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Biochimica et Biophysica Acta 1565 (2002) 1–5

BIOCHIMICA ET BIOPHYSICA ACTA
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Rapid report

Highly efficient over-production in *E. coli* of YvcC, a multidrug-like ATP-binding cassette transporter from *Bacillus subtilis*

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Received 14 June 2002; accepted 26 July 2002

Abstract

ATP-binding cassette (ABC) transporters have often been refractory to over-expression. Using the C41(DE3) *E. coli* as a host strain, membrane vesicles highly enriched (>50%) in YvcC, a previously uncharacterized ABC transporter from *Bacillus subtilis* homologous to P-glycoprotein multidrug transporters, were obtained. The functionality of YvcC was assessed by its high vanadate-sensitive ATPase activity and its ability to transport a fluorescent drug, the Hoechst 33342.
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Keywords: YvcC; ATP-binding cassette; *Bacillus subtilis*

1. Introduction

ATP-binding cassette (ABC) transporters constitute a huge family of proteins which are involved in the import or export of a wide diversity of substrates in all living organisms [1]. In bacteria, their utmost implication in many cellular processes stems from the fact that they often constitute the largest protein family [2–4]. Thus, these transporters are involved in the uptake of essential nutrients, the secretion of either virulence factors, proteases or toxins, the export of drugs like noxious compounds, and in antibiotic resistances developed by pathogenic bacteria [5,6]. In human, many diseases are associated with either a dysfunction of an ABC transporter, for instance the CFTR channel in cystic fibrosis, or the over-expression of others such as P-glycoprotein or "Multidrug Resistance associated Protein" (MRP), thereby allowing cancer cells to escape therapeutic treatments by conferring a multidrug resistance (MDR) phenotype [7]. ABC transporters are constituted of at least four building blocks: two membrane-spanning domains involved in substrate translocation, and two nucleotide-binding domains energizing the transporter. The latter domains are the most conserved in sequence, and notably contain the signature of this family [8,9]. These four domains are found either as separate subunits (for instance, in the maltose transporter), or fused together to form three, two (designated as half-transporters) or even one single polypeptide (most eukaryotic transporters fall in the latter category). Importers, which are found only in bacteria, have an additional subunit involved in the extracellular capture of the substrate [1].

Despite many efforts, functional and structural characterization of membrane proteins have often been hampered by the lack of efficient over-producing systems [10]. Hence, although *E. coli* is usually an organism of choice for the over-expression of proteins, difficulties have been encountered when trying to over-produce bacterial membrane proteins such as ABC transporters. For instance, over-expression of the bacterial full-length HlyB, involved in haemolysin export, could not be obtained in *E. coli* [11]. Also, over-expression of LmrA, the first bacterial multidrug transporter akin to human P-glycoprotein, was only achieved by using a homologous *Lactococcus lactis* over-expression system with a specific nisin-inducible promoter [12]. Here, we show that the use of the C41(DE3) *E. coli*

Abbreviations: ABC, ATP-binding cassette; EDTA, Ethylenediaminetetraacetic acid; IPTG, Isopropyl β-D-thiogalactopyranoside; MDR, Multidrug resistance; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate

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PII: S0005-2736(02)00515-1

mutant strain [13] is particularly well-suited and efficient for the over-production of YvcC, a multidrug-like ABC-transporter from *Bacillus subtilis*. Membrane vesicles highly enriched in YvcC are able to hydrolyze ATP in a vanadate-sensitive manner and to actively transport a fluorescent drug. Therefore, these results show that YvcC is correctly folded in the membrane as a fully functional transporter. The strategy described here might also be applicable to a broader spectrum of membrane proteins, especially when scientists face the challenging goal of purifying membrane proteins in high yield for structural purposes.

2. Materials and methods

The *E. coli* C41(DE3) host strain, a mutant strain of BL21(DE3), has been described previously [13]. The *yvcC* gene was amplified by PCR using a DNA template prepared from *B. subtilis*, and verification of the correct sequence was made by DNA sequencing. The gene was then cloned into a pET23b(+) vector (Novagen), allowing the addition of six consecutive histidine residues on the carboxyl terminus of the protein. For the over-expression, a freshly transformed colony was inoculated into 2 × YT broth medium (Bio 101 Inc.) and 0.7 mM isopropyl β-D-thiogalactopyranoside (IPTG) was added when the $OD_{600\ nm}$ reached 0.6. The culture was further grown for 4 h at 25 °C and bacteria were collected by a low-speed centrifugation (7500 × *g*, 10 min) at 4 °C; all subsequent steps were carried out at the same temperature. Pellets were re-suspended in buffer A (50 mM Tris–HCl pH 8.0, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 μM leupeptine and 5 μM pepstatine) containing 30 U/ml of benzonase, and bacteria were lysed by two successive passages through a French pressure cell (18 000 psi). Ethylenediaminetetraacetic acid (EDTA) was then added at 10 mM, and unbroken cells and debris were removed by centrifugation for 30 min at 15 000 × *g*. Membrane vesicles were collected by centrifugation at 100 000 × *g* for 60 min, re-suspended in buffer A (except that $MgCl_2$ was replaced by 1.5 mM EDTA) and centrifuged again at the same speed. The membrane vesicles were finally suspended in 20 mM Tris–HCl pH 8.0, 300 mM sucrose,1 mM EDTA and stored as small aliquots in liquid nitrogen.

