Characterization of YvcC (BmrA), a Multidrug ABC Transporter Constitutively Expressed in *Bacillus subtilis*†

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ABSTRACT: The involvement of transporters in multidrug resistance of bacteria is an increasingly challenging problem, and most of the pumps identified so far use the protonmotive gradient as the energy source. A new member of the ATP-binding cassette (ABC) family, known in *Bacillus subtilis* as YvcC and homologous to each half of mammalian P-glycoprotein and to LmrA of *Lactococcus lactis*, has been studied here. The *yvcC* gene was constitutively expressed in *B. subtilis* throughout its growth, and a knockout mutant showed a lower rate of ethidium efflux than the wild-type strain. Overexpression of *yvcC* in *Escherichia coli* allowed the preparation of highly enriched inverted-membrane vesicles that exhibited high transport activities of three fluorescent drugs, namely, Hoechst 33342, doxorubicin, and 7-aminoactinomycin D. After solubilization with *n*-dodecyl β-D-maltoside, the hexahistidine-tagged YvcC was purified by a one-step affinity chromatography, and its ability to bind many P-glycoprotein effectors was evidenced by fluorescence spectroscopy experiments. Collectively, these results showed that YvcC is a multidrug ABC transporter functionally active in wild-type *B. subtilis*, and YvcC was therefore renamed BmrA for *Bacillus m*ultidrug *r*esistance *A*TP. Besides, reconstitution of YvcC into liposomes led to the highest, vanadate-sensitive, ATPase activity reported so far for an ABC transporter. Interestingly, such a high ATP hydrolysis proceeds with a positive cooperativity mechanism, a property only found so far with ABC importers.

Since the discovery of antibiotics, many pathogenic bacteria have been shown to develop resistance mechanisms, thereby abolishing the effectiveness of therapy (*1*), whereas other species were identified as being intrinsically resistant to some antibiotics (*2*). Over the years, molecular mechanisms underlying many kinds of resistance have been elucidated, including the well-characterized enzymatic inactivation of antibiotics such as β-lactams (*3*). The first evidence that resistance to antibiotics was caused by an active efflux was put forward in 1980 by Levy and colleagues (*4*). Since then, efflux mechanisms have been recognized as a major player in bacterial drug resistance (*5*, *6*), and this is of greatest concern in numerous pathogenic strains that had developed *m*ulti*d*rug *r*esistance (MDR)[1] phenotypes (*5*). Indeed, whereas some efflux pumps are rather selective for a given substrate, many transporters show a polyspecificity being able to extrude a plethora of structurally unrelated drugs (*7*). Given their ability to pump out a broad spectrum of noxious compounds, these MDR transporters are exquisite bacterial weapons to escape conventional antibiotic therapies and thus constitute prominent targets for the search of new inhibitors able to restore the efficacy of conventional treatments (*8*).

The overwhelming majority of MDR efflux pumps identified so far are energized by a protonmotive force (or a Na^+-driven force) and, depending on their size, similarities in primary structure, and topology, fall into one of the four distinct transporter families (*9*): the major facilitator family

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[1] Abbreviations: ABC, ATP-binding cassette; AMPPNP, 5′-adenylyl imidodiphosphate; DDM, *n*-dodecyl β-D-maltoside; MDR, multidrug resistance; NBD, nucleotide-binding domain; P-gp, P-glycoprotein; TNP-ATP, 2′,3′-*O*-(2,4,6-trinitrophenyladenosine 5′-triphosphate).

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(*10*), the small multidrug resistance family (*11*), the resistance-nodulation-cell division family (*12*), and the multidrug and toxic compound extrusion family (*13*). In 1996, Konings and colleagues made the stunning discovery that a bacterial MDR transporter, called LmrA (for *Lactococcus m*ultidrug *r*esistance *A*TP) and heterogously expressed in *Escherichia coli*, could use the free energy of ATP hydrolysis to pump drugs out of this bacterium (*14*). Recently, LmrA was shown to extrude many unrelated antibiotics from *E. coli*, raising the possibility that this new kind of pump might be involved in resistance, either intrinsic or acquired, to antibiotics in many bacterial species (*15*). This was further supported by analysis of new microbial genomes showing the broad distribution of putative ATP-driven MDR pumps in most pathogenic bacteria (*16*), such as the Gram-positive *Mycobacterium tuberculosis* (*17*). However, evidence is still lacking that such transporters are indeed involved, in vivo, in multidrug efflux of wild-type bacteria.

With the discovery of LmrA, the problem of multidrug resistance in prokaryotes merged with that found in eukaryotes since this transporter was characterized as a structural and functional homologue of human P-glycoprotein (P-gp) (*14*, *18*). The latter is responsible for the MDR phenotype of many cancer cells and is a well-known eukaryotic member of the ATP-binding cassette (ABC) family (*19*). This family contains thousands of members, mostly transporters (*20*, *21*), present from bacteria to man and involved in the efflux or influx of a wide diversity of substrates including ions, sugars, lipids, peptides, or complex organic molecules (*22*, *23*). Apart from P-gp, dysfunction of many ABC transporters plays a causative role in a variety of genetic diseases, including cystic fibrosis and adrenoleukodystrophy (*19*, *24*). The basic architecture of ABC transporters comprises two membrane-spanning domains and two nucleotide-binding domains (NBDs), and the high sequence homology detected between different NBDs is consistent with the common fold observed for several NBD structures of various origins (*25*−*27*). Structural data are also available for two whole ABC transporters (*28*, *29*), but if these first glimpses of structures allowed to hypothesize how ABC transporters may function, additional work is needed to fully understand their molecular mechanism. Thus, although a unifying mechanism of energy coupled to transmembrane transport is believed to occur in all ABC transporters (*30*, *31*), some of them, such as maltose or histidine permeases, display a positive cooperativity for ATP hydrolysis (*32*, *33*) whereas others, such as P-gp, hydrolyze ATP without any apparent cooperativity (*31*, *34*, *35*), a property that has a strong implication on the postulated mechanism of drug extrusion by P-gp (*36*, *37*). Cooperativity between the two NBDs of P-pg was nevertheless demonstrated since mutation in one ATP-binding site, either one of them, fully abolished the activity of the transporter (*38*).

We report here a thorough functional characterization of a new MDR ABC transporter identified from genome sequencing of *Bacillus subtilis* as YvcC (*39*). This transporter was chosen due to its high sequence homology with LmrA and both halves of human P-gp. Expression of the *yvcC* gene was detected throughout the growth of *B. subtilis*, and its disruption led to a slower rate of ethidium efflux in the mutant as compared to the wild-type strain. Overexpression of YvcC in *E. coli* was achieved in high yield and efficiently processed to the membrane (*40*) from which inverted membrane vesicles allowed to measure a high specific transport of three structurally different drugs: Hoechst 33342, doxorubicin, and 7-aminoactinomycin D. Furthermore, additions of low concentrations of vinblastine stimulated the transport of Hoechst 33342. Next, YvcC has been purified to homogeneity and its ability to bind many P-gp effectors demonstrated. Overall, these results showed that YvcC is a new multidrug ABC transporter, and to our knowledge, this is the first evidence that such a transporter is constitutively expressed in a wild-type strain and capable of actively pumping drugs out of *B. subtilis*. Finally, reconstitution of YvcC into liposomes led to the highest, vanadate-sensitive, rate of ATP hydrolysis reported so far for an ABC transporter, allowing us to show that the dependence of its ATPase activity on ATP concentration obeys a positive cooperativity mechanism.

