Multidrug Transport by the ABC Transporter Sav1866 from *Staphylococcus aureus*[†]

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ABSTRACT: Sav1866 is an ATP-binding cassette (ABC) protein from the pathogen *Staphylococcus aureus* and is a homologue of bacterial and human multidrug ABC transporters. Recently, the three-dimensional crystal structure of Sav1866 was determined at 3.0 Å resolution [Dawson, R. J., and Locher, K. P. (2006) *Nature 443*, 180−185]. Although this structure is frequently used to homology model human and microbial ABC multidrug transporters by computational methods, the ability of Sav1866 to transport multiple drugs has not been described. We obtained functional expression of Sav1866 in the drug-sensitive, Gram-positive bacterium *Lactococcus lactis* Δ*lmrA* Δ*lmrCD* lacking major endogenous multidrug transporters. Sav1866 displayed a Hoechst 33342, verapamil, tetraphenylphosphonium, and vinblastine-stimulated ATPase activity. In growing cells, Sav1866 expression conferred resistance to Hoechst 33342. In transport assays in intact cells, Sav1866 catalyzed the translocation of amphiphilic cationic ethidium. Additionally, Sav1866 mediated the active transport of Hoechst 33342 in membrane vesicles and proteoliposomes containing purified and functionally reconstituted protein. Sav1866-mediated resistance and transport were inhibited by the human ABCB1 and ABCC1 modulator verapamil. This work represents the first demonstration of multidrug transport by Sav1866 and suggests that Sav1866 can serve as a well-defined model for studies on the molecular bases of drug−protein interactions in ABC transporters. Our methods for the overexpression, purification, and functional reconstitution of Sav1866 are described in detail.

Multidrug resistance of cancers and infectious pathogenic microorganisms poses a major clinical problem (*1, 2*). It can be associated with the overexpression of multidrug transporters, which mediate the active extrusion of multiple drugs from the cell. Clinically relevant examples in human are the ATP-binding cassette (ABC)[1] multidrug transporters ABCB1 (also termed multidrug resistance P-glycoprotein MDR1), ABCC1 (also termed multidrug resistance-associated protein 1), and ABCG2 (also termed the breast cancer resistance protein) (*2, 3*). ABC multidrug transporters are also present in bacteria; LmrA from *Lactococcus lactis* was the first structural and functional homologue of ABCB1 identified (*4*−*6*) and was the first ABC extrusion system that was functionally reconstituted in proteoliposomes in a transport-active form (*6, 7*), enabling detailed studies on its transport mechanism (*6, 8, 9*). Recently, high-resolution crystallographic structures were determined of the bacterial ABC transporter Sav1866 from the pathogen *Staphylococcus aureus* (*10, 11*). Similar to LmrA, Sav1866 is a "half-transporter" with an N-terminal membrane domain followed by the nucleotide-binding domain. In agreement with the basic architecture of ABC transporters (*1*), and previous observations on LmrA (*6*), Sav1866 crystallized in a homodimeric complex (*10, 11*). The atomic coordinates of the Sav1866 structure have been used to homology model ABC transporters including those known to interact with multiple drugs (*12*−*23*). However, the important question whether Sav1866 can transport multiple drugs has not been addressed.

In this work we describe the functional expression of Sav1866 in *L. lactis* Δ*lmrA* Δ*lmrCD* (*9, 24*), which we previously used for the characterization of LmrA (*9*). Our data demonstrate that Sav1866 can transport structurally unrelated substrates in intact cells, plasma membrane vesicles, and proteoliposomes containing purified and functionally reconstituted protein and that this activity can be inhibited with a known modulator of human ABCB1 and ABCC1.

EXPERIMENTAL PROCEDURES

Genetic Manipulations. The *sav1866* gene was amplified from genomic DNA from *S. aureus* subsp. *aureus* Rosenbach (ATCC 700699D-5) (LGC Promochem) by PCR using the forward primer 5′-CGG CCC ATG GGG CAT CAC CAT CAC CAT CAC GAT GAC GAT GAC AAA ATT AAA CGA TAT TTG CAA TTT GTT AAG CC-3′ to introduce an *Nco*I site and a coding region for a six-histidine tag at the 5′ end and the reverse primer 5′-GCG CGA GCT CTT ATA AGT TTT GAA TGC-3′ to introduce a *Sac*I site at the 3′ end of the gene. The PCR product was digested with *Nco*I and *Sac*I and cloned into the lactococcal pNZ8048 expression vector (*25*) downstream of the *nisA* promoter, giving pNHSav1866. The *galP* gene (*26*) was amplified by PCR from genomic DNA from *Escherichia coli* DH5α using

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[1] Abbreviations: ABC, ATP-binding cassette; ABCB1, multidrug resistance P-glycoprotein MDR1; ABCC1, multidrug resistance-associated protein 1; ABCG2, breast cancer resistance protein; ethidium, 3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; Hoechst 33342, phenol 4-[5-(4-methyl-1-piperazinyl)[2,5′-bi-1*H*-benzimidazol]-2′-yl] trihydrochloride; TBS, Tris-buffered saline.

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the forward primer 5′-CTA GGG CAT ATG CAT CAC CAT CAC CAT CAC ATG CCT GAC GCT AAA AAA CAG GGG CGG-3′ to introduce an *Nde*I site and a coding region for a six-histidine tag at the 5′ end of the gene and the reversed primer 5′-GCC GAC AAG CTT TTA ATC GTG AGC GCC TAT TTC GCG-3′ to insert a *Hin*dIII site at the 3′ end. The PCR product was digested with *Nde*I and *Hin*dIII and cloned into the *E. coli* expression vector pET41A. DNA was sequenced to ensure that no unintended changes were introduced.