ATPase activity was measured by Pi release. Briefly, control or YvcC-enriched membranes (2 μg of proteins) were incubated at 24 °C for 30 min in 50 mM Hepes/KOH pH 8.0, 7 mM ATP, 7 mM $MgCl_2$, 2 mM NaN_3, and in the presence of increasing concentrations of vanadate as indicated in the figure legend (final volume of 50 μl). The reactions were stopped by addition of 1 ml of 20 mM H_2SO_4 and samples were immediately transferred on ice. The Pi release was then measured by a colorimetric method as previously described [14].

For Hoechst 33342 (2′-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5′-bis-1*H*-benzimidazole; Sigma) transport assay, variations in fluorescence intensity were monitored with a Photon Technology International Quanta Master I fluorimeter, using excitation and emission wavelengths of 355 and 457 nm, and slit widths of 2 and 4 nm, respectively. Membrane vesicles (500 μg of protein) were added into a 3 ml cuvette containing 2 ml of 50 mM Hepes/KOH pH 8.0, 2 mM $MgCl_2$, 8.5 mM NaCl, 40 μg pyruvate kinase (E.C.2.7.1.40) and 4 mM phosphoenolpyruvate. After 1 min incubation at 25 °C, 2 μM Hoechst 33342 was added (from a 1 mM stock solution in water), and its fluorescence was recorded for 1–2 min. ATP was then added to a final concentration of 2 mM and the fluorescence intensity was monitored for several minutes.

3. Results and discussion

In order to identify an MDR-like ABC transporter in *B. subtilis*, a computer search was initiated on its whole genome using the Subtilist web server http://genolist.pasteur.fr/SubtiList/. Among all the open reading frames of *B. subtilis*, a putative ABC transporter of unknown function, called YvcC [2], showed the highest sequence homology to LmrA (41.5% of identity) and also a high homology to each half of human P-glycoprotein (28.8% and 27.7% of identity with the N- and C-terminus halves, respectively). The *yvcC* gene was cloned and the corresponding protein was successfully over-expressed in the C41(DE3) *E. coli* strain (Fig. 1, left panel). This strain, which was derived from parental BL21(DE3) strain due to an uncharacterized mutation, has been shown to increase the yield of many over-expressed proteins, notably membrane proteins [13]. A high expression level was also detected in the parental BL21(DE3) strain but, and in contrast to C41(DE3), induction by IPTG stopped the bacterial growth (Fig. 1, right panel). This different behavior between the parental and the mutant strains was previously observed when over-expression of some toxic proteins was attempted [13]. Here, this led to an overall lower yield in YvcC-containing BL21(DE3) cells as compared to YvcC-containing C41(DE3) cells, a drawback for a starting material when the goal is to purify a protein in high yield. Moreover, and especially when using the BL21(DE3) as a host strain, the *yvcC* gene-carrying plasmid was rather unstable when IPTG was added to an exponentially growing culture derived from an overnight grown preculture (not shown). Over-expression of the MDR ABC transporter LmrA, isolated from *L. lactis*, was likewise obtained by using C41(DE3) (not shown here), showing that this special strain might be useful as a host for the over-expression of ABC transporters from various origins. In all cases, the presence of the protein of interest was confirmed by Western blotting using a monoclonal antibody directed against the hexahistidine tag. Recently, over-expression of the b-subunit of *E. coli* F_0F_1 ATP synthase in the C41(DE3) strain was reported to occur through a high proliferation of intracellular membranes [15]. While these intracellular