EXPERIMENTAL PROCEDURES

Unless stated otherwise, chemicals were from Sigma, and restriction or DNA-modifying enzymes were from Promega.

yvcC Gene Interruption. It was performed by a double recombination event leading to deletion of the coding sequence and integration of a tetracycline-resistance cassette. The ligation-independent cloning procedure was used all along the cloning process (*41*). Four different partners, possessing at their extremities particular sequences allowing to generate 5′ overhangs which permit directional cloning, were used for the process. One was derived from the pJM105A cloning vector, which contains a choramphenicol-resistance cassette (*42*), and obtained by PCR amplification using the pjmamp1 and pjmamp2 primers (see below). The two *yvcC* fragments that allow the homologous recombination were obtained by PCR using *B. subtilis* genomic DNA as a template with either yvcc1/yvcc2 or yvcc3/yvcc4 as primers. Finally, the tetracycline cassette was PCR amplified from the pDG1515 plasmid (*43*) using 2rtet1 and 2rtet2 as primers. The cloning procedure was as follows: First, the tetracycline cassette was inserted between the two *yvcC* recombinant DNA fragments using a three-partner PCR amplification. The two *yvcC* DNA fragments and the tetracycline cassette DNA fragment (see above) were mixed together at equimolarity, and a PCR amplification was carried out using yvcc1 and yvcc4 as primers. The resulting fragments were treated by T4 DNA polymerase in the presence of dTTP to generate 5′ overhangs and mixed together with the pjmamp1/pjmamp2 pJM105 amplification product previously treated by T4 DNA polymerase in the presence of dATP. The mixture was used directly to transform *E. coli*. Recombinant plasmid was purified and used to transform competent *B. subtilis* cells. Recombinant clones were selected for their resistance to tetracycline and sensitivity to chloramphenicol and checked for the insertion of the tetracycline cassette at the right location on genomic DNA by PCR using 2rtet1/yvcc_down and yvcc_up/2rtet2 primers. All along the process, PCR-amplified fragments were purified using magnetic beads as described (*44*).

Primers used were the following: pjmamp1, 5′-GGCCGCGAAGACGCTGTCACGACGTTGTAAAACGACGGC-3′; pjmamp2, 5′-CAACCGACGCAGCGTGGGAAAACCCTGGCGTTACCCAAC-3′; 2rtet1, 5′-CCAGGAGGGCACGGATCAATGGTGCAGGTTGTTCTCAATG-3′; 2rtet2,

5′-CCGACACAGCCAAGCTAGGATCAATTTTGAACTCTCTCCC-3′; yvcc1, 5′-GCGTCTTCGCGGCCAACGGACCAATCCTTCTTATGGAAAGCT-3′; yvcc2, 5′-TCCGTGCCCTCCTGGACTTTGACCACCATCGTATCGTTCG-3′; yvcc3, 5′-GCTTGGCTGTGTCGGCGGACAAAGGCAAAGAATCGCGATTG-3′; yvcc4, 5′-CGCTGCTCGGTTGAGTTCAGCAAAATCCCGGTAAAGG-3′; yvcc_up, 5′-TTCTGAACGCTGAAACAGGAGG-3′; yvcc_down, 5′-GAGAAGATTGCATAAGGCGTGAAG-3′.

Real-Time PCR Assays. cDNA was synthesized using SuperScript II from GibcoBRL (Life technology, Cergy, France). For each reverse transcription reaction, 1 μg of RNA was used together with 100 ng of random hexamer (GibcoBRL) as primers. cDNA was then used as a template in real-time PCR assays using a LightCycler system (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. cDNA were amplified in the presence of SYBR-Green (Fast Start DNA Master SYBR Green I) in a 20 μL final volume with optimized primers and $MgCl_2$ concentrations. The amplification procedure comprises a 8 min denaturation step at 95 °C for Taq DNA polymerase activation and 45 cycles including the three following steps: denaturation at 95 °C for 15 s, annealing at 60 °C for 6 s, and extension at 72 °C for 10 s. The set of primers used to amplify cDNA is listed below. They were chosen according to gene sequences described on the NCBI site: yvcC_SG1, 5′-GCCGGTCGATACTTACTCGC-3′; yvcC_SG2, 5′-ACGGGCTTTCCTGTGATACATAC-3′; hbs_SG1, 5′-CGGTGATAAAATCCAACTGATCG-3′; hbs_SG2, 5′-TGCGTCCTTTACGTGCAGAA-3′. Results were expressed as mRNA amount (as arbitrary units) of a gene expressed in bacteria grown in the presence or in the absence of 10 μM ethidium bromide added at time 60 min of the growth curve (see Figure 2).

Cloning, Overexpression, and Purification of Histidine-Tagged YvcC. Cloning, overexpression of *yvcC* in the C41-(DE3) *E. coli* mutant strain, and preparation of inverted-membrane vesicles were performed as described previously (*40*). YvcC was purified from plasma membranes by solubilization with 1% *n*-dodecyl β-D-maltoside (DDM). Briefly, membranes were rapidly defrosted at 37 °C and diluted into solubilization buffer containing 100 mM potassium phosphate (pH 8), 15% glycerol, 100 mM NaCl, 10 mM imidazole, 1% DDM, 5 mM β-mercaptoethanol, and 1 mM PMSF to a final protein concentration of 2 mg/mL. The suspension was stirred for 1 h incubation at 4 °C, and the insoluble material was removed by centrifugation (220000*g*, 1 h, 4 °C). Solubilized membrane proteins were incubated for 30−45 min at 4 °C with Ni-NTA−agarose (2 mL of resin/L of bacterial culture) which was preequilibrated in a buffer containing 100 mM potassium phosphate, pH 8.0, 100 mM NaCl, 15% glycerol, and 5 mM β-mercaptoethanol. After incubation, the resin was transferred to a column (1.5 cm × 4.5 cm) and washed overnight at 4 °C with 100 mL of washing buffer (50 mM potassium phosphate, pH 8.0, 100 mM NaCl, 15% glycerol, 20 mM imidazole, 0.05% DDM, and 5 mM β-mercaptoethanol). The protein was recovered with elution buffer (50 mM potassium phosphate, pH 8.0, 100 mM NaCl, 15% glycerol, 200 mM imidazole, 0.05% DDM, and 5 mM β-mercaptoethanol). The fractions were assayed for protein content by the Coomassie Plus Protein Assay Reagent (Pierce) and dialyzed twice against a buffer containing 50 mM HEPES/KOH (pH 8.0), 50 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol, and 0.05% DDM. Then, aliquots of purified YvcC were frozen and stored in liquid nitrogen. Gel electrophoretograms of the different fractions were analyzed as previously described (*40*).

