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Collection and characterisation of bacterial membrane proteins

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Abstract A general strategy for the amplified expression in *Escherichia coli* of membrane transport and receptor proteins from other bacteria is described. As an illustration we report the cloning of the putative α-ketoglutarate membrane transport gene from the genome of *Helicobacter pylori*, overexpression of the protein tagged with RGS(His)$_6$ at the C-terminus, and its purification in mg quantities. The retention of structural and functional integrity was verified by circular dichroism spectroscopy and reconstitution of transport activity. This strategy for overexpression and purification is extended to additional membrane proteins from *H. pylori* and from other bacteria.

Key words: Membrane transport protein; α-Ketoglutarate; Two-component system; Pathogen; *Helicobacter pylori*

1. 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, which operate by a variety of mechanisms; examples are facilitated diffusion, adenosine triphosphate (ATP)-dependent primary active transport, sugar-H^+ secondary active transport and phosphotransferase [1]. In addition the bacterial membrane contains proteins that sense environmental conditions and, through the 'two-component' sensor/kinase regulator (TCS) system, enable an appropriate response of the cells [2]. 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 three-dimensional (3D) structure and molecular mechanism.

In this article a strategy is described that enables the purification of one protein, which is readily adapted for the purification of other membrane proteins in amounts required for structural studies. Some of these are homologous to transport proteins found in numerous organisms from cyanobacteria, eubacteria, protozoan parasites, fungi, plants and mammals including man [3]; the convenience of structure–activity studies in bacteria then illuminates the molecular mechanism of transporters in numerous organisms. Others 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 one example can be illustrated here, the α-ketoglutarate transport protein of *Helicobacter pylori*. Tomb et al. [4] determined the complete genome sequence of the *H. pylori* strain 26695, later compared with that of the pathogenic strain J99 [5]. One of the genes found was *jhp0334*. This was thought to encode an α-ketoglutarate membrane transport protein, because its predicted amino acid sequence aligned with 40–44% identity to the known 'KgtP' protein from *Escherichia coli* [3,6], homologues of which occur in *Campylobacter jejuni*, *Salmonella typhimurium*, *Pseudomonas putida*, *Salmonella typhi* and *Brucella suis*, and are members of the major facilitator superfamily of transport proteins [7]. The *jhp0334* gene product is predicted to be of M_r 48 865.6 Da, comprised of 437 amino acids, the hydropathic profile of which suggests it is an integral membrane protein possibly arranged in 12 transmembrane α-helices.

2. Materials and methods

Most of the materials and methods used in this work are described in [8,9]. Only the most relevant detail is included here. Genomic DNA from *H. pylori* strains J99 and 26695 was kindly provided by Professor D.E. Berg (St. Louis, MO, USA), from *C. jejuni* and *Neisseria meningitidis* by Dr M.C.J. Maiden (Oxford, UK) and from *Brucella abortus* by Professor R.C. Essenberg (Ohio, USA). *n*-Dodecyl-β-D-maltoside (DDM) was from Melford Labs, Ipswich, Suffolk, UK and nickel nitrilo-triacetic acid (Ni-NTA) was from Qiagen, Ltd., Dorking, Surrey, UK.

E. coli strains XL1-blue Stratagene™ (*recA1, endAI, gyrA96, thi-I, hsdR17, supE44, relA1, lac* [*F'proAB lacI^q ZΔM15, Tn10 (Tet^R)*]) and BL21 Novagen™ (F$^-$ *ompT hsdS$_B$(r_B^- m_B^-) gal dcm (DE3)*) were used as hosts for transformation work. *E. coli* strain 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 on Luria broth (LB) [9] liquid medium, or plates containing 1.5% agar, or minimal salts medium [9] containing 20 mM glycerol. Carbenicillin (100 ug/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 at 37°C 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-l baffled conical

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flasks or from 25-l fermenter cultures. After harvesting the chilled (0–4°C) 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 sucrose and ethylenediamine tetraacetic acid (EDTA) [9].

