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Overexpression and purification of the three components of the *Enterobacter aerogenes* AcrA–AcrB–TolC multidrug efflux pump

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Abstract

The tripartite AcrA–AcrB–TolC system is the major efflux pump of the nosocomial pathogen *Enterobacter aerogenes*. AcrA is a trimeric periplasmic lipoprotein anchored in the inner membrane, AcrB is an inner membrane transporter and TolC is a trimeric outer membrane channel. In order to reconstitute the AcrA–AcrB–TolC system of *E. aerogenes* in artificial membranes, we overexpressed and purified the three proteins. The *E. aerogenes acrA*, *acrB* and *tolC* open reading frames were individually inserted in the expression vector pET24a^{+}, in frame with a sequence coding a C-terminal hexahistidine tag to allow purification by INAC (Immobilized Nickel Affinity Chromatography). The mature AcrA–6His was overproduced in a soluble form in the cytoplasm of *Escherichia coli* BL21(DE3). AcrA–6His was purified under native conditions in two steps using INAC and gel permeation chromatography. We obtained about 25 mg of 97% pure AcrA–6His per liter of culture. AcrB–6His was solubilized from the membrane fraction of *E. coli* C43(DE3) in 300 m*M* NaCl, 5% Triton X-100 and purified in one step by INAC. The AcrB–6His enriched fraction was eluted with 100 m*M* imidazole. The final yield was 1–2 mg of 95% pure AcrB–6His per liter of culture. The membrane fraction of *E. coli* BL21(DE3)pLysS containing TolC–6His was first treated with 2% Triton X-100, 30 m*M* $MgCl_2$ to solubilize the inner membrane proteins. After ultracentrifugation, the pellet was treated with 5% Triton X-100, 5 m*M* EDTA to solubilize the outer membrane proteins. Approximately 5 mg of 95% pure TolC–6His trimers per liter of culture was purified by INAC.
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Keywords: Expression; Purification; *Enterobacter aerogenes*; AcrA–AcrB–TolC multidrug efflux pump

1. Introduction

During the last decade, *Enterobacter aerogenes* has emerged as an important opportunist nosocomial pathogen. Since 1992, this Gram-negative bacterium is the third most prevalent pathogen involved in hospital infections, after *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Today, most of the *E. aerogenes* clinical isolates are multidrug resistant (MDR) [1].

In Gram-negative bacteria, the MDR phenotype results from two main mechanisms. First, the outer membrane (OM) acts as a permeability barrier: the narrow porin channels slow down the penetration of hydrophilic compounds to the periplasmic space, and the low fluidity of the lipopolysaccharidic leaflet

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decreases the diffusion of lipophilic compounds [2,3]. Moreover, porin production can decrease in response to an antibiotic stress [4]. Second, the overexpression of efflux systems can increase resistance levels [4]. Drug efflux is mediated by transport proteins located in the inner membrane (IM) that can recognize and extrude a wide variety of toxic molecules: solvents, detergents, basic stains and lipophilic antibiotics [5]. To date, efflux pumps have been subdivided in five different classes [5,6]. Among these, the resistance-nodulation-cell division (RND) family systems utilize three components: a transporter located in the IM, a periplasmic membrane fusion protein and an OM channel [7,8]. The tripartite complex traverses both membranes and allows the extrusion of drugs directly into the extracellular medium. The well-studied AcrAB–TolC pump of *Escherichia coli* belongs to the RND class [8,9]. The 110 000 AcrB transporter contains 12 transmembrane α-helices and two large periplasmic loops between helices I–II and VII–VIII [10]. AcrA is a 41 000 periplasmic lipoprotein anchored in the IM, which appears to interact with AcrB as a trimer [11,12]. Because of its elongated form, AcrA is thought to span the periplasm and contact TolC [13]. The recent elucidation of the crystal structure of the 51 500 TolC protein revealed that TolC trimers form a channel in the OM, prolonged by a transperiplasmic tunnel, which may function as a bridge to the AcrAB complex [14].

When overexpressed, the AcrAB–TolC system increases *E. coli* resistance to tetracycline and chloramphenicol [15,16].