Fluorescence Experiments. Binding experiments were performed at 25.0 ± 0.1 °C using an SLM-Aminco 8000C spectrofluorometer with spectral bandwidths of 2 and 4 nm for excitation and emission, respectively. The YvcC solution was diluted (0.33 μM final concentration, corresponding to a concentration of 1 μM tryptophan residue) in 1.2 mL of 50 mM HEPES/KOH (pH 8.0), 100 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol, and 0.01% DDM. Emission spectra, upon excitation at 295 nm, were recorded from 310 to 360 nm after each addition of drug from stock solutions. The variations in the fluorescence of purified YvcC were first corrected from the buffer contribution and then from the ligand inner filter effect as measured in parallel experiments using *N*-acetyltryptophanamide (*45*, *46*).

Extrinsic fluorescence of 2′,3′-*O*-(2,4,6-trinitrophenyl-adenosine 5′-triphosphate) (TNP-ATP) was measured upon excitation at 408 nm, and emission spectra were recorded from 530 to 580 nm. They were corrected for buffer fluorescence, dilution (<5%), and free nucleotide fluorescence by control experiments performed in the absence of protein. Curve fitting of the concentration-dependent binding of effectors was performed by using GraFit (Erithacus Software) as described previously (*45*, *46*).

For the transport of fluorescent drugs, experiments were carried out by using a Photon Technology International Quanta Master I fluorometer as described previously (*40*). The excitation and emission wavelengths were respectively 500 and 580 nm for ethidium bromide, 355 and 457 nm for Hoechst 33342, 546 and 647 nm for 7-aminoactinomycin D, and 480 and 590 nm for doxorubicin, with spectral bandwidths of 2 and 4 nm for excitation and emission, respectively.

For the transport of ethidium in whole bacteria, *B. subtilis* wild-type or *yvcC* knockout strains grown overnight in LB medium (supplemented with 10 μg/mL tetracycline for the Δ*yvcC* mutant) were diluted into a fresh LB medium (supplemented with 5 μg/mL tetracycline for the Δ*yvcC* mutant) at an OD_{600nm} of 0.05. When the culture reached an OD_{600nm} of 0.5 at 37 °C, bacteria were centrifuged at 2000*g* for 4 min and resuspended in 0.35 M NaCl. To 1.8 mL of resuspended bacteria were added 50 mM KP_i (pH 7.2), 25 mM glucose, and 5 mM $MgSO_4$ into the fluorometer cuvette, and transport of ethidium bromide (10 μM) was monitored at 25 °C; 50 μM reserpine was added to the cuvette where indicated. For the ethidium efflux, when the freshly grown culture reached an OD_{600nm} of 0.5 at 37 °C, 10 μM ethidium bromide and 10 μM reserpine were added to the samples, which were further incubated for 5 min at 25 °C. Bacteria were then centrifuged at 2000*g* for 4 min and resuspended in 0.35 M NaCl. To 1.8 mL of resuspended bacteria were added 50 mM KP_i (pH 7.2), 25 mM glucose, and 5 mM $MgSO_4$ into the fluorometer cuvette, and efflux of ethidium was monitored at 25 °C.

Reconstitution of Purified YvcC into Liposomes. Proteoliposomes were reconstituted according to the method previously reported (*47*).

ATPase Activity. The ATPase activity of YvcC reconstituted into proteoliposomes was monitored at 37 °C unless stated otherwise by using an ATP-regenerating system coupled to the disappearance of NADH recorded at 340 nm, as described previously (*48*). YvcC (5 μg) were used, and the rate of ATP hydrolysis was monitored for several minutes. The initial rate of ATP hydrolysis was plotted against the ATP concentration, and curve fitting was performed by using GraFit 4.0 (Erithacus Software). To avoid any interference with the enzymes in the coupled enzymatic assay, the inhibition by vanadate of the ATPase activity was measured by a colorimetric assay of P_i release, as previously described (*40*).

RESULTS

A New Putative MDR ABC Transporter Constitutively Expressed in B. subtilis. We previously reported that a search for a new putative bacterial MDR transporter identified YvcC as the *B. subtilis* ABC transporter exhibiting the highest homology to each half of P-gps (MDR1) and LmrA (~42% identity for the latter; see ref *40*). YvcC is also homologous to HorA (~40% identity), another multidrug ABC transporter from *Lactobacillus brevis* (*49*), and to uncharacterized putative transporters from Gram-positive bacteria such as Q8Y3T6 from *Listeria monocytogenes* (~56% identity) and Q8NXS2 from *Staphylococcus aureus* (~42% identity; Figure 1). Among Gram-negative bacteria, YvcC shares a significant homology with MsbA (~30% identity), the lipid A transporter from *E. coli* whose 3-D structure was solved at 4.5 Å (*29*).

The expression of *yvcC* in *B. subtilis* was evaluated by real-time PCR in comparison to *hbs*, a histone-like gene that showed a relatively constant level of expression in *B. subtilis* (*50*). Figure 2 shows that *yvcC* was constitutively expressed throughout the growth phases of *B. subtilis*, although its level of expression seemed to decline once the culture reached the stationary phase. Addition of either ethidium bromide (Figure 2) or Hoechst 33342 (not shown) to the culture medium did not significantly modify the expression pattern of *yvcC* all along the growth curve.

YvcC Is a MDR Transporter. To assess whether YvcC is functionally active in vivo, a *B. subtilis* Δ*yvcC* mutant strain was made. The accumulation of ethidium, a typical substrate of most MDR pumps which exhibits increased fluorescence upon binding to DNA (*14*, *49*, *51*), was monitored in both wild-type and mutant strains. Despite some variation in the initial rate of ethidium accumulation depending on the experiments, the moderate accumulation seen in the wild-type strain was strongly and consistently increased upon reserpine addition (Figure 3A; slope ratio measured after addition versus before addition of reserpine = 2.15). Since this compound is a well-known inhibitor of many MDR pumps as a whole, including P-gp and LmrA (*14*), its effect indicates that reserpine-sensitive pump(s) efficiently efflux(es) ethidium in the wild-type *B. subtilis*. In contrast, addition of reserpine to the Δ*yvcC* mutant strain afforded a very little increase in the rate of ethidium accumulation (slope ratio measured after addition versus before addition of reserpine = 1.2). These results show that, in wild-type *B. subtilis*, YvcC is involved in the reserpine-sensitive efflux of ethidium. It should be emphasized that under the conditions used here, i.e., addition of glucose to energize the bacteria, the protonmotive MDR pump known as Bmr did not appear to be involved in ethidium efflux since a Δ*bmr B. subtilis* strain (provided by A. Neyfakh; cf. ref *51*) behaved similarly to the wild-type strain (not shown). Direct efflux of ethidium was also measured after the bacteria were preloaded with the dye. As shown in Figure 3B, a rapid efflux of ethidium was observed from wild-type *B. subtilis*, which was greatly reduced by the presence of reserpine (slope ratio measured before addition versus after addition of reserpine = 1.75). The Δ*yvcC* mutant effluxed ethidium at a much slower rate than the wild-type strain, and addition of reserpine brought the rate further down to a level approaching that of the wild-type rate inhibited by reserpine (slope ratio measured before addition versus after addition of reserpine = 1.37). Thus, although YvcC appears to be involved in the reserpine-sensitive efflux of ethidium in wild-type *B. subtilis*, the remaining sensitivity to reserpine of the Δ*yvcC* mutant suggests that (an)other unidentified multidrug pump(s) different from Bmr is (are) also functional in the wild-type strain.