For both small-scale and inner membrane production of *E. coli* strains, growth at 37°C was allowed to continue until the cell density reached an A_{680} of approximately 0.6 when the expression of the cloned gene was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) 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.

2.1. Recombinant DNA procedures

The plasmid constructs used in this project were pTTQ18 [10] (Fig. 1) and pNorAH$_6$ [11]. The *jhp0334* gene from *H. pylori* J99 was isolated by polymerase chain reaction (PCR) amplification and cloned into the vector pTTQ18 to form pTTQ18KgtPRGSH$_6$ (Fig. 1 and see text). Primers were synthesised by MWG-Biotech Ltd (Milton Keynes, UK). DNA was resuspended in sterile Milli-Q H_2O at a concentration of 5 pmol/μl. The primers were stored at −20°C. Forward primer was 5′-GCGATGAATTCGCATATGAACCCCCAGATTCAACCCGCCACTAA-3′ (44-mer); reverse primer was 5′-GCAGCCACGACCGCCTGCAGCCTCCAAATAGGTTTTTTTAGGGAAT-3′ (46-mer). 'Touchdown' PCR [12] was used as it simplifies the complicated process of determining optimal annealing temperatures. The size of the amplified product(s) was determined by agarose gel electrophoresis to confirm that the major product was as expected. The DNA was cleaned by phenol/chloroform extraction and ethanol precipitation.

2.2. Purification of protein

Ni-NTA affinity chromatography was used to purify the membrane proteins, which had been genetically modified with RGS(His)$_6$ at their C-terminus. The method is based on the affinity of Ni-NTA resin for proteins and peptides that contain six consecutive histidine residues (hexahistidine affinity tag) at either their N- or C-terminus [13,14]. The purification method was based on that of Racher et al. [15].

The inner membrane containing the overexpressed transport protein was solubilised as follows. Membranes (5 mg/ml) in 5-ml volumes were resuspended in 50 mM potassium phosphate buffer pH 8.0, 10% glycerol and 2 mM β-mercaptoethanol. DDM was added dropwise to give a final concentration of 1.5% w/v and the mixture stirred for 30 min at 0°C. Solubilised protein was recovered from the supernatant after centrifugation at 100 000×*g* for 1 h at 4°C.

Ni-NTA agarose (stored in 30% ethanol) was washed with at least three, 5-fold volumes of Milli-Q H_2O and twice with wash buffer 1 consisting of 50 mM potassium phosphate buffer pH 8.0, 10% glycerol, 10 mM imidazole pH 8.0, 0.05% w/v DDM, 2 mM β-mercaptoethanol and 300 mM NaCl. The solubilised membrane protein was mixed with the Ni-NTA resin for 45 min to 1 h at 4°C and loaded into a mini-disposable polystyrene column (Pierce Biotechnology). Unbound material was recovered for analysis and non-His$_6$-tagged protein was removed by washing the Ni-NTA agarose with 30 vol per ml of resin with wash buffer 1. This was followed by a further wash with 25 vol per ml Ni-NTA agarose with wash buffer 2 containing 50 mM potassium phosphate buffer pH 8.0 (KPi), 10% glycerol, 30 mM imidazole pH 8.0, 0.05% w/v DDM, 2 mM β-mercaptoethanol and 300 mM NaCl.

Bound protein was eluted with five times 1-ml aliquots of 50 mM potassium phosphate buffer pH 8.0, 10% glycerol, 200 mM imidazole pH 8.0, 0.05% w/v DDM, 2 mM β-mercaptoethanol and 300 mM NaCl (elution buffer).