Some *E. aerogenes* MDR clinical isolates exhibit an active antibiotic efflux [17]. Recently, the *E. aerogenes acrAB* and *tolC* loci have been cloned, and shown to play a major role in *E. aerogenes* MDR [18]. *E. aerogenes* AcrA, AcrB and TolC share more than 80% sequence identity with their *E. coli* homologs, suggesting an identical molecular organization in the envelope. In order to purify and reconstitute the *E. aerogenes* AcrA–AcrB–TolC efflux pump in artificial membranes, we produced the three proteins individually in *E. coli*. The *acrA*, *acrB* and *tolC* coding sequences were inserted in the expression vector pET24a^{+}, in frame with a sequence encoding a C-terminal hexahistidine (6His) tag to allow purification by INAC (Immobilized Nickel Affinity Chromatography). We overproduced a soluble non-lipid form of AcrA–6His in the cytoplasm as AcrA acylation is not required for its function [19]. Recombinant AcrB–6His and TolC–6His were produced in their native form and were located in the IM and OM, respectively.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

The strains and plasmids used in this work are listed in Table 1. *E. coli* strains were grown at 37 °C in Luria–Bertani medium (LB) or on solid agar obtained by addition of 1.5% (w/v) Bacto-Agar. Antibiotics were added in the growth media at the following final concentrations: chloramphenicol (Cm), 30 μg/ml; kanamycin (Km), 30 μg/ml.

2.2. DNA techniques

Plasmid DNA was routinely isolated by the alkaline lysis method [19] or purified by using a Wizard® Plus SV Minipreps DNA Purification System kit (Promega, Madison, WI, USA) for sequencing. PCRs were carried out with Vent® polymerase (New England Biolabs, Beverly, MA, USA). DNA fragments were sequenced using a dRho Terminator cycle Sequencing Ready reaction kit and an ABI PRISM sequencer (Perkin-Elmer, Norwalk, CT, USA) and a combination of universal and custom-synthesized primers. DNA fragments were gel-extracted by using a Concert Rapid Extraction kit (Invitrogen, Cergy Pontoise, France).

2.3. Plasmids construction

acrA was amplified by polymerase chain reaction (PCR) without its signal sequence and the N-terminal cysteine residue of the mature sequence, from pEP709 (*acrAB*) with primers *Nde*I–*acrA* (5′-ATATCATATGGACGATAAACCGGCAA-3′) and *acrA*–*Xho*I (5′-ATATCTCGAGAGACTTGGTT-TGTTCAGA-3′). The *acrB* and *tolC* open reading frames were PCR-amplified using pEP709 or pEP710 (*tolC*) as template, and primers *Nde*I–*acrB* (5′ - CATATGCCTAATTTCTTTATCGATCG - 3′)

Table 1
Bacterial strains and plasmids

Strains or plasmids	Relevant features[a]	Source or reference
XL1-Blue	*E. coli* K-12 *recA* β*lac* F′lacZ M15, Tc^r high efficiency transformation	Stratagen, La Jolla, CA, USA
BL21(DE3)	*E. coli* B *lonompT* expression strain	Novagen, Madison, WI, USA
BL21(DE3)pLysS	pLysS encodes T7-lysozyme, Cm^r	Novagen
C41(DE3)		
C43(DE3	BL21(DE3) derivatives	[20]
Plasmids		
$pBCSK^+$	High copy number cloning vector, Cm^r	Stratagen
$pET24a^+$	T7 promoter expression vector, Km^r	Novagen
pEP709	$pBCSK^+$ containing *E. aerogenes acrAB*	[18]
pEP710	$pBCSK^+$ containing *E. aerogenes tolC*	[18]
pMM759	$pBCSK^+$ containing *E. aerogenes acrA*	This work
pMM760	$pBCSK^+$ containing *E. aerogenes tolC*	This work
pMM761	$pET24a^+$ containing *E. aerogenes acrA*	This work
pMM762	$pET24a^+$ containing *E. aerogenes tolC*	This work
pMM763	$pBCSK^+$ containing *E. aerogenes acrB*	This work
pMM797	$pET24a^+$ containing *E. aerogenes acrB*	This work

[a] Cm^r, Km^r and Tc^r, resistance to chloramphenicol, kanamycin and tetracycline, respectively.

and *acrB–Xho*I (5′-CTCGAGATGATGCTCAACCGGATG-3′), or *Nde*I–*tolC* (5′-ATATCATATGCAAATGAAGAAACTTTTTC-3′) and *tolC–Xho*I (5′-ATATCTCGAGGTGGATAAACGGATTAGAACC-3′), respectively. The forward primers contain an *Nde*I restriction site (underlined) to generate the ATG initiation codon, and the reverse primers contain a *Xho*I restriction site (underlined) to clone the amplification products in frame with the 6His sequence in $pET24a^+$. PCR reactions were 30 cycles of 30 s at 96 °C, 1 min at 58 °C, and 1 min (for *acrA* and *tolC*) or 3 min (for *acrB*) at 72 °C. Each PCR product was gel-purified and cloned into $pBCSK^+$. The inserts were sequenced to ensure that no mutation had been introduced. The *Nde*I–*Xho*I fragments were then individually inserted into *Nde*I–*Xho*I restricted $pET24a^+$ to obtain pMM761 (*acrA*), pMM797 (*acrB*) and pMM762 (*tolC*). The encoded proteins will be named AcrA–6His, AcrB–6His and TolC–6His hereafter.