Expression of Sav1866. L. lactis strain NZ9000 Δ*lmrA* Δ*lmrCD* (*9, 24*) was used as a host for pNZ8048 derived plasmids in this study. The cells were grown at 30 °C in sterile M17 medium (Oxoid) supplemented with 0.5% (w/v) glucose. Chloramphenicol (5 μg/mL) was added where appropriate. Overnight cultures of *L. lactis* NZ9000 Δ*lmrA* Δ*lmrCD* harboring pNZHSav1866 or empty control plasmid were inoculated into fresh medium by 50-fold dilution. Protein expression was induced for 1 h by the addition (1:1000) of the supernatant of the nisin-A producing *L. lactis* strain NZ9700 (*25*) (containing approximately 10 ng nisin-A/mL). For this purpose nisin-A was added to the culture at an optical density A660 of 0.5 for transport studies in intact cells, or 0.8 for the preparation of inside-out membrane vesicles.

Preparation of Sav1866-Containing Inside-Out Membrane Vesicles. Sav1866-expressing *L. lactis* cells were harvested by centrifugation at 13000 *g* for 15 min, 4 °C, and washed and resuspended in 100 mM KP_i (pH 7.0) containing complete protease inhibitor mixture (Roche). Lysozyme was then added to a final concentration of 10 mg/mL, and the suspension was incubated at 30 °C for 30 min to digest the cell wall (*27*). The cells were lysed by three passages through a Basic Z 0.75 kW Benchtop cell disruptor (Constant Systems) at 20000 psi. Subsequently, DNase I (10 μg/mL) and 10 mM $MgSO_4$ were added, and the suspension was further incubated for 30 min at 30 °C. After the addition of 15 mM K-EDTA (pH 7.0), unbroken cells and debris were removed by a low-spin centrifugation at 13000*g* for 15 min at 4 °C. Inside-out membrane vesicles were collected by a high-spin centrifugation at 125000*g* for 40 min at 4 °C and resuspended in 50 mM KP_i (pH 7.0) supplemented with 10% (v/v) glycerol to a protein concentration of about 50 mg/mL. Membrane vesicles were prepared in 50 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid which was pH adjusted with KOH (K-HEPES) (pH 7.0) rather than 100 mM KP_i (pH 7.0) and finally resuspended in 50 mM K-HEPES (pH 7.0) containing 10% (v/v) glycerol when used in ATPase measurements. The protein concentration was determined by the Bradford method in the Bio-Rad DC protein assay using bovine serum albumin as a standard. The membrane vesicles were stored in 100 μL aliquots in liquid nitrogen.

Purification and Reconstitution of Sav1866. For the purification of Sav1866 by Ni^{2+}-affinity chromatography (*28*), inside-out membrane vesicles were resuspended to a protein concentration of 40 mg/mL in 50 mM KP_i (pH 8.0), 10% (v/v) glycerol, and 0.2 M NaCl. After the addition of 1.5% (w/v) *n*-dodecyl β-D-maltoside (DDM) (Calbiochem), membrane proteins were solubilized for 3 h at 4 °C with gentle agitation (*7*). Unsolubilized protein was removed by ultracentrifugation at 164000*g* for 40 min at 4 °C. The supernatant was incubated in 15 mL Falcon tubes with Ni^{2+}-nitrilotriacetic acid (Ni-NTA) resin (Sigma) (1 mL of resin per 10 mg of histidine-tagged protein) which was previously washed three times with 5 volumes of ultrapure H_2O and then two times with 5 volumes of wash buffer A [50 mM KP_i, 10% (v/v) glycerol, 0.2 M NaCl, 0.05% (w/v) DDM, and 20 mM imidazole (pH 8.0)]. After overnight incubation the resin was collected by centrifugation for 1 min at 175*g* and washed with 20 volumes of wash buffer A, followed by 30 volumes of wash buffer B [50 mM KP_i (pH 7.0), 10% (v/v) glycerol, 0.2 M NaCl, 0.05% (w/v) DDM, and 20 mM imidazole (pH 7.0)]. The resin was transferred to a 4 mL polysterene minicolumn (Pierce), and wash buffer B was drained. Histidine-tagged protein was eluted in 2.5 resin volumes of elution buffer [50 mM KP_i, 5% (v/v) glycerol, 0.2 M NaCl, 0.05% (w/v) DDM, and 200 mM imidazole (pH 7.5)], of which the first 0.5 volumes were discarded. The protein concentration was determined using the Bio-Rad DC assay. The purity of protein was determined on a silver-stained PAGE gel.

For the preparation of liposomes, commercially available *E. coli* phospholipids (Avanti Polar Lipids) were further purified by extraction with acetone−ether (*29*). *E. coli* phospholipids (100 mg) were suspended in 3 mL of chloroform and then slowly dripped into 10 mL of ice-cold acetone containing 4 μL of β-mercaptoethanol. The mixture was stirred for 3 h at 4 °C and centrifuged in glass tubes for 15 min at 3000*g*. The pellet was dried under nitrogen gas and dissolved in 15 mL of diethyl ether containing 4 μL of β-mercaptoethanol for 10 min at room temperature. Following centrifugation for 10 min at 1600*g* the supernatant was evaporated to dryness in a rotary evaporator. The residue was weighed and dissolved in chloroform, and egg phosphatidylcholine (PC) (Avanti Polar Lipids) was added to the acetone−ether-washed lipids at a PC to *E. coli* lipid ratio of 1:3 (w/w). Once mixed, the lipids were dried under nitrogen gas and stored at −20 °C until required.

For the reconstitution of purified Sav1866 into Triton X-100-destabilized liposomes (*30*), the lipids were hydrated at a concentration of 4 mg/mL in 100 mM KP_i (pH 7.0) containing 2 mM $MgSO_4$ and extruded 11 times through a 400 nm polycarbonate filter using a 1 mL LiposoFast-Basic extruder (Avestin) (*31*). The liposomes were destabilized by the addition of 3−3.5 mM Triton X-100 (or 3.5−4 mM DDM in Figure 1D) at 0.25 mM increments until the OD_{540} of the liposome suspension reached a maximum (*32, 33*). Purified Sav1866 in elution buffer was then added to a protein to lipid ratio of 1:100 (w/w). After incubation for 30 min at room temperature, 80 mg of hydrated polystyrene Bio-Beads (Bio-Rad) were added per milliliter of liposome suspension to remove the detergent (*34, 35*). Following incubation for 2 h at 4 °C, these beads were replaced twice by fresh Bio-Beads (80 mg/mL), which were incubated at 4 °C for 2 h and overnight, respectively. Before use, Bio-Beads were hydrated by one wash in methanol, followed by one wash in ethanol and five washes with ultrapure H_2O. The proteoliposomes were collected by centrifugation at 164000*g* for 30 min and resuspended to 1 mg of membrane protein/mL in 100 mM KP_i buffer (pH 7.0) supplemented with 2 mM $MgSO_4$. Proteolipsomes were immediately used in transport assays.