2.3. Characterisation of the protein

Transport assays using intact bacteria were carried out as described in [9,16]. Reconstitution of purified KgtP protein into preformed extruded liposomes [9,17,18] prepared from *E. coli* lipids (Avanti Polar Lipids Inc., Alabaster, AL, USA) was accomplished by the rapid detergent dilution method. Liposomes were destabilised by titration with aliquots of 13.6% (w/v) octyl-β-D-glucoside (OG) monitoring light scattering at 540 nm. The purified protein was added to give a final lipid to protein ratio of 100:1 (w/w) and mixing was continued for 15 min at 4°C. The detergent was removed by the rapid dilution technique where the protein:lipid:detergent mixture (2 ml) was diluted to 130 ml with 50 mM KPi pH 7.6, 1 mM dithiothreitol (DTT), 20 mM α-ketoglutarate, which takes the OG below its critical micelle concentration (CMC). The solution was then separated into two Ti45 tubes and centrifuged at 100 000×*g* for 1 h at 4°C. The supernatant was removed and the pellet of proteoliposomes resuspended in maximum 1 ml (total volume) 50 mM KPi pH 7.6, 1 mM DTT, 20 mM α-ketoglutarate. Counterflow assays employing added [^{14}C]α-ketoglutarate were conducted as described [9,17].

When membrane histidine protein kinases associated with TCS systems were assayed using membrane preparations or purified proteins, autophosphorylation of the proteins was measured directly using [γ-^{33}P]ATP or [γ-^{32}P]ATP [19].

Circular dichroism (CD) measurements [20,21] on purified protein or reconstituted proteoliposomes were made as described [9,17].

3. Results

3.1. Introduction of the gene encoding a putative transport protein into the plasmid pTTQ18 vector

In order to clone the 1.3-kb *jhp0334* gene into the pTTQ18 vector (Fig. 1), the plasmid pNorAH6 (pTTQ18 containing the gene *norAH$_6$* [11]) was isolated from *E. coli* strain BLR and digested with the restriction endonucleases *Eco*RI and *Pst*I to yield two DNA fragments of 4.59 and 1.2 kb, respectively. The larger fragment of 4.59 kb (pTTQ18 with the RGSH$_6$-coding DNA sequence) was isolated from an agarose gel.

The gene putatively encoding an α-ketoglutarate transport protein was amplified from the *H. pylori* genomic DNA, using mutagenic oligonucleotides (Section 2). They were designed to introduce an *Eco*RI site at the 5′ end and a *Pst*I site at the 3′ end to promote subsequent ligation with the 4.59-kb pTTQ18/RGSH$_6$ fragment. The PCR product was isolated from an agarose gel and then digested with *Eco*RI and *Pst*I.

Ligation reactions were performed using the *Eco*RI–*Pst*I-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 *Hin*dIII, *Nco*I uniquely cutting restriction enzymes and automated DNA sequencing of the 5′ end to confirm the correct size of the plasmid and the presence of the gene [17]. The size and DNA sequence of the insert in the new plasmid (Fig. 1) 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 *Eco*RI or *Pst*I restriction sites. If these sites are present in the coding region then *Eco*RI and *Pst*I can still be introduced as flanking sites and partial digestion used to obtain a fragment uncut at the internal site(s). Alternatively, as in the case of *regB* (*prrB*), which contains an internal *Eco*RI site, a two-step cloning procedure was adopted, followed by sequencing of the full final insert [19]. Also, other flanking restriction sites can be chosen compatible with the multi-cloning site in pTTQ18 [10].

3.2. Expression and activity of the cloned histidine-tagged transport protein in E. coli

Transport proteins are readily assayed using radioisotope-labelled substrate [16]. *E. coli* BL21(DE3) (pTTQ18KgtPRGSH$_6$) was grown on LB plus 20 mM glycerol in the presence or absence of 0.5 mM IPTG, the cells were harvested and washed and transport assays were performed.