2.4. Protein expression in E. coli

Overnight cultures of BL21(DE3) or its derivatives bearing pMM761, pMM797 or pMM762 were diluted 1:100 in LB and incubated at 37 °C until OD_{600} reached 0.6. At this point, cells were induced with 1 m*M* isopropyl-β-D-thiogalactoside (IPTG), incubated for 4 h at 37 °C, then harvested by centrifugation. The cellular pellets were washed in HEPES 10 m*M* (pH 7.4) and stored at −80 °C.

2.5. Purification of AcrA–6His

BL21(DE3)(pMM761) cells from a 100 ml culture were thawed on ice and resuspended in 2 ml of 50 m*M* NaH_2PO_4, 300 m*M* NaCl, 10 m*M* imidazole, pH 8.0 (column buffer) containing 1 mg/ml lysozyme. The mixture was sonicated on ice and centrifuged at 8000 *g* for 30 min at 4 °C to pellet unbroken cells and cellular debris. The supernatant containing soluble proteins was clarified by centrifugation (40 000 *g*, 20 min, 4 °C) and filtered through a 0.22 μm filter. The resulting lysate was loaded onto a 5 ml HiTrap Chelating column (Amersham Biosciences, Freiburg, Germany) charged with Ni^{2+} and equilibrated with 10 column volumes (CV) of column buffer, at a flow-rate of 1 ml/min. The column was washed with 50 m*M* imidazole in column buffer and eluted with 250 m*M* imidazole in the same buffer. Fractions of 1 ml were collected and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Column fractions containing purified AcrA–6His were pooled. To

remove imidazole, the protein pool was dialyzed against two changes of 50 m*M* NaPi (pH 7.4), 150 m*M* NaCl. The protein was stable for several weeks at 4 °C. Fractions enriched in AcrA–6His were concentrated to 10–20 mg/ml using a Centriprep® YM30 (Millipore, Bedford, MA, USA). Samples of purified AcrA–6His in a 0.5 ml maximum volume were injected onto a Superose 12 HR 10/30 column (Amersham Biosciences, Freiburg, Germany). Isocratic elution was performed with 4 CV of 50 m*M* NaPi (pH 7.4), 150 m*M* NaCl, at 0.5 ml/min. Fractions of 1 ml were collected and analyzed by SDS–PAGE. Fractions containing highly purified AcrA–6His were pooled.

2.6. Membrane preparation

AcrB–6His and TolC–6His were extracted and purified from the membrane fraction of C43-(DE3)(pMM767) and BL21(DE3)pLyS(pMM762), respectively. Cell pellets from 250 ml cultures were thawed on ice and resuspended in 10 ml of 20 m*M* Tris–HCl (pH 8.0), 100 m*M* NaCl (buffer A) containing 1 mg/ml lysosyme. From this step, all solutions were supplemented with 1:100 (v/v) of protease inhibitor cocktail for His-tagged proteins (Sigma, St Louis, MO, USA). After sonication, cell fragments were removed by centrifugation (8000 *g* for 30 min at 4 °C) and the whole membrane fraction was recovered by ultracentrifugation at 110 000 *g* for 60 min at 4 °C. Pellets were stored at −80 °C until use.

2.7. Membrane solubilization and purification of AcrB–6His and TolC–6His

AcrB–6His purification was performed at 4 °C. The C43(DE3)(pMM797) membranes were resuspended in 5 ml of 20 m*M* Tris–HCl (pH 8.0), 300 m*M* NaCl, 10 m*M* imidazole (binding buffer) containing 5% (v/v) Triton X-100. After overnight storage at 4 °C, the mixture was ultracentrifuged (110 000 *g* for 60 min at 4 °C). The supernatant was loaded onto the 5 ml HiTrap Chelating column recharged with Ni^{2+} and equilibrated with 10 CV of binding buffer containing 0.2% (v/v) Triton X-100 (buffer B), at a flow-rate of 1 ml/min. The column was washed with 5 CV of buffer B and the bound protein was eluted with a linear gradient from 10 m*M* to 250 m*M* imidazole. The fractions containing AcrB–6His were identified by SDS–PAGE.

