# Active membrane transport and receptor proteins from bacteria

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## Abstract

**A general strategy for the expression of bacterial membrane transport and receptor genes in *Escherichia coli* is described. Expression is amplified so that the encoded proteins comprise 5–35% of *E. coli* inner membrane protein. Depending upon their topology, proteins are produced with $RGSH_6$ or a Strep tag at the C-terminus. These enable purification in mg quantities for crystallization and NMR studies. Examples of one nutrient uptake and one multidrug extrusion protein from *Helicobacter pylori* are described. This strategy is successful for membrane proteins from *H. pylori*, *E. coli*, *Enterococcus faecalis*, *Bacillus subtilis*, *Staphylococcus aureus*, *Microbacterium liquefaciens*, *Brucella abortus*, *Brucella melitensis*, *Campylobacter jejuni*, *Neisseria meningitides*, *Streptomyces coelicolor* and *Rhodobacter sphaeroides*.**


## Introduction

The lipid cell membrane of bacteria is inherently impermeable to nutrients required for metabolism. Uptake of nutrients (and secretion of wastes) therefore depends on the presence of transport proteins, activities of which are typically coupled to metabolic energy to drive transport against the prevailing electrochemical gradient of solute. Examples are ATP-dependent primary active transport, sugar-$H^+$ or antibiotic/$H^+$ secondary active transport and phosphotransferase [1] (Figure 1). In addition, the bacterial membrane contains proteins that sense environmental conditions and, through the TCS ('two-component' sensor/response system), facilitate an appropriate response of the cells [2] (Figure 1). In most cases, the low abundance of these membrane proteins and their hydrophobic nature make them difficult to isolate in amounts required for elucidation of their 3D structure. Determination of structure is a major bottleneck in understanding the molecular mechanisms of membrane transport and sensor proteins, which comprise 5–15% of genomic potential in all organisms.

In this paper, a strategy is described that enables the amplified expression and purification of bacterial membrane transport and receptor proteins in amounts required for structural studies; the strategy is successful for many such proteins (Table 1). Many of these are prokaryotic proteins homologous to those found in numerous organisms from cyanobacteria, eubacteria, protozoan parasites, fungi, plants and mammals [3]; the convenience of structure–activity studies in bacteria then illuminate the molecular mechanism of transporters in numerous organisms including human. Other transport systems and TCSs are unique to bacteria and critical for growth of pathogenic organisms during infection; the availability of the purified active protein may then be useful for discovery of novel antibacterials.

Only two examples of amplified expression are illustrated here, a KgtP ($\alpha$-ketoglutarate transport protein) and a putative Mdr (multidrug resistance protein), both originating from *Helicobacter pylori*. Tomb et al. [4] determined the complete genome sequence of the *H. pylori* strain 26 695, later compared with that of the pathogenic strain J99 [5]. One of the genes found was *JHP0334*. This was thought to encode a KgtP, because its predicted amino acid sequence aligned with 40–44% identity to the known 'KgtP' protein from *Escherichia coli* [3,6]. Homologues of KgtP also occur in *Campylobacter jejuni*, *Salmonella typhimurium*, *Pseudomonas putida*, *Salmonella typhi* and *Brucella suis*. The *JHP0334* gene product is predicted to comprise 437 amino acids. Similarly, the *JHP1092* gene product is thought to encode an efflux protein for drugs and/or antibiotics of 386 amino acids. Both proteins belong to the widespread Major Facilitator Superfamily of transport proteins [7] and their hydropathy profiles suggest that they are integral membrane proteins arranged in 12 transmembrane $\alpha$-helices. Their transport is likely to be energized by the trans-membrane electrochemical potential of $H^+$, but this is not proven; wherever the nature of the cation linkage is thought to be established, it is indicated in Table 1.

## Materials and methods

The materials and methods used in this work are described in [8–12]. Genomic DNA of *H. pylori* strains J99 and 26 695 was kindly provided by D.E. Berg (Washington University

**Key words:** bacteria, *Helicobacter pylori*, His tag, membrane transport protein, pathogen, two-component system.

**Abbreviations used:** DDM, n-dodecyl-$\beta$-D-maltoside; IPTG, isopropyl-$\beta$-D-thiogalactopyranoside; KgtP, $\alpha$-ketoglutarate transport protein; LB broth, Luria–Bertani broth; Mdr, multidrug resistance protein; Ni-NTA, $Ni^{2+}$-nitrilotriacetate; TCS, two-component sensor/response system.