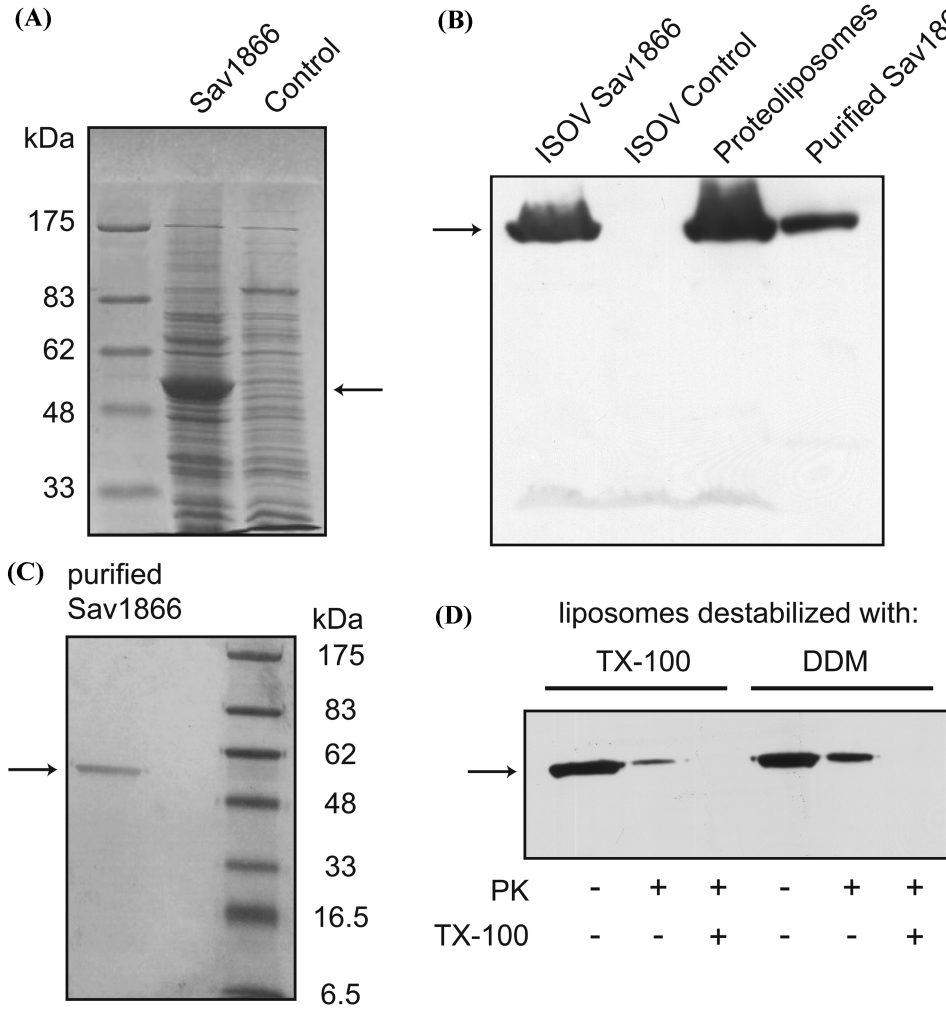

FIGURE 1: Expression, purification, and reconstitution of histidine-tagged Sav1866. (A) Detection of Sav1866 in the cytoplasmic membrane of *L. lactis*. Total membrane proteins in inside-out membrane vesicles (15 μg of protein/lane) were separated on a Coomassie brilliant blue stained 10% (w/v) SDS−PAGE gel. Membrane vesicles were prepared from cells harboring plasmid pNHSav1866 (Sav1866) and from cells harboring empty vector pNZ8048 (Control). (B) Western blot of inside-out membrane vesicles (ISOV) with Sav1866 or without the protein (Control), Sav1866 protein purified by Ni-NTA affinity chromatography, and Sav1866 protein reconstituted in proteoliposomes (each 15 μg of protein/lane), probed with anti-six-histidine tag antibody. (C) Purified Sav1866 (250 ng of protein) applied on a silver-stained PAGE gel. (D) Orientation of Sav1866 in proteoliposomes generated from Triton X-100 (TX-100) or *n*-dodecyl β-D-maltoside (DDM) destabilized liposomes. The orientation of reconstituted Sav1866 was determined by cleavage of the histidine tag at the external side by proteinase K (PK), and the detection of residual histidine-tagged Sav1866 by Western blotting (7.5 μg of protein/lane). In the presence of 1% (v/v) TX-100, proteinase K gained access to histidine tags on both sides of the membrane. Where required, the migration of molecular mass markers (kDa) and Sav1866 protein (arrow) is indicated.

To study the orientation of Sav1866 in the proteoliposomal membrane, proteoliposome suspensions were diluted to a protein concentration of 0.5 mg/mL in 100 mM KP_i buffer (pH 7.0) and kept on ice. Where required, proteinase K was added to a final concentration of 1 unit/mL. Control samples also received 1% (v/v) Triton X-100 to permeabilize the proteoliposomal membrane. After incubation for 4 h on ice, the reaction was terminated by the addition of 1.25× SDS-containing loading buffer. Protein in 15 μL samples was subjected to SDS−PAGE followed by immunoblotting using the monoclonal anti-six-histidine tag antibody (Sigma).

Expression and Purification of GalP. The expression of histidine-tagged GalP in *E. coli* BL21(DE3) (Novagen) in Luria broth supplemented with 0.5% (w/v) glucose and 50 μg/mL kanamycin was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (Sigma). The protein was solubilized from inside-out membrane vesicles and affinity purified by established methods (*26*).