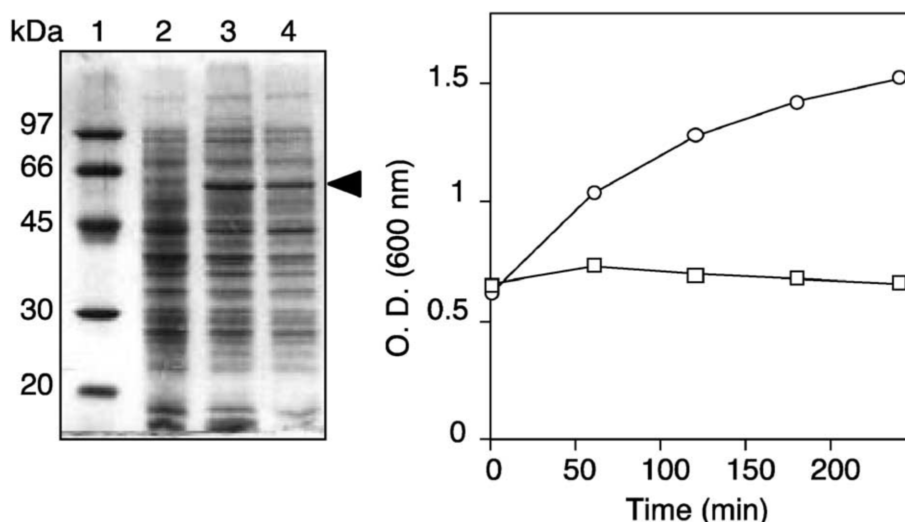

Fig. 1. Over-expression of YvcC in the mutant C41(DE3) or parental BL21(DE3) *E. coli* host strains. Left panel, Coomassie brilliant blue-stained SDS-PAGE (12%) of C41(DE3) (lanes 2 and 3) or BL21(DE3) (lane 4) *E. coli* strains transformed with pET23b(+)/*yvcC*. Lane 2 was a sample withdrawn before IPTG induction, and lanes 3 and 4 were samples obtained after IPTG induction. Lane 1 corresponded to the molecular weight markers. The position of YvcC is indicated by an arrowhead. Right panel, growth of C41(DE3) (○) or BL21(DE3) (□) *E. coli* strains transformed with pET23b(+)/*yvcC*, after induction by IPTG (added at time 0).

membranes were pelleted by a low-speed centrifugation [15], the YvcC-enriched membrane vesicles were collected by a high-speed centrifugation suggesting that a different scenario occurred here.

As shown in Fig. 2 (left panel), YvcC was by far the most abundant protein in the membrane vesicles. A quantification of YvcC in this fraction, by densitometry analysis of the Coomassie brilliant blue-stained gel, allowed us to estimate that YvcC amounted for about 50% of the whole membrane proteins. To our knowledge, such a high yield of recovery in the membrane fraction has never been achieved previously for any MDR-like ABC transporter, neither of eukaryotic origin [16–18], nor in the case of LmrA. The latter amounted to about 30–35% of total membrane proteins by using a nisin-inducible promoter specific for a homologous over-expression in *L. lactis* [12].

The functionality of over-produced YvcC was assessed first by measuring its ATPase activity. YvcC-enriched

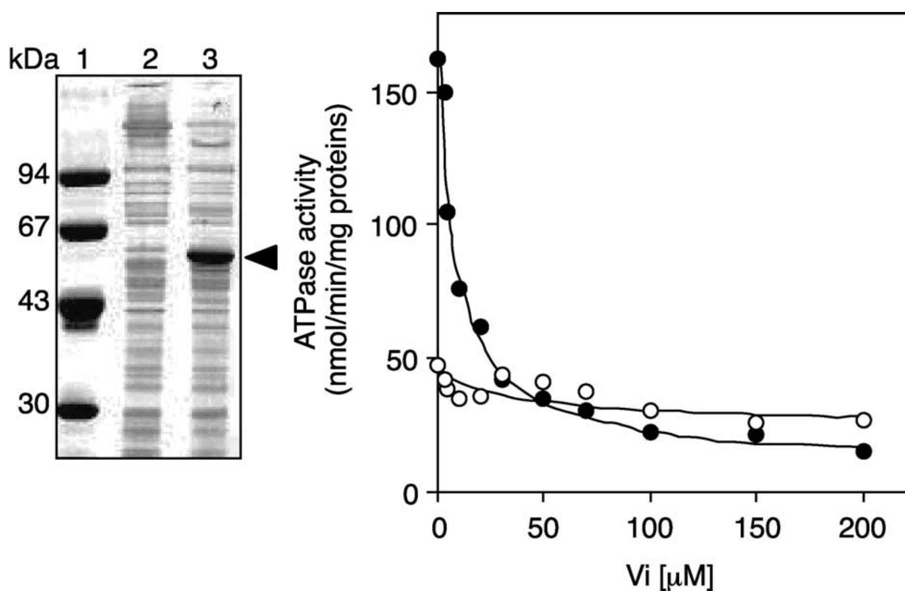

Fig. 2. Accumulation of YvcC in the membrane vesicles and its vanadate-sensitive ATPase activity. Left panel, Coomassie brilliant blue-stained SDS-PAGE (12%) of membrane vesicles prepared from the C41(DE3) strain transformed with either the pET23b(+) (lane 2) or the pET23b(+)/*yvcC* (lane 3). Lane 1 corresponds to the molecular weight markers. The position of over-expressed YvcC is indicated by the arrowhead. Right panel, inhibition by vanadate of ATPase activities of control or YvcC-containing vesicles. ATPase activities of control (○) or YvcC-containing vesicles (●) were measured by Pi release as described in Materials and methods.