In a previous report, we had shown that overexpression of YvcC could be successfully achieved in *E. coli*. This allowed us to obtain inverted-membrane vesicles highly enriched in YvcC that catalyzed a specific transport of Hoechst 33342 as compared to vesicles prepared from the native *E. coli* strain (*40*). It is shown here that ATP hydrolysis is required to fulfill the Hoechst transport since a YvcC mutant, which was altered in the Walker A-motif (K380R) and therefore deficient in ATP hydrolysis (ATPase activity of the K380R mutant <3% of that of the wild-type protein), was unable to carry out such a transport (Figure 4A). A similar result was also obtained with the K380A mutant, which is totally devoid of ATPase activity (not shown). It should be noted that both mutant proteins were overexpressed and addressed to the plasma membrane as efficiently as the wild-type protein, as revealed by electrophoresis of the membrane preparation (not shown). Also, prior addition of vanadate or reserpine (cf. Figure 3) to wild-type YvcC prevented the transport of the dye (not shown). Interestingly, addition of low concentrations of either vinblastine (Figure 4B, curve b) or doxorubicin (not shown) stimulated the Hoechst transport, whereas higher concentrations of vinblastine prevented Hoechst transport (Figure 4B, curve c).

Two other fluorescent compounds were assayed as potential substrates of YvcC, namely, doxorubicin and 7-aminoactinomycin D, the fluorescence of which is quenched for the former upon DNA binding inside the vesicles (*52*) and most likely autoquenched for the latter upon binding to intravesicular DNA.[2] Importantly, the inverted-membrane vesicles contained enough naturally entrapped *E. coli* DNA fragments, broken during the French press treatment, so that it was unnecessary to add exogenous DNA fragments to carry out these experiments (not shown). Addition of ATP to both control and YvcC-enriched vesicles provoked a sudden drop of doxorubicin fluorescence, as also found with the nonhydrolyzable ATP analogue 5′-adenylyl imidodiphosphate (AMPPNP), which is indicative of a direct interaction between doxorubicin and nucleotide (Figure 5A; see also ref *52*). Then, a time-dependent quenching of fluorescence

[2] O. Dalmas, A. Di Pietro, and J.-M. Jault, manuscript in preparation.

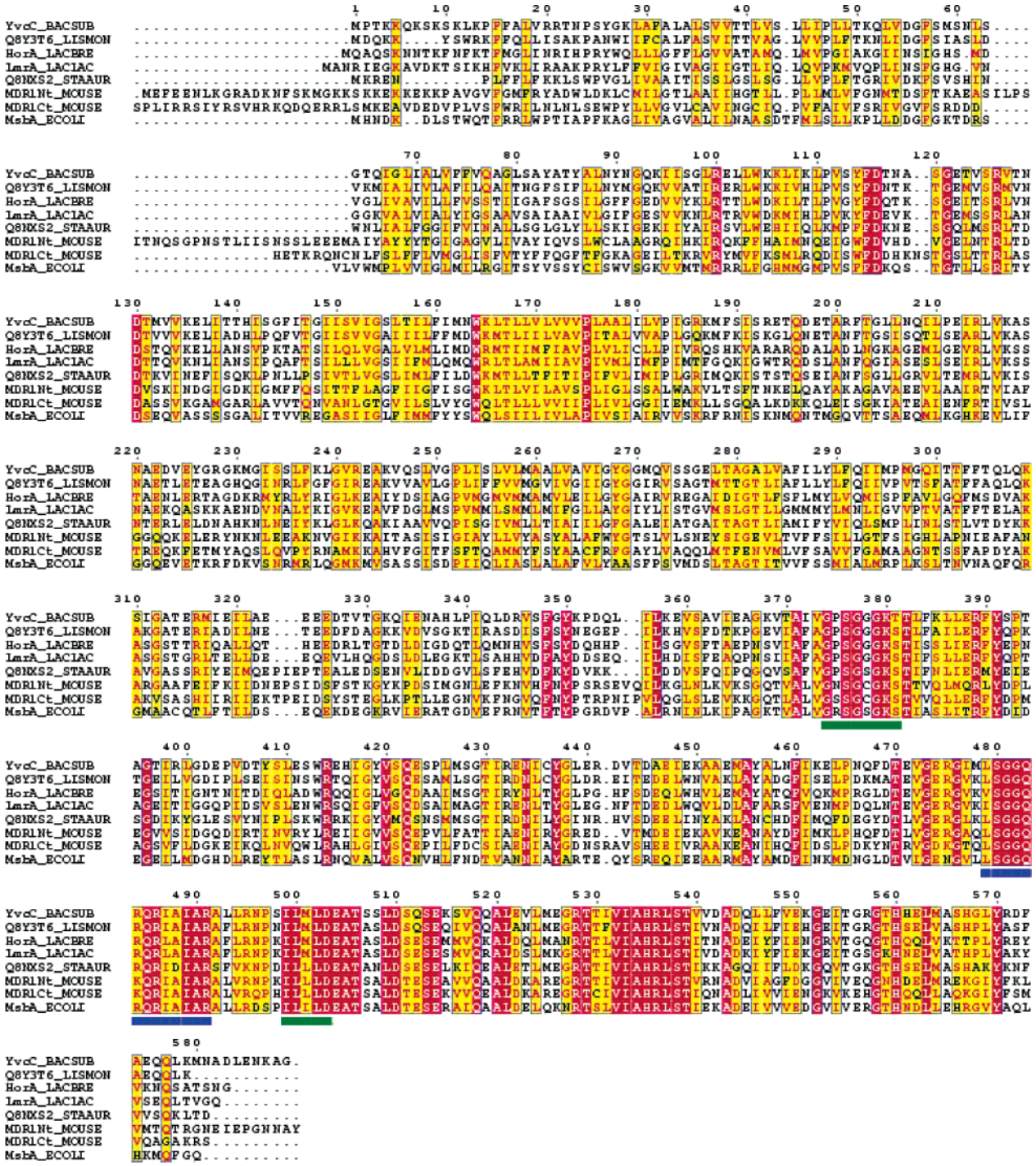

FIGURE 1: Sequence alignment of ABC transporters, MDRs or related. Sequences were obtained on the ExPASy server (http://us.expasy.org/), and the alignment was generated by Clustal W (1.8) on the NPS@ server (http://www.ibcp.fr/). Abbreviations: BACSUB, *B. subtilis*; LISMON, *L. monocytogenes*; LACBREV, *L. brevis*; LACLAC, *L. lactis*; STAAUR, *S. aureus*; ECOLI, *E. coli*. The TrEMBL entry name is given for two sequences (Q8Y3T6 and Q8NXS2). The figure was made with ESPript 2.0 (http://prodes.toulouse.inra.fr/ESPript/). Blue frames were drawn when at least 70% of the residues were conserved, with white characters in red boxes for strict identity and fully conserved residues and red characters in yellow boxes for similarity. The position of Walker A- and B-motifs is underlined in green, and that of the ABC signature is underlined in blue.

was observed in the presence of ATP for YvcC-enriched vesicles only, indicating that YvcC was indeed able to pump the doxorubicin inside the vesicles. Similar results were obtained when the transport activity of 7-aminoactinomycin D was studied. No transport was detected with the control vesicles, whereas a high ATP-dependent transport activity was seen with YvcC-enriched vesicles (Figure 5B). When AMPPNP was used instead of ATP, no transport activity was observed with wild-type YvcC, and likewise the YvcC K380R mutant was unable to carry out drug transport in the presence of ATP. Using the same approach, a specific transport by YvcC of two additional drugs, mitoxantrone and a fluorescent derivative of vinblastine (the bodipy-vinblastine), was also detected (not shown).