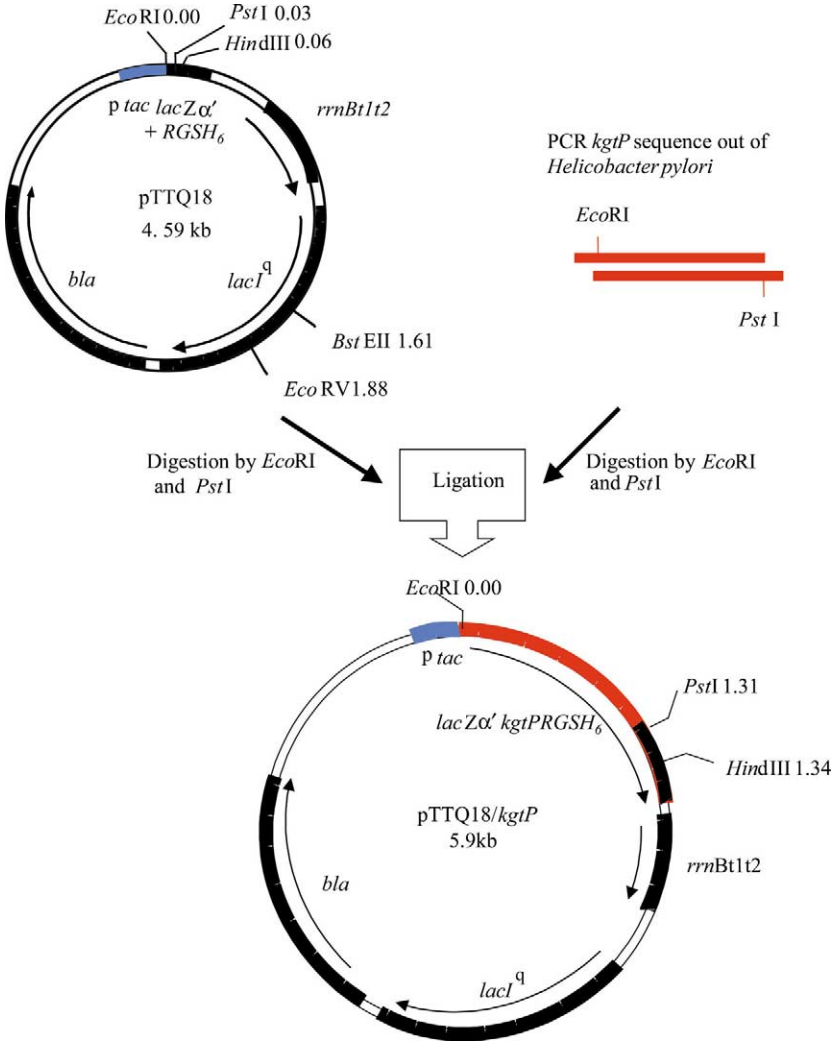

Fig. 1. Cloning strategy for membrane proteins using plasmid pTTQ18 [10].

The IPTG-induced cells exhibited a prolonged uptake of labelled α-ketoglutarate, which was absent from the uninduced bacteria (Fig. 2, inset). This was not due to α-ketoglutarate transport activity of the host cells as no activity was observed in the IPTG-induced BLR(DE3) host strain without the plasmid [17] (not shown). Thus, the protein encoded by the *H. pylori* gene on the plasmid was confirmed to be an α-ketoglutarate transporter.

In the case of cloned TCS membrane receptor proteins their ATP-dependent autophosphorylation activity can similarly be checked in membrane preparations from IPTG-induced cells and compared with activities from uninduced cells [19].

3.3. *Detection of expressed histidine-tagged transport protein in* E. coli *membrane preparations*

E. coli BL21(DE3) cells harbouring each plasmid were cultured in Luria–Bertani (LB) broth plus glycerol and expression trials performed with concentrations of IPTG of 0.0–1.0 mM [17]. 0.5 mM IPTG is sufficient for maximal expression of the putative KgtP(His)$_6$ protein.

After preparing membrane samples [9], sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis and staining with Coomassie brilliant blue (Fig. 2), an