Extraction of TolC–6His from the OM of BL21(DE3)pLysS(pMM762) was carried out in three steps. Membranes were resuspended in 10 ml of buffer A containing 2% (v/v) Triton X-100 and 30 m*M* $MgCl_2$. After 20 min at 4 °C, the mixture was ultracentrifuged as above. The supernatant containing the solubilized IM proteins was removed, and the OM pellet was resuspended in 5 ml of binding buffer containing 5% (v/v) Triton X-100 and 5 m*M* EDTA (pH 8.0). The solution was stirred on ice for 60 min, and the solubilized material was separated by ultracentrifugation as above. This extraction step was repeated, the supernatants were pooled and dialyzed against two changes of binding buffer to remove EDTA. This fraction was loaded onto the 5 ml HiTrap Chelating column recharged with Ni^{2+} and equilibrated with 10 CV of buffer B, at a flow-rate of 1 ml/min. The column was washed with 5 CV 100 m*M* imidazole in buffer B and eluted with 5 CV with 250 m*M* imidazole in the same buffer. Column fractions were collected and analyzed by SDS–PAGE. Fractions containing TolC–6His were pooled and stored at −20 °C.

2.8. SDS–PAGE and Western blotting

SDS–PAGE was performed on a discontinuous SDS gel system [19], with a 3% stacking gel and a 10% resolving gel. Gels were stained with Coomassie Brilliant Blue R-250, or silver nitrate. Samples were mixed with an equal volume of 2× concentrated sample buffer, heated for 5 min at 96 °C for those containing AcrA–6His or TolC–6His, or incubated for 20 min at 37 °C for those containing AcrB–6His. For Western blots, proteins were electrotransfered onto a nitrocellulose membrane (Schleicher & Schull, Keene, NH, USA) in buffer 20 m*M* Tris, 150 m*M* glycine, 20% isopropanol, 0.05% SDS. Membranes were probed with a 1:1000 dilution of Ni–NTA AP conjugate (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions and developed by colorimetric detection.

2.9. Protein microsequencing

For N-terminal microsequencing, proteins were electrotransfered onto a Immobilon polyvinydilene

difluoride membrane (Millipore, Bedford, MA, USA) in 20% methanol buffer. Microsequencing of proteins was performed by automated Edman degradation (492A protein sequencer, PE Applied Biosystems, Foster City, CA, USA).

2.10. Mass spectrometry

Samples were precipitated with 15% trichloroacetic acid, washed with acetone, dried and resuspended in formic acid. Molecular weights were determined by ''Matrix Assisted Laser Desorption Ionisation, Time of Flight'' (Reflex III MALDI–TOF, Bruker–Franzen Analytic, Bremen, Germany) using 2,5-DHB as template.

2.11. Protein determination

Protein concentrations were determined by using the Micro-BCA protein assay (Pierce Chemical Co., Rockford, Illinois, USA) using bovine serum albumin as a standard.