Purified YvcC Interacts with Many P-gp Effectors. After solubilization from *E. coli* membranes by DDM, the hexahistidine-tagged YvcC was purified by a one-step affinity chromatography (Figure 6). Four minor bands with apparent

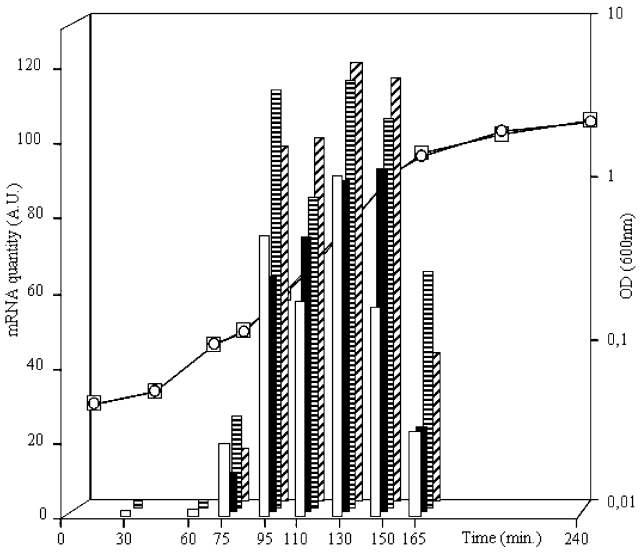

FIGURE 2: Quantification by real-time PCR of the expression of the *B. subtilis yvcC* gene. Total RNA was extracted from *B. subtilis* grown in LB medium, in the presence or the absence of ethidium bromide. The results are expressed as the amount of mRNAs produced under each condition: mRNA of *yvcC* obtained in the absence (white bars) or in the presence (black bars) of ethidium bromide. As a control, mRNA levels were also shown for a housekeeping gene, *hbs*, obtained in the absence (horizontally hatched bars) or in the presence (diagonally hatched bars) of ethidium bromide. Growth curves obtained in LB media in the absence (squares) or in the presence (circles) of ethidium bromide are also presented and expressed as optical density measured at 600 nm.

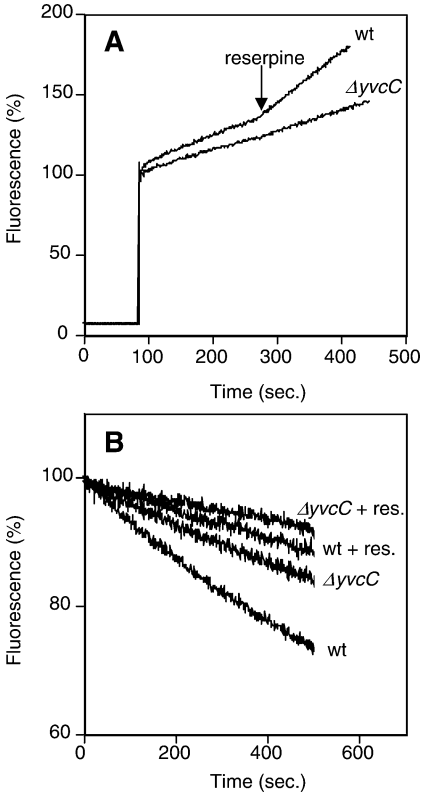

FIGURE 3: Transport of ethidium in *B. subtilis* wild-type or Δ*yvcC* mutant strains. Panel A: Washed *B. subtilis* wild-type (wt) or Δ*yvcC* mutant bacteria (Δ*yvcC*) were incubated at time zero in the presence of 50 mM KP_i (pH 7.2), 25 mM glucose, and 5 mM $MgSO_4$, and the fluorescence was recorded at 25 °C. Ethidium bromide (10 μM) was then added at ~80 s, and where indicated 50 μM reserpine was added to the cuvette containing either the wild-type or the Δ*yvcC* mutant strain. Panel B: After prior incubation in the presence of 10 μM ethidium bromide, 50 mM KP_i (pH 7.2), 25 mM glucose, and 5 mM $MgSO_4$ were added to *B. subtilis* wild-type (wt) or Δ*yvcC* mutant bacteria (Δ*yvcC*), and efflux of ethidium was immediately monitored at 25 °C in the absence or the presence of 50 μM reserpine (+ res.). Six and three independent experiments were performed for panel A and panel B, respectively, and typical results obtained in one of these experiments are shown here.

molecular masses lower than that of the full-length YvcC were found in the preparation that corresponded to proteolysis products from YvcC as they were recognized by an antibody directed toward the histidine tag (not shown). The purified protein, obtained with a yield of ~2 mg/L of culture medium, was then used to probe the binding of different P-gp substrates or effectors. First, the binding of TNP-ATP, a fluorescent analogue of ATP, was analyzed, and the propensity of ATP to compete with TNP-ATP was checked. Figure 7 shows that TNP-ATP bound to YvcC with a K_D value of 0.85 ± 0.06 μM, an affinity value slightly higher than that reported for purified P-gp (*53*). Displacement of TNP-ATP from YvcC was readily achieved by subsequently adding ATP (Figure 7, inset), allowing estimation of a K_D value of ~1.55 mM for ATP (*54*), a value about 6 times lower than that found with purified P-gp (*53*). Conversely, prior incubation of YvcC with increasing concentrations of ATP led to a decrease in the apparent affinity for TNP-ATP (not shown). Altogether, these results support the idea that the "native" conformation of YvcC was maintained throughout the purification protocol. Next, the ability of purified YvcC to bind various drugs, either substrates or inhibitors of P-gp, was assessed. To this end, the quenching of intrinsic fluorescence of YvcC was followed upon addition of different drugs, since this technique was previously used with purified P-gp (*55*, *56*). YvcC contains three Trp residues in its sequence, two located in the transmembrane domains (Trp-104 and Trp-164) and one in the NBD (Trp-413). The binding to YvcC of most of the drugs that did not interfere with Trp fluorescence was thus visualized, and their estimated K_D values are reported in Table 1. Interestingly, LY 335979 and GF 120918X, which showed the highest affinities for YvcC, are known to be powerful third-generation inhibitors of P-gp (*57*). As reported in the case of verapamil for P-gp (*53*), some of the drugs that did not significantly modify the intrinsic fluorescence of YvcC were nevertheless shown to bind to YvcC, as evidenced by a modification of the fluorescence properties of bound TNP-ATP. This includes notably colchicin, taxol, miltefosine, verapamil, and two antibiotics, namely, clindamycin or spiramycin (not shown). Therefore, YvcC is able to interact with, and possibly transport, many P-gp substrates and antibiotics and is likely to be inhibited by most P-gp inhibitors.