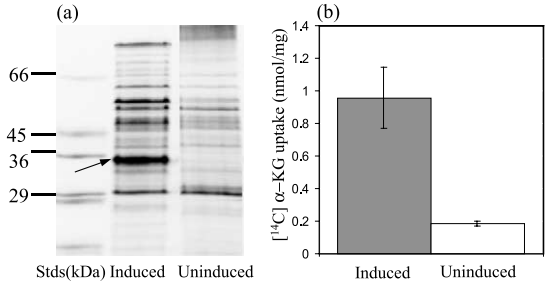

Fig. 2. a: Putative identification of the expressed KgtP(His)$_6$ membrane protein. Membranes were prepared from *E. coli* BL21 (pTTQ18*kgtPRGSH*$_6$) grown in the presence (induced) or absence (uninduced) of 0.5 mM IPTG (see Section 2). The proteins were separated by SDS–PAGE and stained with Coomassie blue. The arrow indicates the position of the protein putatively identified as the product of the *H. pylori kgtp* gene, which was confirmed by Western blotting (Fig. 3). b: Confirmation of activity of the *H. pylori* KgtPRGS(His)$_6$ protein expressed in *E. coli*. *E. coli* BL21 (pTTQ18*kgtPRGSH*$_6$) was grown in the presence (induced) or absence (uninduced) of 0.5 mM IPTG, harvested and washed (Section 2 and [9]). The transport of [^{14}C-α]ketoglutarate was then measured into each for 2 min in triplicate.

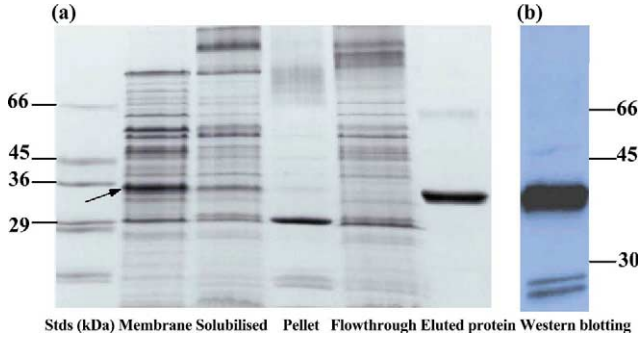

Fig. 3. a: Purification (and identification) of the *H. pylori* KgtPRGS(His)$_6$ protein. Membrane preparations were solubilised in 1.5% DDM and centrifuged (Section 2). Samples of the original preparation ('Membrane', with an arrow indicating the KgtPRGS(His)$_6$ protein), the soluble ('Solubilised') and insoluble ('Pellet') material were examined by SDS–PAGE (Coomassie 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 ('Eluted protein') were processed in the same gel. b: Confirmation of the identity of the KgtPRGS(His)$_6$ protein. The purified protein from a was blotted and tested for reactivity with an anti-RGSH$_6$ antibody by Western blotting (Section 2).

IPTG-inducible protein is observed migrating at approximately 35 kDa. It is typical for membrane transport proteins to migrate at 65–75% of their true molecular weight, possibly as a result of their hydrophobicity, high binding of SDS or the retention of secondary structure accelerating passage through the gel mixture [9]. The predicted molecular weight of KgtP(His)$_6$ is 50 245.3 Da, so the migrating band would be expected at 32–37 kDa. Scanning densitometry analysis showed that the induced protein is expressed at 20%, indicating that overexpression has occurred, whereas the protein at the same position in the uninduced sample is expressed at 3% of inner membrane protein. Its identity was further confirmed by Western blotting (see below).

The induced protein migrating at 35 kDa appeared in the inner membrane sample and was absent from the outer membrane sample [17], consistent with the location expected of a transport protein.

3.4. Solubilisation and purification of histidine-tagged transport protein

This initial purification using Ni-chelate affinity chromatography to exploit the C-terminal His-tag (Section 2) shows a protein migrating at 35 kDa in the eluted fractions following staining with Coomassie brilliant blue (Fig. 3), similar to the IPTG-inducible protein migrating at this M_r in the SDS–PAGE gel of all the membrane proteins (Fig. 2).

In order to reinforce identification of the 35-kDa protein as KgtP(His)$_6$ the gels were subjected to Western blot analysis using an antibody to the C-terminal RGS(His)$_6$-tag. Fig. 3 shows that the purified 35-kDa protein reacted with the antibody, confirming that the protein migrating at this position is KgtP(His)$_6$. N-terminal sequencing (MNSH MNPQIQ, the first four amino acids are from the LacZα peptide) of the purified protein further confirmed its identification and its integrity. Some minor contaminants (hardly visible on silver staining) of lower M_r were positive with the antibody (Fig. 3), suggesting that some breakdown of the purified protein may have occurred.