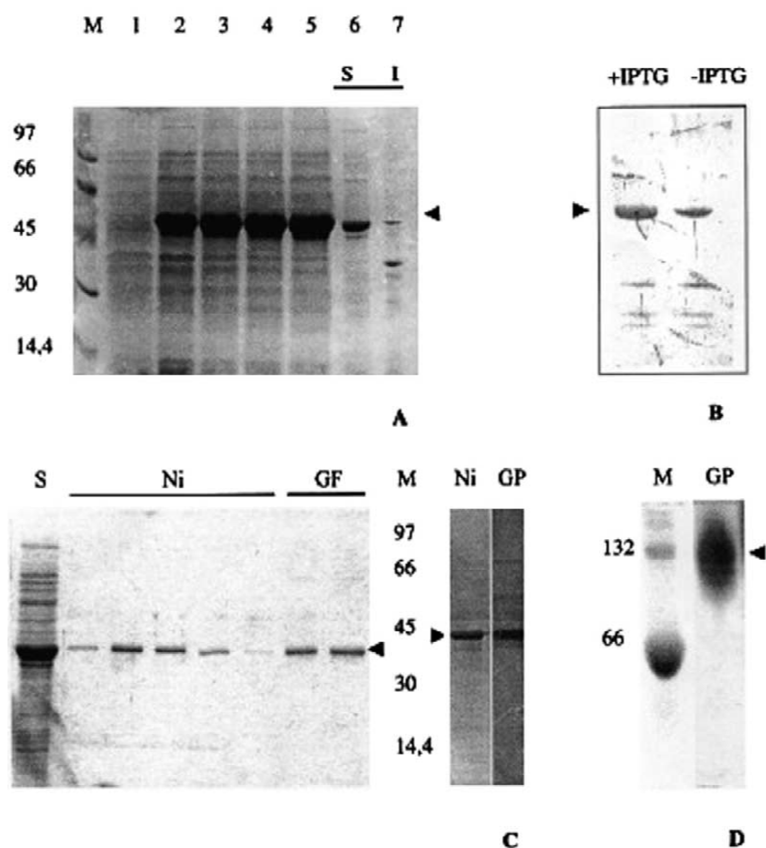

Fig. 1. Overproduction and purification of AcrA–6His. (A) Lanes 1–5: at $t=0$ and at 1 h intervals after IPTG induction, 500 μl samples were withdrawn from the culture, cells were harvested by centrifugation and whole cell lysates were analyzed by SDS–PAGE. Lanes 6 and 7: BL21(DE3)(pMM761) cells were sonicated. Following a low speed (8000 *g*) centrifugation, the soluble (S) and insoluble (I) fractions were analysed by SDS–PAGE. (B) Western blot analysis using Ni–NTA conjugates. The soluble protein fractions were prepared from induced (+IPTG) and non-induced (−IPTG) cells grown for 4 h at 37 °C. (C) Samples of different steps of the purification procedure were analyzed by SDS–PAGE, Coomassie Blue (left panel) and silver staining (right panel). Lane S: soluble fraction after cell lysis. Lanes Ni: proteins eluted with 250 m*M* imidazole from the nickel-affinity column. Lanes GP: gel permeation chromatography. Samples were mixed with 1.5% SDS sample buffer and heated at 96 °C for 5 min prior to electrophoresis. Molecular mass of standards (*M*) is indicated. The position of AcrA–6His corresponds to 45 000 (arrowhead). (D) Native-PAGE analysis of the purified AcrA–6His. An aliquot was mixed with 0.1% Triton X-100 sample buffer without β-mercapto-ethanol nor dithiothreitol, and was loaded on a 5% polyacrylamide gel containing 0.1% Triton X-100.

3. Results and discussion

3.1. Overproduction of E. aerogenes AcrA, AcrB and TolC in E. coli

Previous studies had shown that a C-terminal 6His tag has no effect on the drug efflux properties of *E. coli* AcrA and AcrB [20,13]. Thus, we PCR-amplified and cloned the *E. aerogenes acrA*, *acrB* and *tolC* open reading frames into pET242a$^+$ in frame with such a tag, to allow purification by INAC.

When *E. coli* BL21(DE3) carrying pMM761 (*acrA*) was induced with IPTG, AcrA was overexpressed in a soluble form in the cytoplasm (Fig. 1A, lanes 1–6). Very few inclusion bodies were produced (Fig. 1A, lane 7). Detection with Ni–NTA conjugates confirmed that the overexpressed protein is AcrA–6His (Fig. 1B). A densitometric analysis of