YvcC Hydrolyzes ATP According to a Positive Cooperativity Mechanism. Reconstitution of YvcC into liposomes was performed from lipid−protein−detergent micellar solution using SM-2 Bio-Beads as the detergent removing agent (*58*). This was found to be the most efficient technique to regain a high ATPase activity at 30 °C, which increased up to 10 times after reconstitution of YvcC in liposomes prepared from the *E. coli* extract. Interestingly, the high ATPase activity obtained at 37 °C [$V_{max} \sim 7$ μmol min^{-1} (mg of protein)$^{-1}$] displayed a positive cooperativity with a Hill number $n_H = 1.37 \pm 0.06$ (Figure 8A, full curve). A

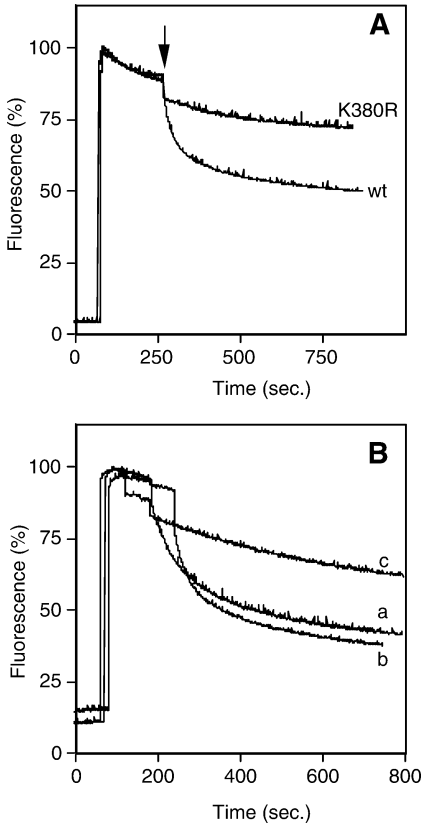

FIGURE 4: Transport of Hoechst 33342 by inverted *E. coli* membrane vesicles and effect of vinblastine. Panel A: Inverted-membrane vesicles (200 μg) containing either wild-type YvcC (wt) or the K380R YvcC mutant (K380R) were added to the cuvette and after ~1 min incubation at 37 °C, 2 μM Hoechst 33342 was added. Where indicated by the arrow, 2 mM ATP was added to initiate Hoechst transport. Panel B: Same as (A) except that 500 μg of inverted-membrane vesicles containing overexpressed wild-type YvcC was used and the Hoechst transport was monitored at 25 °C. Curve a: control experiment. Curves b and c: same as curve a but a prior addition of 0.25 μM vinblastine (curve b) or 25 μM vinblastine (curve c) was made ~1 min before the addition of 2 μM Hoechst 33342.

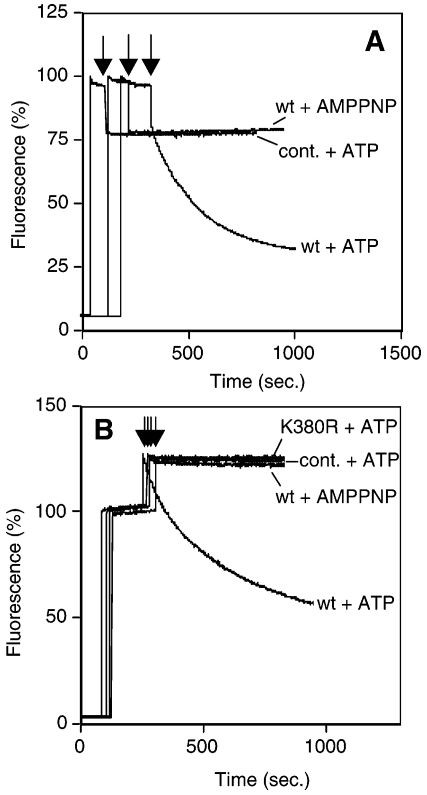

FIGURE 5: ATP-dependent transport of doxorubicin and 7-aminoactinomycin D by inverted *E. coli* membrane vesicles. Panel A: Inverted-membrane vesicles (500 μg) prepared from either control C41(DE3) bacteria (cont.) or C41(DE3) bacteria overexpressing YvcC (wt) were used, and the doxorubicin transport was monitored at 30 °C. After addition of 10 μM doxorubicin (1−2 min), 2 mM ATP or 2 mM AMPPNP was added where indicated by the arrow. Panel B: Same as (A) except that 400 μg of inverted-membrane vesicles prepared from either control C41(DE3) bacteria (cont.) or C41(DE3) bacteria overexpressing either wild-type YvcC (wt) or the K380R YvcC mutant (K380R) were used, and the 7-aminoactinomycin D transport was monitored at 37 °C. After addition of 10 μM 7-aminoactinomycin D (1−2 min), 2 mM ATP or 2 mM AMPPNP was added where indicated by the arrow.

positivecooperativity was also detected when GTP hydrolysis by YvcC was monitored by the same technique or when ATPase activity was measured by a colorimetric assay of P_i release. Furthermore, no ATPase activity was detected when purified E504A, E504Q [corresponding to the catalytic base of ABC transporters (*47*)], or K380A YvcC mutant was used, ruling out the possibility that the ATPase activity seen with the wild-type YvcC might be due to a contaminant. Accordingly, this high ATPase activity could be fully inhibited by addition of vanadate (Figure 8B), and the concentration producing an inhibition by 50% (~10 μM) is similar to the value previously found for the ATPase activity of membrane-bound YvcC (*40*). On the other hand, this ATPase activity was only moderately stimulated (~20−30%) by low concentrations of various drugs, such as reserpine (Figure 8C), vinblastine, or verapamil; it is important to note, however, that a similar limited extent of activation was found when the same drugs were tested on the ATPase activity of YvcC incorporated into inverted membrane vesicles (not shown), i.e., in a conformation of YvcC fully competent for drug transport. Therefore, the moderate stimulation of YvcC ATPase activity afforded by different drugs appears to be an intrinsic property of this new MDR ABC transporter. As also observed for some drugs with P-glycoprotein, further increasing the drug concentration above a certain threshold inhibited the ATPase activity of BmrA (Figure 8C).

DISCUSSION

Although the role of protonmotive-dependent transporters is now widely admitted in the emergence of multidrug resistance in bacteria (*5*, *59*), the more recent finding that MDR transporters could also use the energy of ATP hydrolysis to drive drug extrusion underlines the need for a deepened characterization of this new family of bacterial transporters. In this context, a new member belonging to the bacterial MDR half-ABC transporters has been identified here. YvcC is shown to be able to transport at least five different drugs, namely, ethidium, Hoescht 33342, doxorubicin, 7-aminoactinomycin D, and mitoxantrone and a fluorescent analogue of vinblastine. Moreover, the stimulation of Hoechst 33342 transport by low concentrations of either vinblastine or doxorubicin revealed that two drug-binding sites (or one huge drug-binding site able to accommodate simultaneously two different drugs) coexist on YvcC, a feature typical of MDR transporters, notably ABC pumps (*60*−*62*).