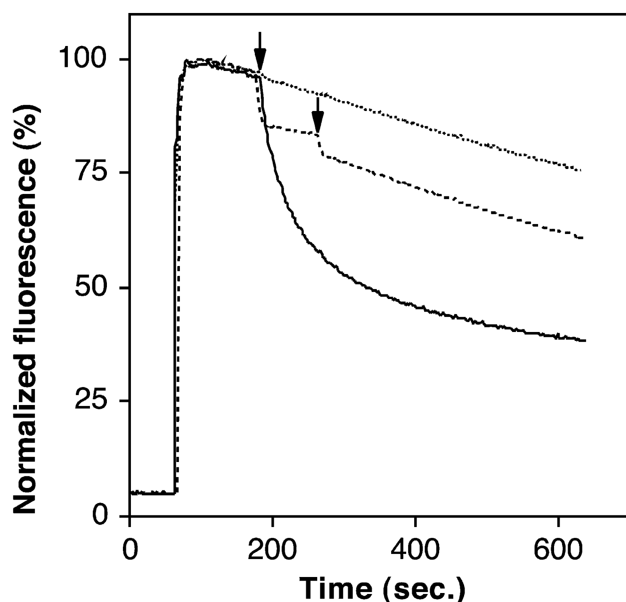

Fig. 3. Transport activity of Hoechst 33342. Addition of 2 μM Hoechst 33342 to either "control" inside–out membrane vesicles (dotted line) or YvcC-enriched inside–out membrane vesicles (plain or dashed lines) led to an increase in fluorescence intensity due to its accumulation into the membrane [26]. Subsequent addition of 2 mM ATP (first arrow, plain or dotted line) induced a rapid drop in fluorescence intensity indicative of active transport towards the inside of YvcC-containing vesicles (plain line), whereas it did not modify the steady-state diffusion towards the inside of control vesicles (dotted line). A prior addition of 50 μM vinblastine to YvcC-enriched vesicles (dashed line, first arrow) fully prevented the ATP-dependent Hoechst transport (second arrow).

vesicles exhibited a high ATPase activity of about 160 nmol/min/mg of total proteins corresponding to an activity of about 270 nmol/min/mg of YvcC. This activity and in contrast to the much lower activity of control vesicle was highly sensitive to vanadate inhibition (Fig. 2, right panel). A concentration of about 10 μM vanadate produced an inhibition of about 50% of YvcC ATPase activity, which is similar to the value reported for the inhibition of P-glycoprotein ATPase activity [19]. Furthermore, the ability of YvcC to transport a fluorescent drug, the Hoechst 33342 previously shown to be transported by P-glycoprotein [20] or LmrA [12], was investigated. Fig. 3 shows that, whereas inside–out control vesicles were unable to transport the Hoechst 33342, YvcC-enriched vesicles were quite efficient to actively transport this drug upon ATP addition. This transport was fully prevented by prior addition of vinblastine, another substrate of P-glycoprotein [21] and LmrA [22].

4. Conclusions

A high over-expression of an ABC transporter from *B. subtilis* previously uncharacterized, YvcC, was achieved here by using the C41(DE3) *E. coli* as a host strain, and the membrane vesicles derived thereof were astonishingly enriched in this transporter. This property allowed us to show that YvcC bears, like its bacterial or eukaryotic counterparts (LmrA or P-glycoprotein, respectively), the ability to extrude the fluorescent dye Hoechst 33342. Thus, YvcC is embedded in the membrane in a fully functional conformation and, consequently, this membrane preparation constitutes a suitable material for purification of YvcC in high yield (Steinfels et al., manuscript in preparation). This feature is a prerequisite for any biochemical studies, especially for structural purposes, and we have recently taken advantage of this opportunity to determine the YvcC 3-D structure at a resolution of about 25 Å [23].

Given the preponderance of the ABC transporter family, especially in the bacterial world [24], the C41(DE3) strain should be useful for the study of many ABC transporters yet uncharacterized. Additionally, it might be suitable for the over-expression of other bacterial MDR pumps, including for instance those belonging to the Major Facilitator Superfamily [25]. Even beyond the MDR scope, over-expression of any bacterial membrane protein is a challenging step and, when successful, represents a major breakthrough towards its characterization.

Acknowledgements

We are grateful to Dr. John E. Walker to have kindly provided us with the C41(DE3) *E. coli* mutant strain. This work was supported by the Centre National de la Recherche Scientifique (PCV-2000). E.S. was the recipient of a fellowship from the Ligue Nationale contre le Cancer (comité de Haute-Savoie) and C.O. and O.D. from the Ministère de la Recherche et de l'Enseignement Supérieur.

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