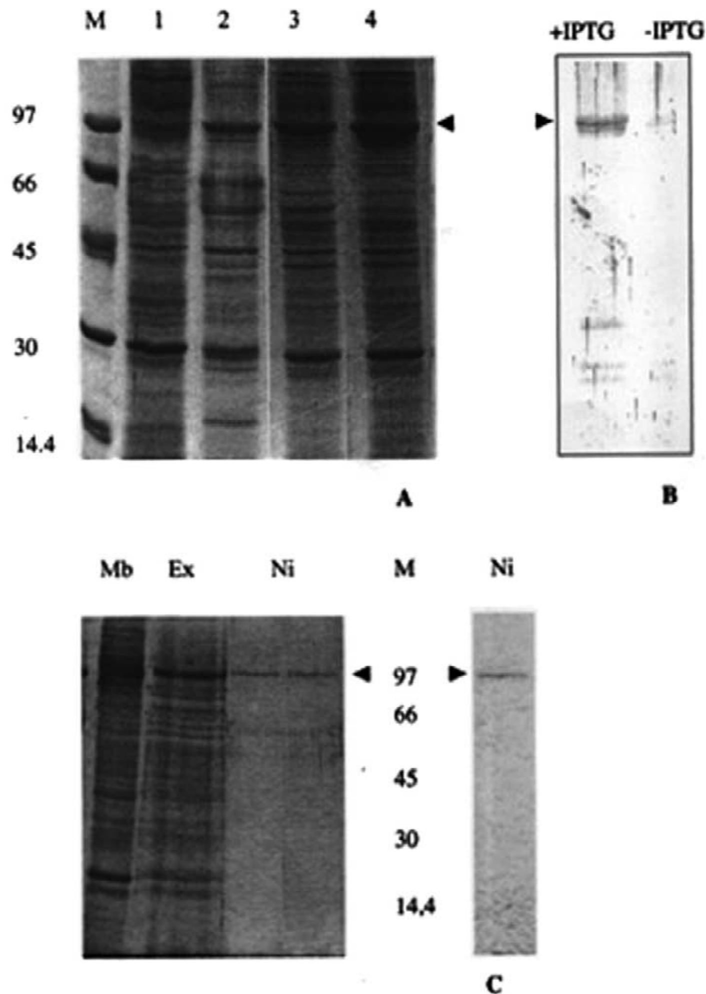

Fig. 2. Overproduction and purification of AcrB–6His. (A) SDS–PAGE analysis of insoluble fractions enriched in membrane proteins prepared from BL21(DE3)(pMM797) (lane 1); BL21(DE3)pLysS(pMM797) (lane 2); C41(DE3)(pMM797) (lane 3) and C43(DE3)(pMM797) (lane 4). (B) Western blot analysis using Ni–NTA conjugates. The insoluble fractions were prepared from induced (+IPTG) and non-induced (−IPTG) C43(DE3)(pMM797) cells grown for 4 h at 37 °C. (C) Representative samples from different steps of the purification procedure were analyzed by SDS–PAGE. Gels were stained with Coomassie Blue (left panel) or silver (right panel). Lane Mb: total membrane proteins of C43(DE3)(pMM797). Lane Ex: Triton X-100 solubilized membrane proteins. Lanes Ni: INAC purified AcrB–6His. Samples were solubilized in 3% SDS sample buffer and incubated for 20 min at 37 °C. The position of AcrB–6His corresponds to 97 000 (arrowhead).

this gel revealed that AcrA–6His represents up to 80% of the protein content.

Attempts to overexpress AcrB–6His in BL21(DE3) or BL21(DE3)pLysS resulted in a very weak production of the recombinant protein (Fig. 2A, lanes 1 and 2). We then evaluated the production of AcrB–6His in C41(DE3) and C43(DE3). When we plated C41(DE3)(pMM797) and C43(DE3) (pMM797) on LB agar containing 1 m*M* IPTG, we observed small and large colonies. Mirroux and Walker had shown that only cells from the small colonies continue to grow and produce the target protein in liquid cultures in the presence of inducer [21]. We examined C41(DE3)(pMM797) and C43(DE3)(pMM797) presenting a small colony phenotype for their ability to express AcrB–6His. The production of AcrB–6His in these strains was nhanced five-fold relative to BL21(DE3)pLysS-(pMM797) and was higher in C43(DE3) than in C41(DE3) (Fig. 2A, lanes 3 and 4). AcrB–6His represents 20% of the total membrane proteins in C43(DE3)(pMM797) (Table 2). Decreasing the temperature to 25 °C or extending the course of expression to 18 h did not significantly increase the yield of AcrB–6His in C43(DE3) (data not shown). We confirmed the identity of AcrB–6His by Western blot analysis using Ni–NTA conjugates (Fig. 2B).

Table 2
Purification of the AcrA–6His, AcrB–6His and TolC–6His recombinant proteins

	Protein[a] (mg/l of culture)	Protein[b] (%)	Yield (%)
AcrA–6His			
Soluble fraction	320	70	100
Ni^{2+} column	40	90	18
Gel permeation chromatography eluates	25	97	12
AcrB–6His			
Total membrane fraction	27	20	100
Solubilized membrane	12	32	61
Ni^{2+} column	1.65	95	30
TolC–6His			
Total membrane fraction	32	25	100
OM fraction	17	43	95
Solubilized OM	10	53	65
Ni^{2+} column	4.8	95	54

[a] Determined by BCA assay.
[b] Determined by densitometry on SDS–PAGE.

To produce TolC–6His, BL21(DE3) and BL21(DE3)pLysS were transformed with pMM762. The presence of pLysS, which encodes the T7 lysosyme, increases the tolerance of BL21(DE3) for toxic genes by reducing the basal activity of the T7 RNA polymerase [22]. SDS–PAGE analysis of the whole cell extracts showed that TolC–6His is produced only in the presence of pLysS (Fig. 3A, lanes 1–4). SDS–PAGE analysis of the insoluble fraction prepared from BL21(DE3)pLysS(pMM762) revealed the presence of two species of TolC–6His with close molecular masses (Fig. 3A, lane 6). They could correspond to the precursor and mature TolC–6His. Both proteins react with Ni–NTA conjugates (Fig. 3B). We estimated the amount the TolC–6His at 25% of the total membrane proteins (Table 2).

3.2. *Purification of AcrA–6His*

AcrA–6His was purified under native conditions in two steps: INAC and gel permeation chromatography. The clarified soluble fraction from BL21(DE3)(pMM761) was loaded on a HiTrap Chelating nickel-affinity column. SDS–PAGE analysis of the first fractions eluted with 250 m*M* imidazole showed that AcrA–6His purification was imperfect as several contaminants were detected on silver-stained gels (Fig. 1C, lanes Ni). These AcrA–6His enriched fractions were then loaded onto a Superose gel permeation column. The elution profile contained a major protein peak (data not shown). SDS–PAGE analysis indicated that the corresponding fractions yielded a single broad band when stained with silver (Fig. 1C, lanes GP). In native-PAGE analysis (Fig. 1D), purified AcrA–6His solubilized in 0.1% Triton X-100 presented an apparent molecular mass (M_{app}) of 130 000 consistent with the trimeric structure of the native protein.