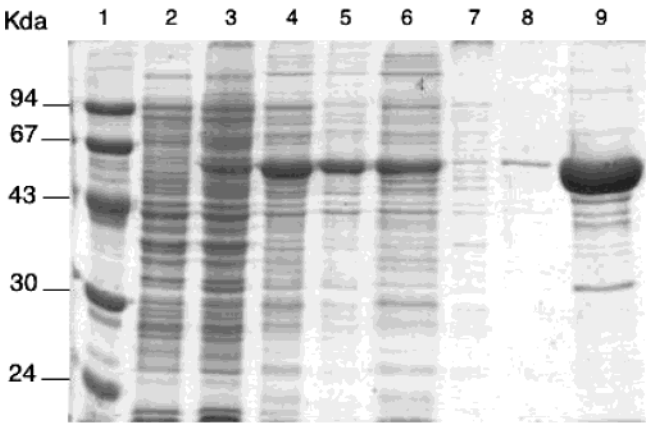

FIGURE 6: Overexpression in *E. coli* and purification of YvcC as analyzed by gel electrophoresis. Lanes: 1, molecular mass markers; 2 and 3, C41(DE3) *E. coli* extract before and after induction by 0.7 mM IPTG, respectively; 4, plasma membranes; 5 and 6, insoluble and soluble fractions obtained after solubilization by 1% DDM, respectively; 7, proteins not retained on the Ni-NTA−agarose column; 8, proteins eluted from the Ni-NTA−agarose column during the washing step; 9, proteins eluted from the Ni-NTA−agarose column by the elution buffer.

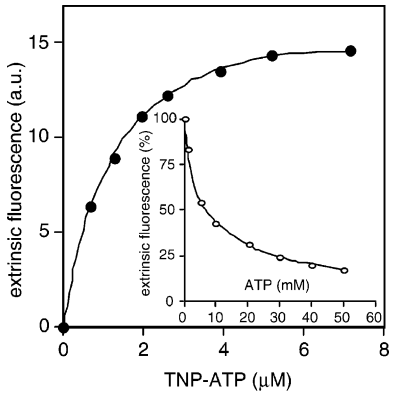

FIGURE 7: Binding of TNP-ATP to purified YvcC and displacement by ATP. Binding of increasing concentrations of TNP-ATP to 0.4 μM YvcC was monitored by the increase in fluorescence intensity of TNP-ATP and plotted as a function of the TNP-ATP concentration added. The inset shows the decrease of TNP-ATP fluorescence upon addition of increasing ATP concentrations.

Table 1: Quenching of YvcC Intrinsic Fluorescence by Interaction with Different Drugs[a]

drug	K_D (μM)	maximal quenching of fluorescence (%)
ethidium bromide	10.6 ± 3	45.8
doxorubicin	22.1 ± 4.5	17.8
daunomycin	12.2 ± 3.5	38
Rhodamine 6G	22.4 ± 5.1	44.3
vinblastine	5 ± 1.1	20.2
tetraphenylphosphonium	15.4 ± 2.7	52.6
Hoechst 33342	9.5 ± 2.3	56.5
LY 335979	4.4 ± 0.7	49.3
GF 120918X	0.44 ± 0.02	46.5

[a] Data were fitted by using GraFit 4.0 (Erithacus Software) and the estimated K_D and maximal quenching values are reported.

To the best of our knowledge, this is the first time that this kind of transporter is shown to be expressed and functionally active in a wild-type strain. Indeed, the prototype of this subfamily, LmrA, has been shown to be a MDR transporter in a drug-hypersensitive *E. coli* strain (*14*, *15*). HorA, first identified as a hop transporter from a hop-tolerant

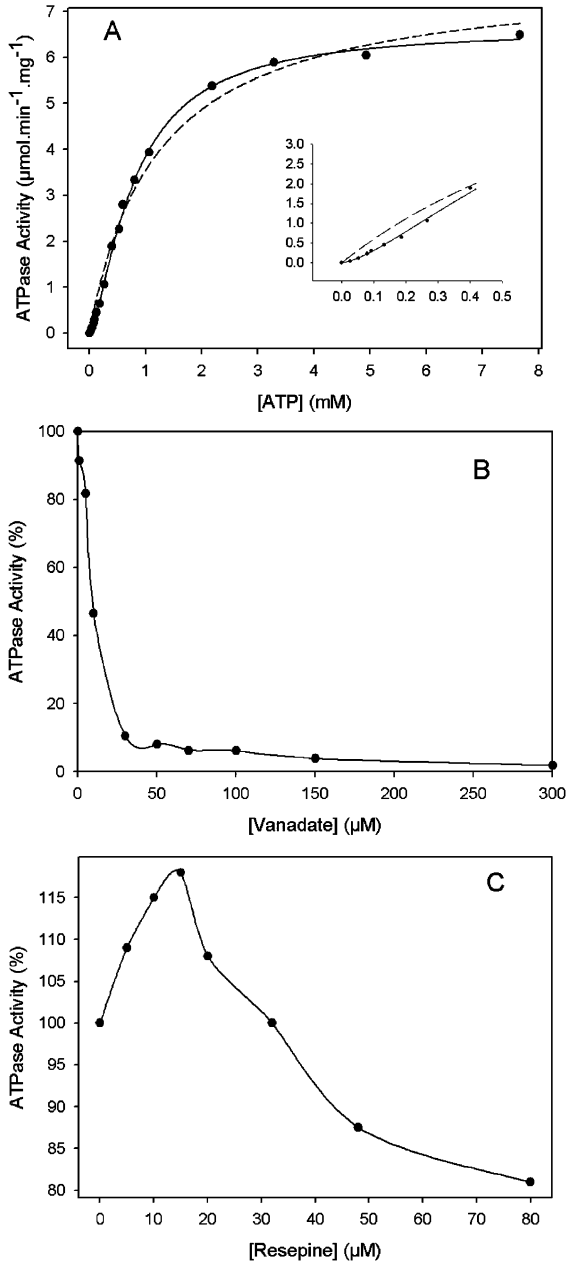

FIGURE 8: Cooperativity, vanadate sensitivity, and reserpine sensitivity of the ATPase activity of YvcC reconstituted into liposomes. Panel A: YvcC (5 μg) reconstituted into liposomes was added to an ATP-regenerating system. The initial rate of ATP hydrolysis was plotted against ATP concentrations, and curve fittings were performed by using GraFit 4.0 (Erithacus Software) with cooperativity (solid line, reduced $\chi^2 = 0.0068$) or with Michaelis−Menten parameters (broken line, reduced $\chi^2 = 0.0688$). This experiment was performed at least five times, and typical results obtained in one of these experiments are shown here. Panel B: The inhibition of ATPase by orthovanadate was monitored by a colorimetric assay of P_i release. 100% of ATPase activity represents 6.5 μmol of ATP hydrolyzed min^{-1} (mg of protein)$^{-1}$. This experiment was performed three times, and typical results obtained in one of these experiments are shown here. Panel C: The effect of reserpine on the ATPase activity was monitored by using the ATP-regenerating system with 5 mM ATP and 10 mM $MgCl_2$. No effect of reserpine on the ATP-regenerating system was observed at the concentrations used here. This experiment was performed three times, and typical results obtained in one of these experiments are shown here.