Immunoblotting. Membrane proteins in inside-out membrane vesicles and proteoliposomes were subjected to 10% (w/v) SDS−PAGE and subsequent electroblotting to Hybond-P membrane (GE Healthcare Life Sciences). Following the blocking in Tris-buffered saline (TBS) (pH 7.4) containing 50 mM Tris-HCl, 0.1% (v/v) Tween-20, and 5% (w/v) semiskimmed milk powder, Sav1866 proteins were detected in the presence of a 1:1000 dilution of the monoclonal anti-six-histidine tag antibody in accordance with the manufacturer's recommendations (Qiagen). Hybond-P membranes were washed four times in TBS containing 0.05% (v/v) Tween-20, after which a 1:10000 dilution of anti-mouse secondary horseradish peroxidase-conjugated antibody (GE Healthcare Life Sciences) was applied for 1 h at room temperature. Signals on the Western blot were detected by the enhanced chemiluminescence system (ECL) (GE Healthcare Life Sciences) according to the manufacturer's instructions.

Hoechst 33342 Transport in Membrane Vesicles and Proteoliposomes. Transport of phenol 4-[5-(4-methyl-1-piperazinyl)[2,5′-bi-1*H*-benzimidazol]-2′-yl] trihydrochloride (Hoechst 33342) (Invitrogen) was measured as described previously (*36*). Inside-out membrane vesicles (0.5 mg of total membrane protein) were diluted in 2 mL of 100 mM KP_i buffer (pH 7.0) containing 5 mM $MgSO_4$, 0.1 mg/mL creatine kinase (Sigma), and 5 mM phosphocreatine (Sigma). After approximately 1 min of incubation at 30 °C, Hoechst 33342 in KP_i buffer was added to a final concentration of 0.25 μM, and the binding of the dye to the membrane vesicles was followed by fluorometry in an LS 55B luminescence spectrometer (Perkin-Elmer) at excitation and emission wavelengths of 355 and 457 nm, respectively, and slit widths of 2.5 nm each, until a steady state was reached. Subsequently, ATP (or AMP-PNP in the control experiments) in KP_i buffer was added to a final concentration of 2 mM, and the fluorescence intensity was followed until a new steady state was reached. Similar techniques were used for measurements of Hoechst 33342 transport in proteoliposomes. The experimental details of these measurements are summarized in the legend to Figure 4B.

Ethidium Transport in Intact Cells. The transport assays were carried out as described previously (*37, 38*) with modifications. Sav1866-expressing cells were harvested by centrifugation (2800*g* for 10 min at 4 °C) in mid-exponential phase and ATP-depleted by washing in ice-cold 50 mM KP_i (pH 7.0) containing 0.5 mM protonophore 2,4-dinitrophenol for 30 min at 30 °C. Cells were washed and were then resuspended in this buffer to an OD_{660} of 5.0 and kept on ice until needed. Cells were diluted to a final OD_{660} of 0.5 in a cuvette containing 2 mL of buffer. After 100 s, 2 μM ethidium bromide (Invitrogen) was added, allowing the detection of facilitated ethidium influx into the cell. For active ethidium efflux, cells were preequilibrated with 2 μM ethidium for 20 min, and efflux was initiated by the addition of 20 mM glucose. Fluorescence was measured at excitation and emission wavelengths of 500 and 580 nm, respectively, and slit widths of 5 and 10 nm, respectively.

Cytotoxicity Assays. Cells were grown at 30 °C to OD_{660} of about 0.3. Subsequently, Sav1866 expression was induced by the addition of 40 pg/mL nisin. The cells were aliquoted in a 96-well plate in the presence of a range of concentrations of Hoechst 33342. Growth was followed at 30 °C by measuring the OD_{660} every 15 min for 7 h in a VersaMax microplate reader (Molecular Devices).

ATPase Activity Measurements. To measure the ATPase activity of Sav1866 in inside-out membrane vesicles in the NADH oxidation method (*39*), the reaction buffer [50 mM K-HEPES, pH 8.0, containing 0.3 mM NADH (Sigma), 4 mM phosphoenolpyruvate (Roche), 50 μg/mL pyruvate kinase (Sigma), 10 μg/mL lactate dehydrogenase (Roche), 3.3 mM $MgCl_2$, and 3.3 mM ATP (both Sigma)] was preincubated for 5 min at 30 °C in a VersaMax microplate reader. The ATPase reaction was started by the addition of 4 μg/mL inside-out membrane vesicles in 50 mM K-HEPES, pH 8.0, and monitored for 30 min an absorbance of 340 nm. An NADH standard curve with a range of NADH concentrations between 0 and 300 μM was determined in the same buffer. The ATPase activity of Sav1866 in inside-out membrane vesicles was also determined by colorimetric measurement of released P_i in a malachite green−molybdate binding reaction and is described elsewhere (*9*). To test for substrate stimulation of the Sav1866-ATPase activity, the drugs were added to the reaction buffer before the addition of the membrane vesicles.

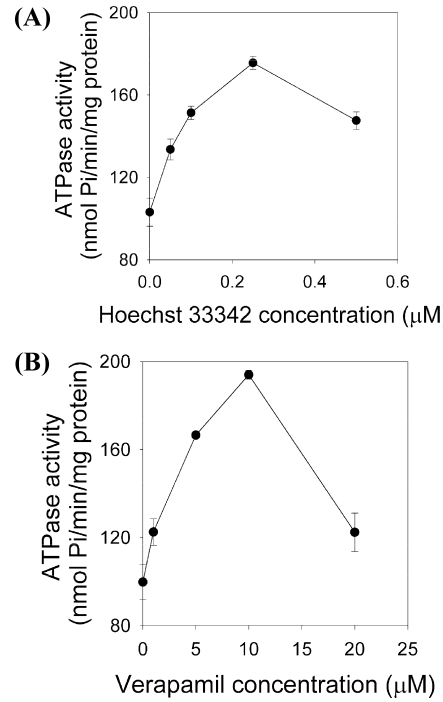

FIGURE 2: Effect of drugs on the Sav1866 ATPase activity. ATPase activity in inside-out membrane vesicles containing Sav1866, based on the liberation of P_i, in the presence of increasing concentrations of Hoechst 33342 (A) or verapamil (B). The data represent the means ± SE of three independent experiments using different batches of membrane vesicles.

