

## Transport of Multidrug Resistance Substrates by the *Streptococcus agalactiae* Hemolysin Transporter

Birgit Gottschalk, Gerd Bröker, Melanie Kuhn, Simone Aymanns,  
Ute Gleich-Theurer, and Barbara Spellerberg\*

Department of Medical Microbiology and Hygiene, University of Ulm, Robert Koch Strasse 8, D-89081 Ulm, Germany

Received 25 May 2005/Accepted 26 May 2006

*Streptococcus agalactiae* (group B streptococcus [GBS]) causes neonatal sepsis, pneumonia, and meningitis, as well as infections of the bovine udder. The *S. agalactiae* hemolysin is regarded as an important virulence factor, and hemolysin expression is dependent on the *cyl* gene cluster. *cylA* and *cylB* encode the ATP binding and transmembrane domains of a typical ATP binding cassette (ABC) transporter. The deduced proteins contain the signature sequence of a multidrug resistance (MDR) transporter, and mutation of the genes results in a nonhemolytic and nonpigmented phenotype. To further elucidate the function of the putative transporter, nonpolar deletion mutants of *cylA* were constructed. These mutants are nonhemolytic and can be complemented by the transporter genes. Wild-type strain and nonhemolytic *cylA* and *cylK* deletion mutants were exposed to known substrates of MDR transporters. Mutation of *cylA* significantly impaired growth in the presence of daunorubicin, doxorubicin, and rhodamine 6G and resulted in a decreased export of doxorubicin from the cells. The mutation of *cylK*, a gene of unknown function located downstream from *cylA*, caused a loss of hemolysis but had no effect on the transport of MDR substrates. Furthermore, the hemolytic activity of the wild-type strain was inhibited by reserpine in a dose-dependent manner. We conclude that CylAB closely resembles an ABC-type MDR transporter and propose that the GBS hemolysin molecule represents a natural substrate of the transporter.

Multidrug resistance (MDR) transporters were first identified in eukaryotic organisms before bacterial homologues were found and characterized (22). In humans they are responsible for the failure of certain chemotherapeutics in cancer treatment. MDR transporters have the ability to extrude a wide variety of amphiphilic substrates with no apparent structural similarity. While the majority of eukaryotic MDR transporters are ATP binding cassette (ABC) transporters, most bacterial MDR transporters are proton-dependent multidrug efflux systems.

Bacterial ABC-type multidrug transporters displaying the typical structure of prokaryotic ABC proteins consist of two genes, encoding a nucleotide binding domain and a transmembrane domain. ABC transporters are one of the largest of all protein families, and almost 5% of the *Escherichia coli* genome codes for this type of transporter (12). In many bacterial genomes putative MDR transporters were found, as for example 34 in the *Enterococcus faecalis* genome (5). Despite this abundance, only a few bacterial ABC-type MDR transporters have been described. LmrA of *Lactococcus lactis* (3), HorA of *Lactobacillus brevis* (25), and MsbA of *E. coli* (13) belong to this group. Natural substrates of these transporters have not been identified in all cases. MsbA encodes the lipid A translocator of *E. coli*, and due to overlapping substrate specificities with MsbA, a role for LmrA in lipid trafficking has been suggested (23), while the natural substrate of HorA remains unknown.

*Streptococcus agalactiae* is a leading cause of sepsis, meningitis, and pneumonia in newborns (15, 32) and is an emerging pathogen in immunocompromised adult patients (9). The  $\beta$ -hemolysin represents an important virulence factor of *S. agalactiae*. Its ability to damage erythrocytes, lung epithelial cells (7), and brain microvascular endothelial cells (8) is regarded as an initial step in invasive disease.

In earlier studies we identified the *cyl* operon in *S. agalactiae*, which is essential for the production of the *S. agalactiae* hemolysin (28) and pigment (26). It contains 12 genes, and the deduced proteins of numerous genes are similar to fatty acid biosynthesis enzymes (*cylD*, *cylG*, *acpC*, *cylZ*, and *cylI*) (Fig. 1A). CylF shows similarities to an aminomethyltransferase, and CylJ carries the conserved domain of a glycosyltransferase. Two of the genes (*cylX* and *cylK*) display no significant similarities to genes of known function in the GenBank database. *cylE* coding for a 78-kDa deduced protein, has been proposed to encode the hemolysin molecule (21). The genes *cylA* and *cylB* display homology to typical ABC transporters, with CylA as the ATP-binding domain and CylB as the transmembrane protein. More precisely, this complex shows significant homology to DrrA, the daunorubicin transporter of *Streptomyces peuceeticus* (11).