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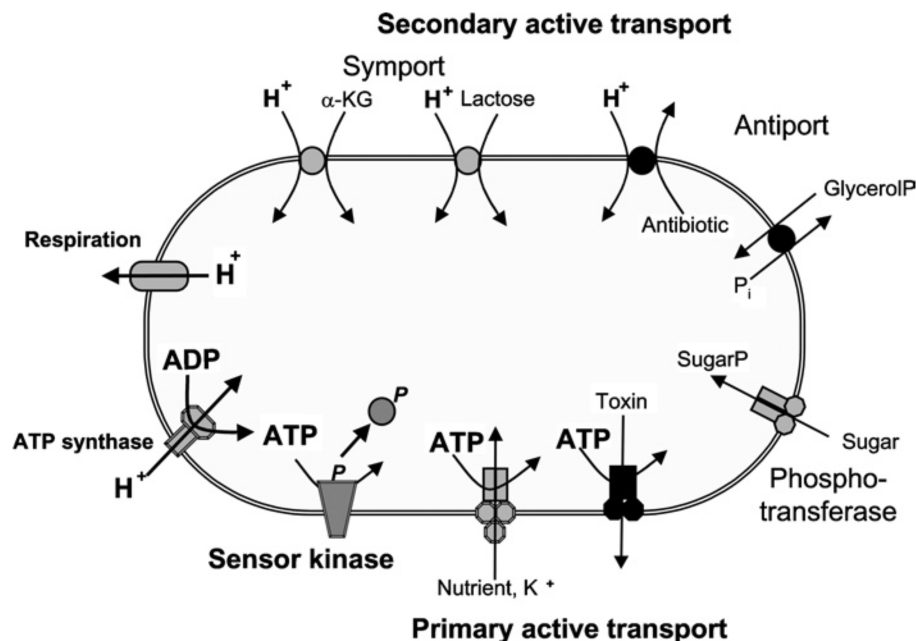
**Figure 1 | Scheme for transport mechanisms and sensor kinases in bacteria**

The oval represents the cytoplasmic membrane of a bacterium containing the enzymes of ATP synthesis and respiration on the left and in a clockwise direction: two symporters, two antiporters, a phosphotransferase, two ABC transporters for efflux and influx and a TCS sensor kinase.




School of Medicine, St. Louis, MO, U.S.A.), from *C. jejuni* and *Neisseria meningitidis* by M.C.J. Maiden (University of Oxford, Oxford, U.K.), from *Brucella abortus* by R.C. Essenberg (Oklahoma State University, Stillwater, OK, U.S.A.) and from *Brucella melitensis* by K. Walravens (Veterinary and Agrochemical Research Centre, Ukkel, Belgium). *Bacillus subtilis* was kindly provided by A. Moir (University of Sheffield, Sheffield, U.K.). DDM (n-dodecyl-$\beta$-D-maltoside) was obtained from Melford Laboratory (Chelsworth, Ipswich, Suffolk, U.K.), Ni-NTA ($Ni^{2+}$-nitrilotriacetate) and an antibody against the $RGSH_6$ epitope were obtained from Qiagen (Dorking, Surrey, U.K.).

*E. coli* strains XL1-Blue Stratagene$^{TM}$ (*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1*, *lac* [*F′proAB lacI$^q$ Z$\Delta$M15*, *Tn10* (*Tet$^R$*)]) and BL21 Novagen$^{TM}$ [$F^-$ *ompT* *hsdS$_B$*($r_B^-$ $m_B^-$) *gal dcm* (*DE3*)] were used as hosts for transformation work. *E. coli* BL21(DE3) was also used for small- and large-scale isolation of plasmids, and for overexpression and purification of transport proteins.

Maintenance and growth of these *E. coli* strains was achieved by culturing the bacteria in LB (Luria–Bertani) broth [9] liquid medium or in minimal salts medium [9] containing 20 mM glycerol or on plates containing 1.5% agar. Carbenicillin (at least 100 $\mu$g/ml) was used throughout all stages of growth to maintain plasmid integrity.

For small-scale investigation of protein expression, 50 ml cultures in 250 ml flasks were used. Total membranes were prepared from sphaeroplasts by the water lysis method [9]. Inner membrane vesicles were prepared from 500 ml cultures in 2 litre baffled conical flasks or from 25 litre fermentor cultures. After harvesting, the cells were disrupted by explosive decompression using a French pressure cell. The inner and outer cell membranes were separated by sucrose density centrifugation, followed by washing in buffer to remove the sucrose and EDTA [9].