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 protein migrating at about 35 kDa is the one desired and has not been degraded during isolation.

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

3.5. 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

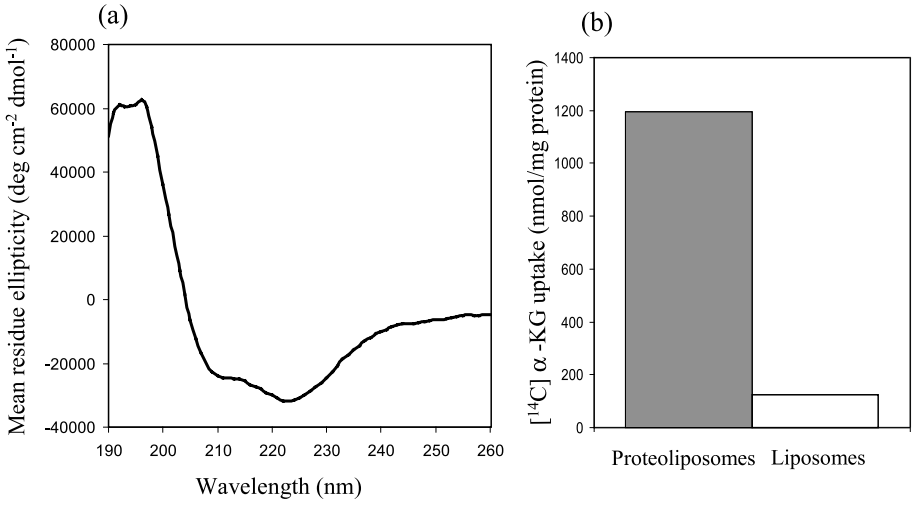

Fig. 4. a: The KgtPRGS(His)$_6$ protein has predominantly α-helix secondary structure. The CD spectrum of the KgtPRGS(His)$_6$ protein was determined in 10 mm KPi, pH 8, after reconstitution into liposomes of *E. coli* lipid (see Section 2). b: The KgtPRGS(His)$_6$ protein is functional after purification. The purified protein was reconstituted into liposomes of *E. coli* lipid and a counterflow transport assay performed for 10 min (see Section 2 and [9]) in duplicate for both proteoliposomes and liposomes.

secondary structure within proteins, although the quantitation of the proportions of different structural elements is severely limited for membrane proteins [20,21]. The CD spectrum obtained for the purified reconstituted KgtP(His)$_6$ protein (Fig. 4) reveals a predominantly α-helix content, confirming retention of the secondary structure during purification of the protein. Furthermore, the purified reconstituted protein catalysed α-ketoglutarate counterflow (Fig. 4) into liposomes [9].

3.6. Wider application of the strategy for amplified expression and purification

The same strategy, with minor modifications in growth and purification conditions, has been used for overexpression of other membrane proteins from *H. pylori* (e.g. [22]), *C. jejuni*, *N. meningitidis*, *B. abortus*, *Staphylococcus aureus*, *Bacillus subtilis*, *Rhodobacter sphaeroides*, and *Streptomyces coelicolor* (Table 1). Out of 39 attempts, 36 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 RGS(His)$_6$-tag was added at the C-terminus the protein has been purified successfully by Ni-NTA chromatography. In one case, the *nupC* gene of *E. coli*, addition of an RGS(His)$_6$-tag could not be accomplished; this is thought to be symptomatic of the C-terminus of this protein being exported to the periplasm. 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.

Growth conditions should be optimised in 1–25-l cultures of *E. coli* host strains, testing both minimal and complex media at temperatures between 25 and 37°C to maximise expression of each protein [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-l fermenter 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-l baffled flasks. Further examination of the parameters regulating growth and protein production in these conditions may enhance our understanding of expression.