3.3. *Solubilization and purification of AcrB–6His*

Zgurskaya et al. demonstrated that the *E. coli* AcrB–6His can be solubilized in a functional form with Triton X-100 and reconstituted into liposomes [20]. This procedure produced a membrane fraction highly enriched in AcrB–6His (Fig. 2C, lane Ex),

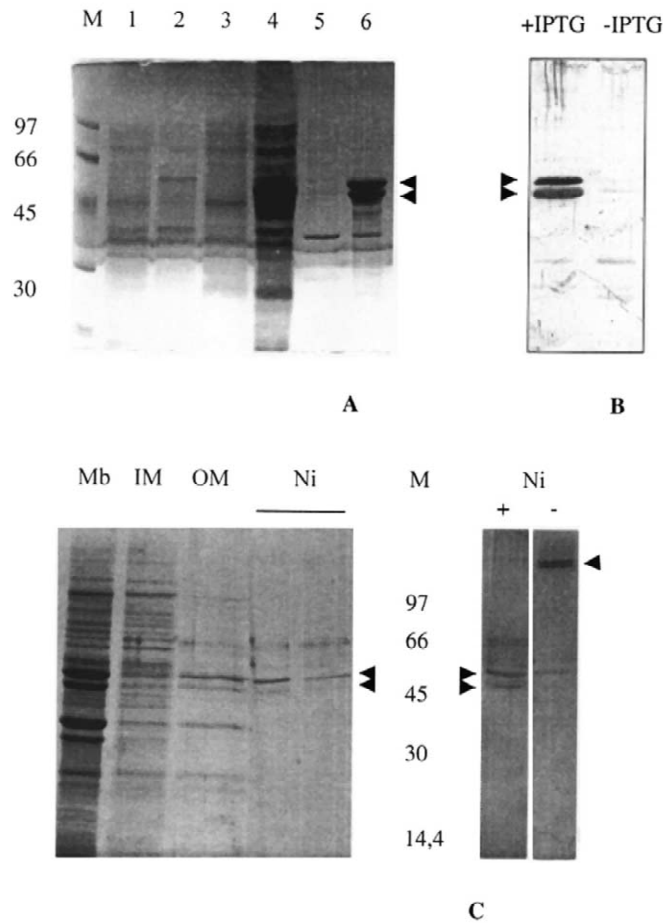

Fig. 3. Overproduction and purification of TolC–6His. (A) SDS–PAGE analysis of BL21(DE3)(pMM762) (lanes 1 and 2) and BL21(DE3)pLysS(pMM762) (lanes 3–6). Whole cell lysates at $t=0$ (lanes 1 and 3) and $t=4$ h (lanes 2 and 4) after IPTG induction. The insoluble fraction prepared from non-induced (lane 5) and induced (lane 6) BL21(DE3)pLysS(pMM762) cells were analyzed. (B) Western blot analysis using Ni–NTA conjugates. The insoluble fractions were prepared from induced (+IPTG) and non-induced cells (−IPTG) grown for 4 h at 37 °C. (C) Representative samples from different steps of the purification procedure were analyzed by SDS–PAGE. Gels were stained with Coomassie Blue (left panel) and silver (right panel). Lane Mb: total membrane proteins of BL21(DE3)pLysS(pMM762). Lane IM: IM proteins solubilized in 2% Triton X-100, 30 m*M* $MgCl_2$. Lane OM: OM proteins solubilized in 5% Triton X-100. Lanes Ni: INAC purified TolC–6His with (+) or without (−) heating at 96 °C in SDS sample buffer.

and resulted in the solubilization of 60% of AcrB–6His (Table 2). The Triton X-100 extract was then loaded on the HiTrap Chelating nickel-affinity column. The elution was done in presence of 0.2% (v/v) Triton X-100 by applying a 10–250 m*M* imidazole gradient. AcrB–6His was eluted with 100 m*M* imidazole and was more than 90% pure in a single-step chromatography (Fig. 2C, lanes Ni). The eluted protein represented 30% of the protein, or approximately 1.5 mg per liter of culture. However, we observed that AcrB–6His was stable only a few days in this state due to its tendency to aggregate

(data not shown). AcrB–6His of *E.coli* is also unstable and must be used for reconstitution right after elution [20].