For both small-scale and inner membrane production of *E. coli* strains, growth was allowed to continue until the cell density reached an $A_{680}$ of approx. 0.6 when the expression of the cloned gene was induced by the addition of IPTG (isopropyl-$\beta$-D-thiogalactopyranoside) to a final concentration of 0.5 mM. Growth was continued for 3–4 h following the induction of the *tac* promoter, thus producing optimal yields of protein.

## Results

### Introduction of the gene encoding a putative symport or antiport protein into the plasmid pTTQ18

The genes putatively encoding the $\alpha$-ketoglutarate or the Mdr transport proteins were amplified from the *H. pylori* genomic DNA, using appropriate mutagenic oligonucleotides. These were designed to introduce an EcoRI site at the 5′-end and a PstI site at the 3′-end to promote subsequent ligation with the 4.56 kb pTTQ18/$RGSH_6$ fragment. The PCR product was isolated from an agarose gel and then digested with EcoRI and PstI.

In order to clone each of the genes into the pTTQ18 plasmid vector [13], the plasmid pNorAH6 (pTTQ18 containing

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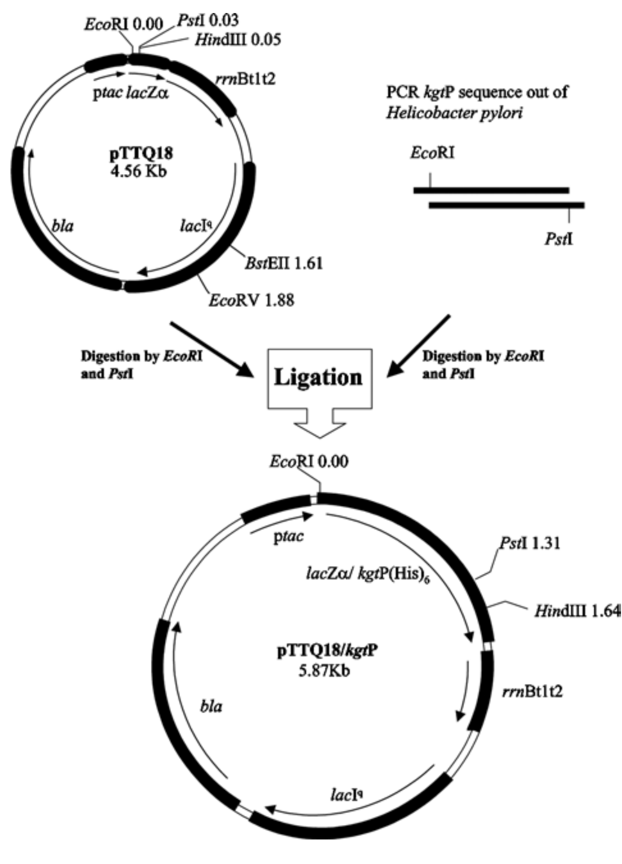
**Table 1** | Examples of 34 overexpressed proteins and their 12 organisms of origin

| Protein | Organism | Major substrate |
|---|---|---|
| KgtP (JHP0334) | *H. pylori* | Ketoglutarate |
| 'ProP' | *H. pylori* | ? |
| GluP | *H. pylori* | Glucose |
| NupC | *H. pylori* | ? |
| PutP | *H. pylori* | Proline-$Na^+$ |
| NixA | *H. pylori* | Nickel |
| Hp1092 | *H. pylori* | Multidrugs |
| Hp1181 | *H. pylori* | Multidrugs |
| AraE | *E. coli* | Arabinose-$H^+$ |
| XylE | *E. coli* | Xylose-$H^+$ |
| GalP | *E. coli* | Galactose-$H^+$ |
| ProP | *E. coli* | Proline-$H^+$ |
| PutP | *E. coli* | Proline-$Na^+$ |
| Bcr | *E. coli* | Bicyclomycin |
| FucP | *E. coli* | Fucose-$H^+$ |
| GusB | *E. coli* | Glucuronide-$H^+$ |
| 'ProP' (Cj0250c) | *C. jejuni* | ? |
| FucP (Cj0486) | *C. jejuni* | Fucose |
| Nma2100 | *N. meningitidis* | Sugar? |
| GluP (Nma0714) | *N. meningitidis* | Glucose |
| 'Bcr' (Nma2040) | *N. meningitidis* | Multidrugs? |
| GluP | *Brucella abortus* | Glucose |
| KgtP | *B. melitensis* | Ketoglutarate |
| LmrB | *B. melitensis* | Lincomycin |
| NorA | *S. aureus* | Multidrugs |
| Mj 1560 | *Methanococcus janaschii* | Multidrugs? |
| Mhp1 | *M. liquefaciens* | Hydantoins |
| AraE | *B. subtilis* | Arabinose |
| Bmr | *B. subtilis* | Multidrugs |
| Blt | *B. subtilis* | Multidrugs |
| VicK | *E. faecalis* | Signal? |
| $VanS_A$ | *E. faecalis* | Signal? |
| PrrB | *R. sphaeroides* | Redox potential |
| SpdB | *S. coelicolor* | DNA? |