Table 1
Overexpressed proteins and organisms

Protein	Organism	Major substrate
KgtP (Jhp0334)	*H. pylori*	ketoglutarate
'ProP'	*H. pylori*	?
GluP	*H. pylori*	glucose
NupC	*H. pylori*	?
Hp1181	*H. pylori*	multidrugs
PutP	*H. pylori*	proline
'ProP' (Cj0250c)	*C. jejuni*	?
'ProP' (Cj1588c)	*C. jejuni*	?
FucP (Cj0486)	*C. jejuni*	fucose
Nma2100	*N. meningitidis*	sugar?
GluP (Nma0714)	*N. meningitidis*	glucose
'Bcr' (Nma2040)	*N. meningitidis*	multidrugs?
GluP	*B. abortus*	glucose
NorA	*S. aureus*	multidrugs
Mj 1560	*Methanococcus janaschii*	multidrugs?
Bmr	*B. subtilis*	multidrugs
Blt	*B. subtilis*	multidrugs
RegB	*R. sphaeroides*	signal?
SpdB	*S. coelicolor*	DNA?

4. Conclusions

The prime purpose of this study was to consolidate a generic procedure for obtaining sufficient quantities of undenatured transport and receptor protein(s) from a variety of microorganisms, including pathogens, for future structural studies. This has been achieved for nine organisms, both Gram-positive and Gram-negative, and for 19 proteins (Table 1). The general strategy for cloning genes encoding membrane proteins into plasmid pTTQ18, heterologous expression in *E. coli*, and addition of an RGS(His)$_6$-tag for purification using DDM [9] cf. [22–27] is shown to be successful. It is possible to proceed from identification of a gene encoding a membrane protein in a bacterial genome to the production of 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 optimisation of conditions, especially for detergent extraction of protein from the membrane. The yields are easily enough for trials to form two-dimensional (2D) ordered arrays for electron crystallography [28,29] and sufficient to start 3D crystallisation trials for X-ray crystallography (Rutherford, Shibayama, Suzuki, Byrne, and Iwata, unpublished) and for NMR studies [30].

In addition, the purified protein can be examined by a variety of biophysical techniques – mass spectrometry for precise M_r and sequence determination [31,32], FTIR, fluorimetry, calorimetry and EPR that elucidate the structure–activity relationship, especially when performed in conjunction with directed mutagenesis and genetic recombination.

In recent landmark papers the 3D structures of the lactose and α-glycerophosphate transport proteins of *E. coli* have been determined by X-ray crystallography [33,34]. Future elucidation of the structures of transport proteins from *H. pylori*, *C. jejuni*, *S. aureus*, *N. meningitidis*, and other pathogens may uncover means of preventing or treating bacterial infections.

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References

[1] Henderson, P.J.F. (1997) in: Transporters Factsbook (Griffith, J.K. and Sansom, C.E., Eds.), pp. 3–29, Academic Press, London.
[2] Hoch, J.A. and Silhavy, T.J. (1995) in: Two-Component Signal Transduction (Hoch, J.A. and Silhavy, T.J., Eds.), ASM Press, Washington, DC.
[3] Griffith, J.K. and Sansom, C.E. (1998) in: Transporter Factsbook (Griffith, J.K. and Sansom, C.E., Eds.), pp. 335–363, Academic Press, London.