L. brevis mutant strain (*63*), was the second MDR ABC transporter to be characterized after overexpression in

Lactococcus lactis (*49*). Therefore, our results highlight the potential in vivo implication of ABC MDR transporters in antibiotic resistance in wild-type pathogenic strains. On the other hand, and despite the high sequence homology existing between YvcC and these two former ABC members, striking functional differences are found among them. Thus, HorA has a much narrower spectrum of transported drugs than LmrA (*9*). The latter is a functional homologue of human P-gp (*18*) and, as such, is able to interact and/or to extrude many structurally unrelated compounds. In this regard, LmrA appears more closely related to YvcC than HorA. Yet, the ATPase activity of LmrA reconstituted into liposomes is at least 10 times lower than that reported here for YvcC (*64*), showing that each MDR ABC transporter has very peculiar properties. In addition, the gene encoding for HorA is borne on a plasmid whereas that encoding for LmrA (*65*) or YvcC is borne on the genomic DNA (*39*). A plasmid-borne inheritance might favor the sharing with other bacteria, a property that would rapidly lead to a widespread dissemination of MDR phenotypes among many bacterial species (*3*). Notwithstanding these differences and by analogy to LmrA, we refer hereafter to YvcC as BmrA for *Bacillus* multidrug resistance ATP.

Since BmrA, LmrA, and HorA were all identified in Gram-positive bacteria, one can wonder if this kind of transporter is present in the Gram-negative ones as well? On the basis of sequence similarity, it has been suggested that this might indeed be the case (*66*). Interestingly, a MDR ABC transporter from *Vibrio cholerae* was very recently identified by overexpression in a drug-hypersensitive *E. coli* strain (*67*), showing that this kind of pump is not restricted to Gram-positive bacteria.

As previously reported for many bacterial MDR transporters (*68*), *bmrA* is found here to be dispensable for the normal growth of *B. subtilis*. Its expression is not modulated by addition of either ethidium bromide or Hoechst 33342, although we cannot exclude that the drug concentrations used in these experiments were too low to produce any detectable effect. It should be noted, however, that the ethidium concentration used in these experiments was able to induce the overexpression of another putative ABC gene, *ygaD*, encoding for an uncharacterized ABC transporter (J.-R. Fantino and F. Denizot, unpublished results). If indeed the expression of *bmrA* is not increased by the presence of drugs, this would argue against a physiological role of BmrA as an MDR transporter and might suggest that its drug efflux abilities are in fact coincidental (*68*). Such a case has been reported for instance with the Blt transporter of *B. subtilis*, which was first classified as an MDR transporter (*69*) but was later shown to naturally efflux spermidine (*70*). Nevertheless, it is possible that the expression of BmrA might be increased in the presence of other drugs encountered in the *B. subtilis* natural habitat and that the transport of ethidium occurs only as a "side effect". Indeed, it has been shown that some protonmotive pumps able to extrude several different compounds could have their expression induced by only a few of them (*71*). Likewise, although human P-gp is able to transport many structurally unrelated products, only some of these compounds seem capable to increase its expression level (*72*). If the in vivo function of BmrA is indeed related to a protective role, such an MDR pump might act as a housekeeping transporter to quickly cope with the presence of low levels of deleterious compounds. Then, if the concentration of these compounds rose above a threshold limit, the expression of additional MDR or even more specific pumps could then be turned on. This assumption would explain why no phenotypic effect was seen on the long run when the Δ*yvcC* mutant was grown in the presence of several different drugs as compared to the wild-type strain (not shown). Related to this, it was reported that deletion of *lmrA* in *L. lactis* led to overexpression of LmrP (*73*), a protonmotive MDR transporter with overlapping substrate specificity; if a similar mechanism occurred in *B. subtilis*, this might account for the lack of phenotypic effect seen in the Δ*yvcC* mutant. In support of BmrA being a housekeeping MDR transporter, it is worthy to mention that its eukaryotic counterpart, the mammalian MDR1 P-gp, is naturally expressed in tissues such as the blood−brain barrier where its physiological function is the detoxification of xenobiotics (*74*).

The ATPase activity reported here for BmrA reconstituted into liposomes is one of the highest ever reported for an ABC transporter. In the case of exporters, the maximal ATPase activity obtained was ∼0.15 μmol min^{-1} (mg of protein)$^{-1}$ for MsbA (*75*), ∼0.5 μmol min^{-1} (mg of protein)$^{-1}$ for MRP1 (*76*), and ∼6.5 μmol min^{-1} (mg of protein)$^{-1}$ for P-gp (*77*). Surprisingly, and as mentioned above, the maximal drug-stimulated ATPase activity measured with reconstituted LmrA was rather low, 0.3 μmol min^{-1} (mg of protein)$^{-1}$ (*64*). In the case of bacterial importers, values of ∼0.15 μmol min^{-1} (mg of protein)$^{-1}$ have been reported for either $MalFGK_2$ (*33*) or $HisQMP_2$ (*32*), which reached 4 μmol min^{-1} (mg of protein)$^{-1}$ for $MalFGK_2$ in the presence of both maltose and the extracellular maltose-binding protein (*78*). For P-gp, it has been shown that the lipid environment strongly influences its ATPase activity and consequently the stimulation afforded by many drugs (*79*), and whether this is the case for BmrA is worth considering. The BmrA ATPase activity might be due for instance to its ability to transport many endogenous substrates (e.g., lipids) naturally found in biological membranes (*80*). Thus, a "flippase-like" activity has been demonstrated for both P-gp and LmrA, although the former showed a relatively broad specificity toward lipids (*81*) whereas the latter was specific for phosphatidylethanolamine (*82*). It must be noted, however, that the moderate activation of BmrA ATPase activity afforded by several drugs was observed regardless of the composition or the origin of lipids used for the reconstitution. Most of all, a similar extent of ATPase activation was found when drugs were added to inverted-membrane vesicles containing BmrA. Therefore, the high ATPase activity of BmrA, which is concomitant to a moderate stimulation afforded by different drugs, is not due to an artifact of the reconstitution procedure used here but rather constitutes an intrinsic property of this transporter, possibly related to a transport activity of lipids. A moderate stimulation by drugs of MRP1 ATPase activity has also been reported, although the basal level of ATPase activity was relatively low (*76*).

Taking advantage of the high ATPase activity of BmrA, we have been able to show that this activity exhibits a positive cooperativity mechanism. This is compatible with the low-resolution 3-D structure of BmrA showing that it forms a homodimer in a lipid environment (*83*). Cooperativity between the two nucleotide-binding sites agrees also

with the assumption that each nucleotide-binding site is shared between the two nucleotide-binding domains in different ABC transporters (*84*). Moreover, the recently solved 3-D structure of an ATPase inactive mutant of MJ0796, where two ATP molecules are simultaneously bound per homodimer, gives a strong structural support to the enzymatic mechanism of positive cooperativity (*85*). A positive cooperativity for ATP hydrolysis has been previously detected for bacterial importers (*32*, *33*, *86*), whereas several reports described typical Michaelis−Menten kinetics for P-gp ATPase activity (*37*, *87*−*89*). Therefore, it might be argued that cooperativity occurs only when the two nucleotide-binding domains of an ABC transporter are strictly identical to each other, as is the case for BmrA and some bacterial importers. However, a positive cooperativity for ATP hydrolysis has been reported in one instance in human P-gp and was observed in the presence of saturating concentrations of drugs, i.e., when ATPase activity was maximally stimulated (*90*). Although the authors did not comment further this finding, this strongly suggests that cooperativity of ATP hydrolysis might not be restricted to bacterial ABC transporters but that this is most likely a common property shared by many, if not all, ABC transporters.

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