the gene *norAH*$_6$ [14], Figure 2) was isolated from *E. coli* strain BLR and digested with the restriction endonucleases EcoRI and PstI to yield two DNA fragments of 4.56 and 1.2 kb. The larger fragment (pTTQ18 with the $RGSH_6$ coding DNA sequence) was isolated from an agarose gel.

Ligation reactions were performed using the EcoRI-PstI digested gene and pTTQ18-$RGSH_6$ fragments at various vector/insert molar ratios. After the ligated product was transformed into *E. coli* XL1-blue, recombinant clones were selected on LB plates containing carbenicillin. Plasmid DNA was prepared from carbenicillin-resistant colonies and subjected to restriction analysis with HindIII, NcoI (results not shown) uniquely cutting restriction enzymes and automated DNA sequencing of the 5′-end. To confirm the correct size of the plasmid and the presence of each gene [11], the size and DNA sequence of the inserts in the new plasmids

**Figure 2** | Cloning strategy for membrane proteins using plasmid pTTQ18. Details are given in [8–14].




(Figure 2) were confirmed. The plasmid was then transformed into *E. coli* strain BL21(DE3) for expression studies.

This procedure can be applied to any gene that does not include EcoRI or PstI restriction sites. If these sites are present in the coding region, then EcoRI and PstI can still be introduced as flanking sites and partial digestion was used to obtain a fragment uncut at the internal site(s), or a two-step procedure adopted as in [12]. Alternatively, other flanking restriction sites can be chosen compatible with the multicloning site in pTTQ18 [9,13], for example, NdeI at the 5′-end and HindIII at the 3′-end of the gene. If the C-terminus is predicted to lie in the periplasm, then a Strep tag may well be successful if the $RGSH_6$ tag is not.

## Detection of expressed His-tagged transport protein in *E. coli* membrane preparations

*E. coli* BL21(DE3) cells harbouring each plasmid were cultured in LB broth plus 20 mm glycerol and expression trials were performed with concentrations 0.0–1.0 mM IPTG [11]. For maximal expression of the putative KgtP–$His_6$ and JHP1092 Mdr proteins, 0.5 mM IPTG is sufficient.

After preparing membrane samples [9–11], SDS/PAGE analysis and staining with Coomassie Brilliant Blue, an IPTG-inducible protein is observed to migrate at approx. 35 kDa

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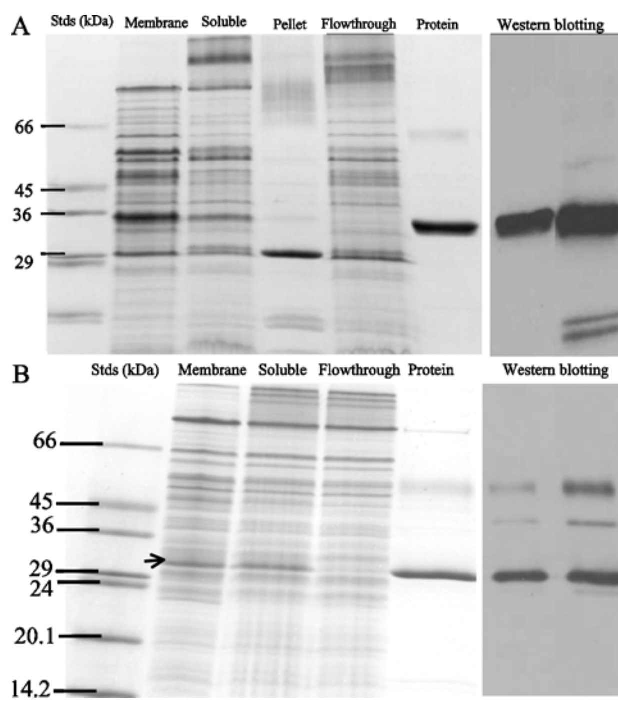
**Figure 3 | Purification of membrane transport proteins**