The aim of this study was to characterize the function of the genes *cylA* and *cylB* and to identify substrates of the putative transporter they encode.

### MATERIALS AND METHODS

**Bacterial strains and media.** Bacterial strains and plasmids used in this study are listed in Table 1. *S. agalactiae* strain 090R was used for growth curves, construction of mutants, and Western blotting experiments. Strain AC475 was used for reserpine inhibition experiments. *E. coli* strain DH5 $\alpha$  served as a host for construction of recombinant pGHost5 and pAT28 plasmids. *S. agalactiae* strains were cultured on tryptic soy agar supplemented with 5% sheep blood, in Todd-Hewitt broth (THB) (Oxoid, Basingstoke, England), or in THB supple-

\* Corresponding author. Mailing address: Department of Medical Microbiology and Hygiene, University of Ulm, Robert Koch Str. 8, D-89081 Ulm, Germany. Phone: 49 731 50024615. Fax: 49 731 50024619. E-mail: barbara.spellerberg@uniklinik-ulm.de.

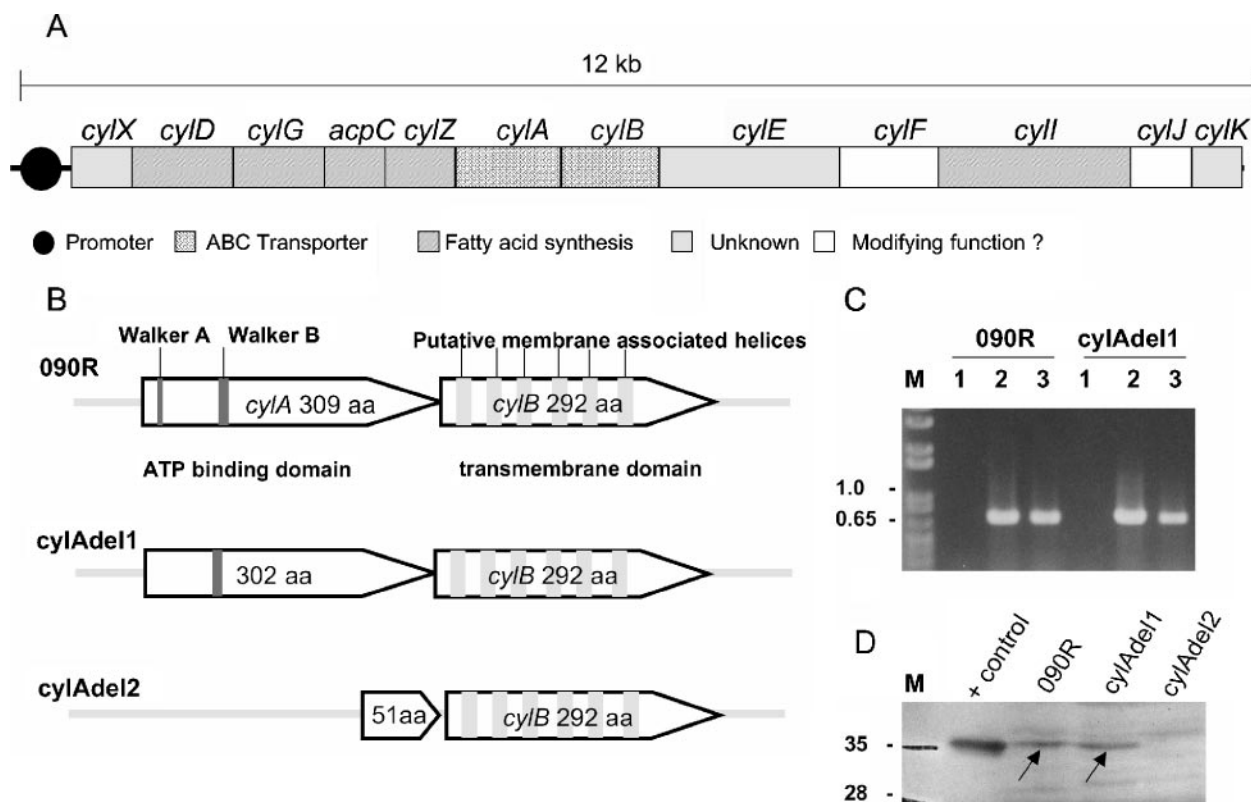


FIG. 1. (A) Genomic map of the *cyl* gene cluster. Depicted are the 12 genes belonging to the cluster. Homologies of the deduced proteins to genes with known function in the GenBank database and the putative promoter region are indicated. Genes that are indicated as open boxes display homologies to genes encoding enzymes such as an aminomethyltransferase (*cylF* annotated in genome sequencing project AAJS01000020 as belonging to protein family PF01571) and a glycosyl transferase (*cylJ* harbors the conserved motif COG1819 of glycosyl transferases), which may modify the structure of an assembled molecule. (B) Graphic representation of the deletion mutants of *cylA*. Indicated are the Walker A and Walker B sites of *cylA* and the six putative transmembrane helices of *cylB* in the wild-type strain. The truncated versions of CylA in both mutants are depicted. (C) Transcription analysis of *cylE* by RT-PCR. Strains 090R and *cylAdel1* were grown to mid-logarithmic phase, and 50 ng of total RNA was used as a template for reverse transcription. The RT primer annealed at nucleotides 765 to 748 of *cylE*, and the subsequent PCR amplified nucleotides 39 to 765 of *cylE* (lane 2). Chromosomal DNA served as a positive control (lane 3), and 50 ng of RNA that had not been subjected to an RT reaction (RT inactivated) was used to control for DNA contamination of RNA samples (lane 1). M, molecular mass marker (kb). (D) Western blot analysis of bacterial membrane fractions with anti-CylA antibodies. *S. agalactiae* wild-type strain 090R and the mutant strains *cylAdel1* and *cylAdel2* were grown to late logarithmic phase in THB supplemented with 1% proteose peptone. Subcellular fractions of all strains were prepared as detailed in Materials and Methods. Western blot analysis was carried out with polyclonal anti-*cylA* (rabbit) antibodies at a dilution of 1:1,000. M, molecular mass marker; lane 1, 2  $\mu$ g of recombinant CylA (+control). For lanes 2 to 4, 40  $\mu$ g of membrane fractions was applied to each lane, as follows: lane 2, 090R; lane 3, *cylAdel1*; lane 4, *cylAdel2*.