Hoechst 33342 Binding. Hoechst 33342 binding to affinity-purified Sav1866 was measured by fluorometry and carried out as described (*40*) in 2 mL reactions containing 20 μg of purified protein in 100 mM KP_i buffer (pH 7.0). Hoechst 33342 was then added to the solution in a stepwise fashion of 0.125 μM, when no major changes in fluorescence were detected (Figure 5B). Measurements were performed at excitation and emission wavelengths of 355 and 457 nm, respectively, and slit widths of 5 and 10 nm, respectively. Nonspecific binding of the amphiphilic drug to hydrophobic regions of Sav1866 was estimated by using half-molar quantities of purified GalP (*40, 41*), which contains twice the number of transmembrane helices as Sav1866 (*26*). Nonspecific binding was less than 20% of total binding in Figure 6B and was subtracted.

RESULTS

Heterologous Expression, Purification, and Reconstitution of Sav1866. For the expression of Sav1866 in *L. lactis* NZ9000 Δ*lmrA* Δ*lmrCD* (*9, 24*), the *Sav1866* gene was PCR amplified from genomic DNA from *S. aureus* and cloned together with an upstream coding region for an N-terminal six-histidine tag into the lactococcal expression vector pNZ8048 (*25*) under the control of nisin-inducible *nisA* promoter, yielding pNZHSav1866. This expression system has been used previously for LmrA (*6*−*8*). The addition of 10 pg/mL nisin-A to exponentially growing *L. lactis* cells harboring pNHSav1866 resulted in the expression of 55 kDa

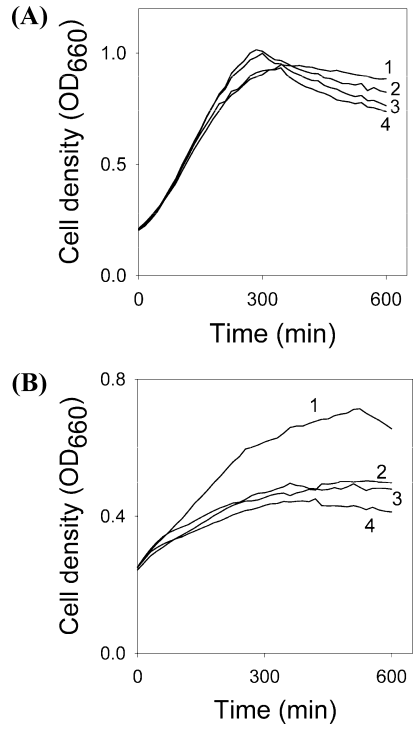

FIGURE 3: Sav1866 expression confers Hoechst 33342 resistance on *L. lactis*. Growth of Sav1866-expressing cells (traces 1 and 2) and control cells (traces 3 and 4) in the absence (A) or presence (B) of 15 μM Hoechst 33342 with (traces 2 and 4) or without (traces 1 and 3) 50 μM verapamil. Shown are representative traces from three independent experiments using different batches of cells.

polypeptide that could be detected in membrane vesicles on a Coomassie brilliant blue stained SDS−PAGE gel (Figure 1A) and Western blot probed with anti-six-histidine antibody (Figure 1B). The protein was expressed at a level between 20% and 25% of total membrane protein, as determined by the densitometric analyses of SDS−PAGE gels (not shown). This 55 kDa band was absent in the control membrane vesicles prepared from cells harboring the empty pNZ8048 vector (Figure 1A).

To study Sav1866 protein in the absence of other membrane proteins, Sav1866 was purified in a single Ni-NTA affinity chromatography step and, when required, functionally reconstituted in proteoliposomes. Our methods are described in detail in the Experimental Procedures. Briefly histidine-tagged Sav1866 was solubilized from inside-out membrane vesicles in the presence of 1.5% (w/v) DDM for 3 h at 4 °C. Total DDM-solubilized protein was mixed with Ni-NTA resin and incubated overnight at 4 °C with gentle agitation. The resin was collected and extensively washed with buffers, pH 8.0 and 7.0, containing 20 mM imidazole to remove impurities. Sav1866 was eluted in buffer (pH 7.5) containing 200 mM imidazole, which competes with the histidine tag for binding to the resin, yielding Sav1866 protein that was purified to apparent homogeneity on a silver-stained PAGE gel (Figure 1C) and Western blot (Figure 1B). The eluted fraction containing Sav1866 was typically at a concentration of 1.8 mg/mL, and the procedure yielded about 4 mg of Sav1866/L of cell culture. Purified Sav1866 was either immediately used in drug binding studies or reconstituted in detergent-destabilized liposomes. During reconstitution, purified Sav1866 was mixed with Triton X100-destabilized liposomes prepared from acetone−ether-washed *E. coli* lipids and egg PC. Subsequently, detergent was removed by adsorption on polystyrene beads, resulting in the incorporation of Sav1866 in the liposomal membrane (Figure 1B). To analyze the orientation of Sav1866 in proteoliposomal membrane, the accessibility of the histidine tag to cleavage by proteinase K was determined. The histidine tags which remained attached to Sav1866 after cleavage were detected by Western blotting using the anti-six-histidine tag antibody. As shown in Figure 1D, about 90% of the histidine tag was removed when proteinase K was added to the outside of the Sav1866-containing proteoliposomes. When the proteoliposomal membrane was permeabilized in the presence of 1% (v/v) Triton X-100, to allow access of proteinase K to both the outside and luminal side of the membrane, the signal was absent on Western blot (Figure 1D). Taken together, these results indicate that Sav1866 was preferentially reconstituted in an inside-out fashion in Triton X-100-destabilized liposomes, with the histidine tag exposed to the external side of the membrane. The unidirectional reconstitution in Triton X-100-destabilized liposomes was previously observed for a wide range of pumps and transporters, including the light-driven proton pump bacteriorhodopsin from *Halobacterium halobium* (*33*), the Ca^{2+} P-type ATPase from skeletal muscle sarcoplasmic reticulum (*42*), the chloroplast phosphate translocator (*43*), the lactose transporter LacS from *Streptococcus thermophilus* (*44*), and the proline transporter PutP (*45*) and proline/glycine-betaine transporter ProP (*46*) from *E. coli*. If, in a control experiment, purified Sav1866 was reconstituted using DDM-destabilized liposomes, about 50% of histidine tags were removed in the presence of proteinase K (Figure 1D), consistent with the 50%-in, 50%-out orientation previously observed for histidine-tagged LmrA in the DDM-based reconstitution method (*7*).

