protein topology and signal peptides. *Nucleic Acids Res*, 43(W1), W401-W407.
- von Heijne, G. (1992). Membrane protein structure prediction. Hydrophobicity analysis and the positive-inside rule. *J Mol Biol*, 225(2), 487-494.
- Wallace, B. A., Lees, J. G., Orry, A. J., Lobley, A., & Janes, R. W. (2003). Analyses of circular dichroism spectra of membrane proteins. *Protein Sci*, 12(4), 875-884.
- Ward, A., O'Reilly, J., Rutherford, N. G., Ferguson, S. M., Hoyle, C. K., Palmer, S. L., Clough, J. L., Venter, H., Xie, H., Litherland, G. J., Martin, G. E., Wood, J. M., Roberts, P. E., Groves, M. A., Liang, W. J., Steel, A., McKeown, B. J., & Henderson, P.

J. (1999). Expression of prokaryotic membrane transport proteins in *Escherichia coli*. *Biochem Soc Trans*, 27(6), 893-899.

- Ward, A., Sanderson, N. M., O'Reilly, J., Rutherford, N. G., Poolman, B., & Henderson, P. J. F. (2000). The amplified expression, identification, purification, assay and properties of hexahistidine tagged bacterial membrane transport proteins. In: Baldwin, S. A. (Ed.) Membrane transport – a Practical Approach. Oxford:Blackwell, pp. 141-166.
- Webber, M. A., & Piddock, L. J. (2003). The importance of efflux pumps in bacterial antibiotic resistance. *J Antimicrob Chemother*, 51(1), 9-11.
- Witholt, B., Boekhout, M., Brock, M., Kingma, J., Heerikhuizen, H. V., & Leij, L. D. (1976). An efficient and reproducible procedure for the formation of spheroplasts from variously grown *Escherichia coli*. *Anal Biochem*, 74(1), 160-170.
- Xie, H., Patching, S. G., Gallagher, M. P., Litherland, G. J., Brough, A. R., Venter, H., Yao, S. Y., Ng, A. M., Young, J. D., Herbert, R. B., Henderson, P. J., & Baldwin, S. A. (2004). Purification and properties of the *Escherichia coli* nucleoside transporter NupG, a paradigm for a major facilitator transporter sub-family. *Mol Membr Biol*, 21(5), 323-336.
- Yılmaz, Ç., & Özcengiz, G. (2017). Antibiotics: Pharmacokinetics, toxicity, resistance and multidrug efflux pumps. *Biochem Pharmacol*, 133, 43-62.
- Zgurskaya, H. I., & Nikaido, H. (2003). Multidrug resistance mechanisms: Drug efflux across two membranes. *Mol Microbiol*, 37(2), 219-225.
- Zgurskaya, H. I., Krishnamoorthy, G., Tikhonova, E. B., Lau, S. Y., & Stratton, K. L. (2003). Mechanism of antibiotic efflux in Gram-negative bacteria. *Front Biosci*, 8, s862-s873.
- Zhao, J., Hellwig, N., Djahanschiri, B., Khera, R., Morgner, N., Ebersberger, I., Wang, J., & Michel, H. (2022). Assembly and functional role of PACE transporter PA2880 from *Pseudomonas aeruginosa*. *Microbiol Spectr*, 10(2), e0145321.