3.4. Solubilization and purification of TolC–6His

Triton X-100 concentration ranging from 0.5% to 2% (v/v), solubilizes both IM and OM. In presence of $MgCl_2$, this detergent solubilizes only the IM [23]. The two-step Triton X-100 extraction (see Materials and methods) yielded TolC–6His samples with very few contaminants and solubilized 65% of TolC–6His (Table 2). However, SDS–PAGE analysis indicated the presence of two bands at 50 000 (Fig. 3C, lane OM). Western blot analysis showed that both bands react with antibodies raised against *E. coli* TolC (data not shown). N-terminal microsequencing confirmed that both bands correspond to *E. aerogenes* TolC and MALDI–TOF mass spectrometry analysis showed the presence of a single protein of 51 600. The observed difference in purified TolC–6His migration in SDS–PAGE may be attributed to a charge difference between two isoforms of the protein. The Triton X-100 extracts containing TolC–6His were pooled and loaded on the HiTrap Chelating nickel-affinity column. SDS–PAGE analysis of the fractions eluted with 250 m*M* imidazole revealed the presence of the two isoforms of TolC–6His (Fig. 3C, lanes Ni). One passage over the Ni^{2+} column purified approximately 50% of TolC–6His. This process yielded 5 mg from 1 l culture (Table 2). TolC–6His from the INAC was mixed with sample buffer containing 3% SDS and was analyzed by SDS–PAGE. When the samples were heated for 5 min at 96 °C TolC–6His migrated at an M_{app} of 51 600 (Fig. 3C, lane Ni +). In contrast, in the absence of heating TolC–6His migrated at an M_{app} greater than 100 000 (Fig. 3C, lane Ni −), indicating that TolC–6His was purified as a trimer.

In this work, we have overproduced and purified each component of the *E. aerogenes* AcrA–AcrB–TolC multidrug efflux pump. In collaboration with biophysicists, we are going to analyze the structure of AcrA by circular dichroism, 2D and 3D crystallography. The purification of the *E. aerogenes* AcrA, AcrB and TolC efflux proteins will allow us to examine the structural and the functional parameters of the system via in vitro reconstitution. First, we plan to study the channel properties of TolC in patch-clamp experiments. Second, the AcrB pump will be reconstituted in proteoliposomes to investigate the drug efflux mechanisms and the putative role of AcrA in the membrane fusion. Third, we will attempt to reconstitute AcrA–AcrB–TolC complexes in vitro to study the mechanistic details of TolC tunnel opening and the interactions between the three components during efflux.

References

[1] C. Bosi, A. Davin-Regli, C. Bornet, M. Mallea, J.M. Pagès, C. Bollet, J. Clin. Microbiol. 37 (1999) 2165.
[2] R.E.W. Hancock, Trends Microbiol. 5 (1997) 37.
[3] H. Nikaido, M. Vaara, Microbiol. Rev. 49 (1985) 1.
[4] H. Nikaido, Science 264 (1996) 382.
[5] H. Nikaido, Mol. Microbiol. 37 (2000) 219.
[6] K. Poole, Antimicrob. Agents Chemother. 44 (2000) 2233.
[7] I.T. Paulsen, M.H. Brown, R.H. Skurray, Microbiol. Rev. 60 (1996) 575.
[8] D. Ma, D.N. Cook, M. Alberti, N.G. Pon, H. Nikaido, J.E. Hearst, J. Bacteriol. 175 (1993) 6299.
[9] J.A. Fralick, J. Bacteriol. 178 (1996) 5803.
[10] E. Fujihira, N. Tamura, A. Tamaguchi, J. Biochem. 131 (2002) 145.
[11] H.I. Zgurskaya, H. Nikaido, J. Bacteriol. 182 (2000) 4264.
[12] T. Kawabe, E. Fujihira, A. Yamaguchi, J. Biochem. 128 (2000) 195.
[13] H.I. Zgurskaya, H. Nikaido, J. Mol. Biol. 285 (1999) 409.
[14] V. Koronakis, A. Sharff, E. Koronakis, B. Luis, C. Hugues, Nature 405 (2000) 914.
[15] D. Ma, D.N. Cook, M. Alberti, N.G. Pon, H. Nikaido, J.E. Hearst, Mol. Microbiol. 16 (1995) 45.
[16] H. Okusu, D. Ma, H. Nikaido, J. Bacteriol. 178 (1996) 306.
[17] M. Mallea, J. Chevalier, C. Bornet, A. Eyraud, A. Davin-Regli, C. Bollet, J.M. Pagès, Microbiology 144 (1998) 3003.
[18] E. Pradel, J.M. Pages, Antimicrob. Agents Chemother. 46 (2002) 2640.
[19] J. Sambrook, E.F. Fritsch, T. Maniatis, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
[20] H.I. Zgurskaya, H. Nikaido, Proc. Natl. Acad. Sci. USA 96 (1999) 7190.
[21] B. Miroux, J.E. Walker, J. Mol. Biol. 260 (1996) 289.
[22] F.W. Studier, A.H. Rosenberg, J.J. Dunn, J.W. Dubendorff, Methods Enzymol. 185 (1990) 60.
[23] C. Filip, G. Fletcher, J.L. Wulff, C.F. Earhart, J. Bacteriol. 115 (1973) 717.