Basal ATPase of Sav1866 Is Stimulated by Drugs. ATPase measurements based on the liberation of P_i indicated that Sav1866-containing membrane vesicles displayed a basal ATPase activity that was stimulated 2-fold in the presence of Hoechst 33342 with a concentration required for half-maximal stimulation (SC_{50}) of about 0.1 μM (Figure 2A) and was stimulated 1.8-fold by verapamil with an SC_{50} of about 5 μM (Figure 2B). Comparable results were observed in ATPase assays in which the hydrolysis of ATP by Sav1866 was enzymatically coupled to the oxidation of NADH, and in these assays the Sav1866 ATPase activity was also found to be stimulated 1.8-fold by 5 μM vinblastine and 2.6-fold by 10 μM tetraphenylphosphonium (data not shown).

Hoechst 33342 Transport by Sav1866. The potential interaction of Sav1866 with Hoechst 33342 was further examined in cytotoxicity assays with control cells and cells expressing Sav1866. In these assays, the growth of cells in liquid medium was followed over time. Whereas no differences were observed between the growth of control cells and Sav1866-expressing cells in the absence of the toxic compound (Figure 3A), the addition of 15 μM Hoechst 33342 significantly reduced growth of the control (Figure 3B). Interestingly, Sav1866-mediated resistance in these experiments was reversed in the presence of 50 μM verapamil. Verapamil had no significant effect on growth of the cells in the absence of Hoechst 33342 (Figure 3A). Hence, heterologously expressed Sav1866 conferred Hoechst 33342

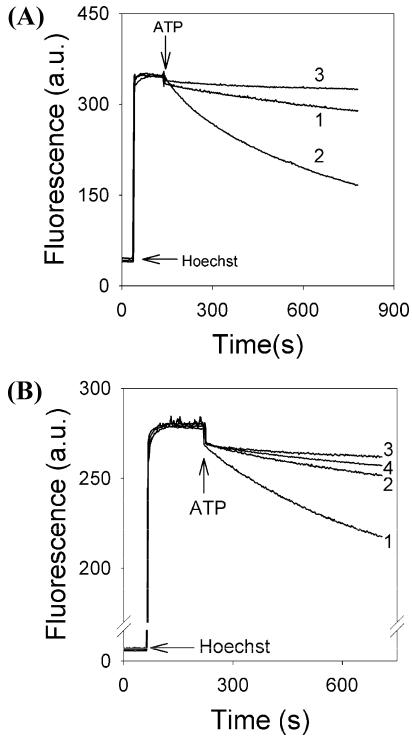

FIGURE 4: Sav1866 mediates the transport of Hoechst 33342. (A) Hoechst 33342 transport in inside-out membrane vesicles without (trace 1) or with (trace 2) Sav1866, which were diluted to a protein concentration of 0.25 mg/mL in 100 mM KP_i buffer (pH 7.0) supplemented with 5 mM $MgSO_4$ and an ATP-regenerating system. Upon the addition of 0.25 μM Hoechst 33342 (first arrow) the increase in the fluorescence of the dye was followed in time until a steady state was reached. Active transport of Hoechst 33342 was initiated by the addition of 2.5 mM Mg-ATP (second arrow). Trace 3 was as described for trace 2 with ATP replaced by the nonhydrolyzable ATP analogue AMP-PNP. (B) Hoechst 33342 transport in proteoliposomes containing purified and functionally reconstituted Sav1866 (traces 1 and 3) or empty control liposomes (traces 2 and 4) in the absence (traces 1 and 2) or presence (traces 3 and 4) of 50 μM verapamil. (Proteo)liposomes were diluted to a phospholipid concentration of 0.5 mg/mL in 100 mM KP_i buffer (pH 7.0) supplemented with 5 mM $MgSO_4$ and an ATP regenerating system. Arrows are as given in (A) using 0.5 μM Hoechst 33342 and 3 mM Mg-ATP. For incubations with verapamil, the proteoliposomes were preincubated for 3 min in the presence of the compound before the addition of Hoechst 33342. Shown are representative traces from three independent experiments using different batches of membrane vesicles and proteoliposomes.

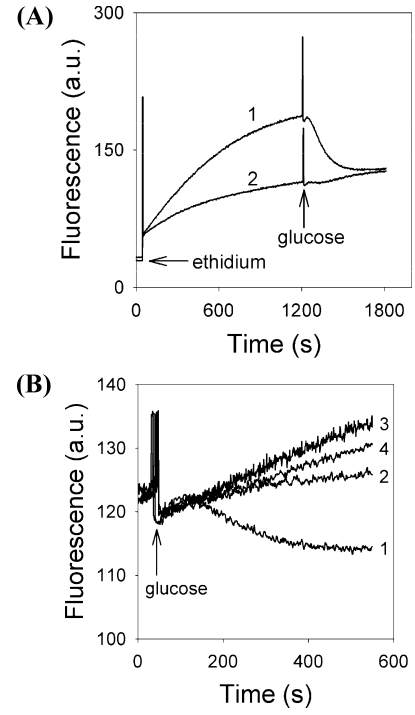

FIGURE 5: Sav1866 mediates transport of ethidium. (A) Ethidium uptake was followed over time in ATP-depleted cells with (trace 1) or without (trace 2) expression of Sav1866. At the first arrow, 2 μM ethidium was added. At the second arrow, 20 mM glucose was added to enable the generation of metabolic energy in the cells. (B) Transport of ethidium in cells with (traces 1 and 2) or without (traces 3 and 4) expression of Sav1866 in the absence (traces 1 and 3) or presence (traces 2 and 4) of 50 μM verapamil. Cells were preequilibrated for 20 min with 2 μM ethidium. Glucose (20 mM) was added at the arrow. Shown are representative traces from nine independent experiments using different batches of cells.

resistance on the lactococcal cells, and the resistant cells were sensitized by verapamil.