(**A**) Purification (and identification) of the *H. pylori* KgtP–$His_6$ fusion protein. Membrane preparations were made from IPTG-induced *E. coli* BL21(DE3) (pTTQ18*kgtP*). Samples of the original preparation ('Membrane'), the proteins solubilized in 1.5% DDM ('Soluble') and insoluble ('Pellet') material were examined by SDS/PAGE (Coomassie Blue-stained). A sample of the material that failed to adhere to Ni-NTA-agarose ('Flowthrough') and the Ni-NTA-bound protein subsequently eluted by 200 mM imidazole ('Protein') were processed in the same gel. The eluted protein, 0.1 and 0.5 $\mu$g, was also tested for reaction with an anti-RGS$H_6$ antibody by 'Western blotting'. (**B**) Purification (and identification) of the *H. pylori* JHP1092–$His_6$ putative Mdr. Samples from membranes of IPTG-induced *E. coli* BL21(DE3) (pTTQ18*jhp1092*) were prepared and are labelled as in (**A**). The purified protein of 1 and 2 $\mu$g was also tested for reaction with an anti-RGS$H_6$ antibody by Western blotting.




for KgtP–$His_6$ and at approx. 31 kDa for JHP1092–$His_6$ (Figures 3A and 3B). It is typical for membrane transport proteins to migrate at 65–75% of their true molecular mass, possibly as a result of their hydrophobicity, high binding of SDS or the retention of secondary structure facilitating passage through the gel mixture [9]. This is also the case for membrane sensor proteins of TCS, though the extent of anomalous migration behaviour is not as great, possibly due to fewer TM regions in these proteins compared with membrane transport proteins. The predicted molecular mass of KgtP–$His_6$ is 50 245.3 Da, so the migrating protein would be expected at 32–37 kDa, and for JHP1092–$His_6$ it is 44 994 Da, so the expected apparent mass is 29–34 kDa. Scanning densitometry analysis showed that the induced proteins were expressed at 20% of total membrane proteins, indicating that overexpression has occurred, whereas the protein at the same position in the uninduced sample is expressed at 3% of inner membrane protein. Their identities in the membranes were further confirmed by Western blotting [10,11].

## Solubilization and purification of His-tagged transport proteins

Initial purification of KgtP–$His_6$ or JHP1092–$His_6$ using Immobilized Metal Affinity Chromatography to exploit the C-terminal His-tag (see the Materials and methods section) show proteins migrating at 35 or 31 kDa, respectively in the eluted fractions following Coomassie Brilliant Blue or silver-staining (Figures 3A and 3B), similar to the IPTG-inducible proteins migrating in the inner membrane fractions (Figures 3A and 3B).

In order to reinforce identification and integrity of the isolated proteins, the gels were subjected to Western-blot analysis using an antibody to the C-terminal RGS–$His_6$ tag. Both purified proteins reacted with the antibody (Figures 3A and 3B). N-terminal sequencing further confirmed their identification and, importantly, the integrity of the amino acid sequences of the purified proteins (MNSH MNPQIQ for KgtP–$His_6$ and MNSH MLRKNILAY for JHP1092–$His_6$, the first four amino acids are from the LacZ$\alpha$ peptide). Some minor contaminants (hardly visible on silver staining) in KgtP–$His_6$ preparations of lower $M_r$ were positive with the antibody (Figure 3), suggesting that some breakdown of the purified protein may have occurred, while bands of higher $M_r$ may be oligomers of each protein (Figures 3A and 3B).

Thus, the uncertainty of the identity of the protein resulting from the anomalous migration on SDS/PAGE gels is overcome by the combined detection of the predicted N-terminal sequence and Western-blot analysis for the C-terminal RGS–$His_6$ tag, confirming that the proteins migrating at 31–35 kDa are the ones desired and that they have not been degraded during isolation. These are generic tests of integrity applicable to any –RGS–$His_6$ tagged proteins.

The conditions for solubilization and purification, i.e. DDM concentration for solubilization and imidazole concentration for washes and elution, vary depending on the characteristics of each of the transport proteins. However, the generic conditions described here for one symporter and one antiporter have proved feasible and provided an initial index of yield and purity.

## Retention of structure and activity is confirmed by CD spectroscopy and activity assays of reconstituted protein

CD spectroscopy is a useful technique for the detection of secondary structure within proteins, although the quantification of the proportions of different structural elements is severely limited for membrane proteins [15,16]. The CD spectra obtained for the purified reconstituted $His_6$-tagged JHP0334 and JHP1092 proteins [10,11] reveal a predominantly $\alpha$-helical content, confirming retention of the secondary

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structure during purification of the protein. Furthermore, the purified reconstituted KgtP–$His_6$ protein catalysed $\alpha$-ketoglutarate counterflow into liposomes [10,11].