mented with 5% yeast extract (THY). To detect pigment production in *S. agalactiae*, strains were cultured on Granada medium (6). Streptococcal mutant strains with chromosomally integrated pGhost5 plasmids were cultured in medium with 1 mg/liter erythromycin, and strains harboring recombinant pAT28 plasmids were cultured on THY plates supplemented with 120 mg/liter spectinomycin. To analyze the influence of MDR substrates on the growth of the strains *cylAdel1*, *cylKdel*, and 090R, overnight cultures in THY broth were diluted 1:100 in fresh THY medium supplemented with various amounts of the indicated substrates. Doxorubicin, daunorubicin, rhodamine 6G, and benzalkonium chloride were obtained from Sigma (Deisenhofen, Germany). Growth was determined by measurement of the optical density at 600 nm ( $OD_{600}$ ).

**General molecular biology techniques.** Standard recombinant DNA techniques were employed for nucleic acid preparation and analysis. PCR was carried out with *Taq* polymerase (Roche Diagnostics, Mannheim, Germany) at amplification steps of 94°C (1 min), 50 to 58°C (1 min), and 72°C (1 to 3 min, depending on product size) for 35 cycles. Genomic streptococcal DNA was isolated as previously described (20). Plasmid DNA was isolated and purified with a Qiaprep Spin Miniprep kit (QIAGEN, Hilden, Germany). PCR products were sequenced on an ABI 310 automated DNA sequencer using the ABI PRISM Big-Dye terminator cycle sequencing kit (Applied Biosystems, Weiterstadt, Germany). Transformation of *S. agalactiae* was carried out according to an

established protocol (24). Southern blot hybridization analysis was performed as previously described (10).

**Construction of mutants.** The plasmid pGhost5 (2) was used for construction of deletion mutants in strain 090R. For construction of *cylAdel1*, genomic regions adjacent to the deletion site were amplified with the following primers (restriction sites are underlined): 5'-GGCGGCGGATCCCTTGATGAATTAA CAGCAGAG-3' and 5'-GGCGGC CTCGAGAAGAAGGCCATAAAATTTT CC-3' (region upstream of the deletion site); 5'-GGCGGCCTCGAGACAAC TTAATTAACCTTTAATTC-3' and 5'-GGCGGCGGAATTCATTAGTACAA CTGTCATCTGCG-3' (region downstream of the deletion site). Amplified PCR products were subcloned into pGhost5 in *E. coli*. The resulting recombinant plasmid was transformed into strain 090R at 30°C. Integration into the chromosome was carried out via insertion duplication mutagenesis by a temperature shift to  $\geq 37^\circ\text{C}$ . To construct deletion mutants by mobilization of the integrated plasmid, the insertion mutant was passaged five times overnight at 30°C in THY without antibiotic pressure and subsequently plated on blood agar plates. Susceptibility to erythromycin was tested by subculturing single colonies on blood agar plates with or without erythromycin (1 mg/liter).