The Sav1866-associated Hoechst resistance in cells points to the ability of Sav1866 to mediate Hoechst 33342 extrusion. The Sav1866-mediated transport of Hoechst 33342 was studied in inside-out membrane vesicles in which the nucleotide-binding domain of Sav1866 was exposed on the outside surface of the membrane. The addition of 0.25 μM Hoechst 33342 resulted in a rapid increase in fluorescence up to a steady-state level due to partitioning of the probe in the hydrophobic environment of the phospholipid bilayer (Figure 4A). The subsequent addition of Mg-ATP resulted in a rapid quenching of Hoechst 33342 fluorescence in membrane vesicles containing Sav1866 compared to control membrane vesicles (Figure 4A), reflecting the Sav1866-mediated transport of Hoechst 33342 from the phospholipid bilayer. Sav1866-dependent transport was absent when Mg-ATP was replaced by the nonhydrolyzable ATP analogue AMP-PNP, suggesting that ATP binding alone was not sufficient to drive the translocation of Hoechst 33342 in inside-out membrane vesicles (Figure 4A).

Additional evidence for the interaction between Sav1866 and Hoechst 33342 was obtained in Hoechst 33342 transport measurements in proteoliposomes containing affinity-purified and functionally reconstituted Sav1866. The addition of 3 mM Mg-ATP resulted in a significant quenching of the fluorescence of 0.5 μM Hoechst 33342 in proteoliposomes containing Sav1866 compared to empty liposomes (Figure 4B). The Hoechst 33342 transport reaction by Sav1866 in proteoliposomes was blocked by the addition of 50 μM verapamil. Verapamil had no effect on Hoechst 33342 fluorescence in control liposomes (Figure 4B). Hence, these findings for purified and functionally reconstituted Sav1866 are consistent with those for intact cells and membrane vesicles and demonstrate that Hoechst 33342 transport by Sav1866 is inhibited by verapamil.

Sav1866 Mediates Ethidium Transport. The ability of Sav1866 in *L. lactis* to transport ethidium was assessed by monitoring the fluorescence of the intracellular ethidium polynucleotide complex. In previous work, we demonstrated that LmrA can mediate the facilitated influx of ethidium in ATP-depleted cells and active efflux from cells in the presence of metabolic energy (*37*). Surprisingly, when 2 μM ethidium was added to a suspension of ATP-depleted lactococcal cells expressing Sav1866, an enhanced rate of ethidium influx was observed compared to the control (Figure 5A). Sav1866 remained functional in these cells, as the

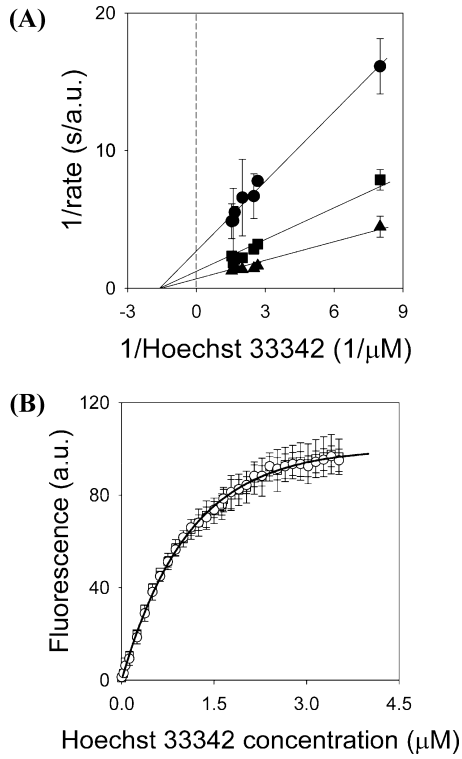

FIGURE 6: Effect of verapamil on interactions of Sav1866 with Hoechst 33342. (A) Kinetic characterization of the inhibition by verapamil of Sav1866-mediated Hoechst 33342 transport in inside-out membrane vesicles (▲, no verapamil; ■, 1 μM verapamil; ●, 20 μM verapamil). The rates obtained for control membrane vesicles in the presence of Mg-ATP were subtracted from those obtained for Sav1866-containing membrane vesicles. (B) Hoechst 33342 binding to purified Sav1866 (10 μg/mL protein) was measured by fluorometry at increasing concentrations of the dye in the absence (□) or presence (○) of 50 μM verapamil. For the determination of Hoechst 33342 binding in the presence of verapamil, the samples were incubated with the drug for 3 min prior to the addition of Hoechst 33342. The data were corrected for nonspecific binding of Hoechst 33342 and were fitted to a hyperbola (solid line). The data represent the means ± SE of three independent experiments using different batches of membrane vesicles and purified Sav1866.

addition of glucose as a source of metabolic energy elicited ethidium efflux in the Sav1866 expressing cells but not in the nonexpressing control (Figure 5A). The addition of glucose also initiated ethidium efflux from Sav1866-containing cells which were preequilibrated with 2 μM ethidium (Figure 5B). Similar to the observations on Hoechst 33342 transport (Figure 5B), the Sav1866-dependent ethidium efflux was blocked by verapamil (Figure 5B). In related work on LmrA, we have demonstrated previously that the fluorescence changes observed in these experiments coincide with the physical movement of ethidium between the intracellular milieu and extracellular buffer (*47*).

Mechanism of Inhibition of Sav1866 Activity by Verapamil. The inhibition of Sav1866-mediated drug transport by verapamil was further studied using Hoechst 33342. The initial rate over the first 45 s of Sav1866-mediated Hoechst 33342 transport in inside-out membrane vesicles was determined at increasing concentrations of Hoechst 33342 in the absence or presence of fixed concentrations of verapamil (Figure 6A). The results of these measurements indicated that the V_{max} of 1.47 ± 0.18 au/s for the Sav1866-mediated Hoechst 33342 reaction was inhibited at increasing verapamil concentrations with a K_i of 9.3 ± 2.4 μM, whereas the K_t of 0.71 ± 0.22 μM Hoechst 33342 remained unaltered. The Lineweaver−Burk plot (Figure 6A) is representative of simple noncompetitive inhibition, pointing to independent binding sites for Hoechst 33342 and verapamil on Sav1866.