In the case of TCS proteins, activity of the protein expressed in membranes and also after purification can be established by direct assays of phosphorylation of the protein [12].

## Wider application of the strategy for amplified expression and purification of membrane proteins

The same strategy, with minor modifications in growth and purification conditions, has been used for overexpression of other membrane transport and TCS proteins from *H. pylori* (e.g. [17]), *E. coli* (e.g. [12,18]) and other bacteria, both Gram-negative and Gram-positive (Table 1). Out of 40 attempts, 34 have so far been successful for amplification of expression, i.e. the induced cloned protein comprised at least 15% of the inner membrane preparations of the *E. coli* host strain, and in every case (so far 16) where the $His_6$ tag was added at the C-terminus, the protein has been purified successfully by Ni-NTA chromatography. In some cases where a parallel construct with the $His_6$ tag at the N-terminus, instead of the C-terminus, was made, the level of expression was substantially reduced.

For each protein, growth conditions should be optimized in 1–25 litre cultures of *E. coli* host strains, testing both minimal and complex media at temperatures between 25 and 37°C to maximize the expression [9]. The concentration of IPTG required is tested between 0.1 and 1.0 mM, and the period of growth before induction varied to obtain as high a cell density as possible, commensurate with optimal protein expression (growth is often diminished or abolished, after induction). Similarly, the period of exposure to IPTG (2–24 h) is investigated in order to promote maximum expression. In many cases, a 25 litre fermentor can conveniently be used without compromising expression, but for some proteins the level of expression is always higher in batch cultures of 500–800 ml in 2 litre baffled flasks. Further examination of the parameters regulating growth and protein production in these conditions may enhance our understanding of expression and enable us to reproduce the complex growth behaviour in flasks in the more controlled environment of a fermentor.

## Conclusions

The prime purpose of this review is to illustrate a generic procedure for obtaining sufficient quantities of correctly folded transport and receptor protein(s) from a variety of micro-organisms, including pathogens, for structural studies. This has been achieved for 12 organisms, both Gram-positive and Gram-negative, and for 34 proteins (Table 1). It is possible to proceed from identification of a gene encoding a membrane protein in a bacterial genome to the production of a mg of purified protein in a few weeks, and the application of higher throughput methods for cloning and purification will hopefully reduce the time required. The yield and purity of protein may well be increased by further optimization of conditions, especially for detergent extraction of protein from the membrane. The yields are easily sufficient for trials to form two-dimensional ordered arrays for electron crystallography [19,20], and sufficient to start 3D crystallization trials for X-ray crystallography (T. Shimamura, B. Byrne, S. Iwata, S. Suzuki, N.G. Rutherford, J. O'Reilly and P.J.F. Henderson, unpublished work) and for NMR studies [21,22].

In addition, the purified protein can be examined by a variety of biophysical techniques, e.g. MS for precise $M_r$ and sequence determination [23,24], FTIR, fluorimetry, calorimetry and EPR that elucidate the structure–activity relationship, especially when performed in conjunction with directed mutagenesis and genetic recombination.

The activity and bioenergetics of each cloned transport protein can be confirmed and/or investigated in IPTG-induced *E. coli* hosts, by measuring transport of radioisotope-labelled substrates (e.g. [10,11]) or phosphorylation kinetics of TCS proteins (e.g. [12]). If there is no significant transport activity in an uninduced strain, but substantial activity in the IPTG-induced one, then any *E. coli* wild-type host and vesicles prepared therefrom can be used to investigate cation dependence, substrate specificity, susceptibility to inhibitors etc. Nevertheless, it is preferable to use an *E. coli* host itself attenuated in the relevant transport system. Expression of genes encoded in the pTTQ18 vector is independent of the *E. coli* host in our experience so far, and movement between host strains by transformation is easily accomplished. By this means we have established, for example, that the PutP–$His_6$ protein from *H. pylori* catalyses proline-$Na^+$ symport [11].

In recent landmark papers, the 3D structures of the lactose and $\alpha$-glycerophosphate transport proteins (Figure 1) of *E. coli* have been determined by X-ray crystallography [25,26]. In future, such elucidation of the structures of transport and other membrane proteins from the additional organisms, as described here, may uncover means of preventing or treating bacterial infections.

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