[4] Tomb, J.F. et al. (1997) Nature 388, 539–547.
[5] Alm, R.A. et al. (1999) Nature 397, 176–180.
[6] Seol, W. and Shatkin, A.J. (1991) Proc. Natl. Acad. Sci. USA 88, 3802–3806.
[7] Pao, S.S., Paulsen, I.T. and Saier, M.H. (1998) Microbiol. Biol. Rev. 62, 1–32.
[8] Clough, J.L. (2001) PhD Thesis, University of Leeds, Leeds.
[9] Ward, A., Sanderson, N.M., O'Reilly, J., Rutherford, N.G., Poolman, B. and Henderson, P.J.F. (2000) in: Membrane Transport – a Practical Approach (Baldwin, S.A., Ed.), pp. 141–166, Blackwell's Press, Oxford.
[10] Stark, M.J. (1987) Gene 51, 255–267.
[11] Hoyle, C.J. (2000) PhD Thesis, University of Leeds, Leeds.
[12] Don, R.H., Cox, P.T., Wainwright, B.J., Baker, K. and Mattick, J.S. (1991) Nucleic Acids Res. 19, 4008.
[13] Hochuli, E., Dobeli, H. and Schacher, A.J. (1987) Chromatography 411, 177–184.
[14] Hochuli, E., Bannwarth, W., Dobeli, H., Gentz, R. and Stuber, D. (1988) BioTechnology 6, 1321–1325.
[15] Racher, K.I., Voegele, R.T., Marshall, E.V., Culham, D.E., Wood, J.M., Jung, H., Bacon, M., Cairns, M.T., Ferguson, S.M., Liang, W-J., Henderson, P.J.F., White, G. and Hallet, F.R. (1999) Biochemistry 38, 1676–1684.
[16] Macpherson, A.J.S. and Henderson, P.J.F. (1986) Methods Enzymol. 125, 387–429.
[17] Saidijam, M., unpublished.
[18] Knol, J., Veenhof, L., Liang, W-j., Henderson, P.J.F., Leblanc, G. and Poolman, B. (1996) J. Biol. Chem. 271, 15358–15366.
[19] Potter, C.A., Ward, A., Laguri, C., Williamson, M.P., Henderson, P.J.F. and Phillips-Jones, M.K. (2002) J. Mol. Biol. 320, 201–213.
[20] Wallace, B.A. and Teeters, C.L. (1987) Biochemistry 26, 65–70.
[21] Wallace, B.A., Lees, J., Orry, A.J.W., Lobley, A. and Janes, R.W. (2003) Protein Sci. 12, 875–884.
[22] Morrison, S., Ward, A., Hoyle, C.J. and Henderson, P.J.F. (2003) Int. J. Antimicrob. Agents 22, 242–249.
[23] Palmer, S.L. (1996) PhD Thesis, University of Leeds, UK.
[24] Aldema, M.L., McMurray, L.M., Walmsley, A.R. and Levy, S.B. (1996) Mol. Microbiol. 19, 187–195.
[25] Racher, K.I., Voegele, R.T., Marshall, E.V., Culham, D.E., Wood, J.M., Bacon, M., Cairns, M.T., Ferguson, S.M., Liang, W.-j., Henderson, P.J.F., Jung, H., White, G. and Hallett, F.R. (1999) Biochemistry 38, 1676–1684.
[26] Ward, A., Hoyle, C.J., Palmer, S., O'Reilly, J., Griffith, J.K., Pos, K.M., Morrison, S., Poolman, B., Gwynne, M. and Henderson, P.J.F. (2001) Mol. Microbiol. Biotech. 3, 193–200.
[27] Wang, D.-N., Safferling, M., Lemiuex, M.J., Griffith, H. and Chen, Y. (2003) Biochim. Biophys. Acta 1610, 23–36.
[28] Yin, C.-C., Aldema-Ramos, M.L., Borges-Walmsley, M.I., Taylor, R.W., Walmsley, A.R., Levy, S.B. and Bullough, P.A. (2000) Molec. Microbiol. 38, 613–625.
[29] Hirai, T., Heymann, J.A.W., Shi, D., Sarker, R., Maloney, P.C. and Subramanian, S. (2002) Nat. Struct. Biol. 9, 597–600.
[30] Patching, S.G., Brough, A.R., Herbert, R.B., Henderson, P.J.F. and Middleton, D.A. (2003) J. Am. Chem. Soc. (in press).
[31] Venter, H., Ashcroft, A.E., Keen, J.N., Henderson, P.J.F. and Herbert, R.B. (2002) Biochem. J. 363, 243–252.
[32] Le Coutre, J., Whitelegge, J.P., Gross, A., Turk, E., Wright, E.M., Kaback, H.R. and Faull, K.F. (2000) Biochemistry 39, 4237–4242.
[33] Abramson, J., Smirnova, I., Kasho, V., Verner, G., Kaback, H.R. and Iwata, S. (2003) Science 301, 610–615.
[34] Huang, Y., Lemieux, M.J., Song, J., Auer, M. and Wang, D.-N. (2003) Science 301, 616–620.