This conclusion was strengthened by experiments in which the equilibrium binding of Hoechst 33342 to Sav1866 at increasing concentrations of the dye was determined from the enhancement of fluorescence upon transfer of the probe from the aqueous buffer to nonpolar binding site(s) on the protein. The data could be fitted to a hyperbola with an R^2 value of 0.993, yielding an apparent K_d of 0.85 ± 0.16 μM and B_{max} of 111 ± 12 au. Hoechst 33342 binding was also carried out in the presence of verapamil at concentrations of up to 50 μM, which was almost 60 times the K_d for Hoechst 33342. No significant differences in the apparent B_{max} and K_d values for Hoechst 33342 binding were obtained with and without verapamil (with verapamil: K_d = 0.88 ± 0.14 μM and B_{max} = 110 ± 14 au) (Figure 6B). Taken together, these experiments indicate that Sav1866 contains nonoverlapping binding sites for Hoechst 33342 and verapamil.

DISCUSSION

In this work, we present important new observations on the ability of Sav1866 from *S. aureus* to interact with and transport multiple drugs. Consistent with earlier findings for Sav1866 which was heterologously expressed in *E. coli* (*10*), the basal ATPase activity of Sav1866 in *L. lactis* was shown to be stimulated up to 2-fold by Hoechst 33342 and vinblastine. In addition, we observed a significant stimulation by other compounds such as verapamil and tetraphenylphosphonium. At concentrations of Hoechst 33342 or verapamil greater than that which stimulated the Sav1866-associated ATPase maximally, the ATPase activity decreased (Figure 2A,B), giving rise to bell-shaped curves. The biphasic pattern of stimulation and inhibition of the ATPase activity was also previously observed for ABCB1 and ABCG2 (*48*−*50*) and might reflect enhanced drug binding to binding sites exposed at the inside surface of the membrane at low drug concentrations and reduced dissociation of these drugs from binding sites at the outside surface of the membrane at high drug concentrations. Alternatively, the inhibition of the Sav1866-associated ATPase activity at high drug concentrations could reflect drug binding to modulator sites in Sav1866, which do not participate in the transport reaction, or to changes in the lipid environment, such as the lateral pressure or membrane fluidity, which might be less optimal for Sav1866 activity. Although these findings on the Sav1866 ATPase activity do indicate a potential interaction of Sav1866 with drugs, they do not provide direct evidence for the ability of Sav1866 to transport these or other drugs.

In cytotoxicity assays in *L. lactis*, the expression of Sav1866 conferred resistance on growing cells to Hoechst 33342 (Figure 3), pointing to its ability to mediate drug efflux. The active transport of Hoechst 33342 by Sav1866 was demonstrated in inside-out membrane vesicles (Figure 4A). In addition, ATP-dependent transport of Hoechst 33342 by Sav1866 was found in proteoliposomes containing purified and functionally reconstituted protein (Figure 4B). In our ethidium transport measurements in intact cells, Sav1866 also clearly exhibited the ability to mediate the transport of

ethidium (Figure 5). However, it should be noted that Sav1866 did not confer resistance on cells to ethidium or tetraphenylphosphonium, indicating that the maximum achievable rate of efflux by Sav1866 in *L. lactis* is insufficient to cope with passive influx during growth and/or that the affinity of Sav1866 for these compounds in *L. lactis* is too low to allow the effective reduction of the cytosolic concentration below levels causing toxicity to the cell. It should also be noted that although the ethidium efflux data displayed in Figure 5B represent the general trend, in a number of experiments in washed cell suspensions, we observed ethidium uptake in Sav1866-expressing cell compared to control in the presence of glucose, giving traces comparable to those in Figure 5A before the addition of glucose. In view of our previous findings for the Sav1866 homologue LmrA from *L. lactis* that drug transport by this system can be affected by transmembrane electrochemical ion gradients (*8, 9*), further experiments are required to study the parameters that can influence the direction of transport by Sav1866.

Verapamil and vinblastine are nontoxic to *L. lactis* when used in the micromolar range of concentrations. Consistent with the verapamil−Sav1866 interactions suggested in the ATPase measurements (Figure 2), verapamil sensitized Sav1866-expressing cells to Hoechst 33342 (Figure 3B) and inhibited the Sav1866-mediated transport of ethidium (Figure 5B) and Hoechst 33342 (Figure 4B). The mechanism of inhibition of transport by verapamil was studied in detail for Hoechst 33342 and was found to be simple noncompetitive (Figure 6A); the inhibition of transport by verapamil ($K_i = 9.3$ μM) was due to a reduction in V_{max} rather than a change in K_t for Hoechst 33342 ($K_t = 0.71$ μM). In agreement with these data, verapamil did not significantly affect Hoechst 33342 binding to purified Sav1866 at concentrations of more than 50-fold the K_d for this dye ($K_d = 0.85$ μM) (Figure 6B). It is interesting to note that K_t and K_d for Hoechst 33342 and K_i for verapamil were in a similar range as the SC_{50} values for the stimulation of Sav1866 ATPase activity by these compounds (Figure 2). Taken together, these results indicate that verapamil and Hoechst 33342 interact at independent sites on Sav1866. Verapamil is a chiral molecule; we used the *R*-isomer in this work. Our observations share interesting similarities with recent findings for human ABCC1 that (*R*)-verapamil can stimulate its ATPase activity and inhibit transport through direct binding to the transporter, resulting in chemosensitization of ABCC1-overexpressing cells to anticancer drugs (*51*). Verapamil is also a well-known inhibitor of ABCB1-associated drug resistance (*52*).

Although the recently crystallized ABC transporter Sav1866 has become one of the paradigms for structural biologists working in the field of ABC (multidrug) transporters, limited biochemical information is available regarding the functionality of Sav1866. As our findings demonstrate for the first time multidrug transport by Sav1866, we conclude that the protein can serve as a well-defined model for detailed studies on the molecular bases of drug−protein interactions in ATP-binding cassette transporters. In addition, our observations raise interesting questions regarding the physiological role of Sav1866.

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