The strains *cylAdel2* and *cylKdel* were created as described above. The fragment upstream of the deletion site in *cylAdel2* was amplified with the primers 5'-GGAATTCCTAATGGTGGATAGAGTGC-3' and 5'-CCCATCCACTAAA

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
<i>E. coli</i> strains		
DH5 $\alpha$	$\lambda^- \phi 80\text{dlacZ}\Delta M15 \Delta(\text{lacZYA-argF})U169 \text{recA1 endA1 hsdR17}(r_k^- m_k^-)$ <i>supE44 thi-1 gyrA relA1</i>	
BL21(DE3)	F <sup>-</sup> <i>ompT hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal dcm</i> (DE3); T7 polymerase gene under control of the <i>lacUV5</i> promoter	Boehringer, Novagen
<i>S. agalactiae</i> strains		
O90R (ATCC 12386)	R. Lancefield grouping strain	ATCC collection
AC475	Clinical isolate serotype III	Aachen collection
cylAde11	O90R derivative; $\Delta$ Walker A motif of <i>cylA</i>	This study
cylAde12	O90R derivative; $\Delta$ <i>cylA</i>	This study
CylKdel	O90R derivative; $\Delta$ <i>cylK</i>	This study
cylAde11 comp1	O90R derivative; $\Delta$ Walker A motif of <i>cylA</i> carrying pBSU224	This study
cylAde12 comp1	O90R derivative; $\Delta$ <i>cylA</i> carrying pBSU224	This study
cylAde11 comp2	O90R derivative; $\Delta$ Walker A motif of <i>cylA</i> carrying pBSU362	This study
cylAde12 comp2	O90R derivative; $\Delta$ <i>cylA</i> carrying pBSU301	This study
Plasmids		
pGhost5	Ery <sup>r</sup> ; ori pBR; ori Ts	Appligene
pAT28	Spec <sup>r</sup> ; oripUC; ori pAmB1	30
pBSU224	pAT28 derivative carrying the complete coding region of <i>cylA</i> and <i>cylB</i>	This study
pBSU362	pAT28 derivative carrying the complete coding region of <i>cylA</i>	This study
pBSU301	pAT28 derivative carrying the complete coding region of <i>cylE</i>	This study
pBSU52	pET21a vector carrying the coding region of <i>cylA</i>	This study

CTTAAACAGAGATTAATGTCTCTCAATGC-3'; the downstream fragment of the deletion site was amplified with the primers 5'-TGTTTAAGTTTAGTGATGGGATTCGAACCTAGAGGCGG-3' and 5'-CCGCGGATCCCTGAATCTCTAGCAAGG-3'. In strain *cylKdel*, primers 5'-TGCCACAAGCTTTTAAACGAGATTGGGTGG-3' and 5'-CCCATCCACTAAACTTAAACAATCTCATCAACCGATCGC-3' were used for the fragment upstream of the deletion, and primers 5'-TGTTTAAGTTTAGTGATGGGAGTTAGTAGTGATATGG-3' and 5'-CCGCGGATCCCTCACATTACTTAAACCG G-3' were used for the downstream region. For each strain, correct deletion of the targeted chromosomal region was confirmed by DNA sequencing of the deletion site and Southern blot analysis.

The genes *cylA* and *cylE* were amplified with the primers 5'-GGCGGCCTCGAGGTTGCCTCAGGAAGGATG-3' and 5'-GGCGGCGGATCCACGCTAACCGATCTCGCGTG-3' and the primers 5'-GGAATTCATTACTAGTTCAGC-3' and 5'-CCGCGGATCCCTCAGCATTTTACTCG-3', respectively. For complementation studies with both transporter genes, *cylA* and *cylB* were amplified with the primers 5'-GGCGGCGAGTTCGAGGTTGCCTCAGGAAGGATG-3' and 5'-GGCGGCGGATCCCTAATGTTGTAAGGAAGCTTCAG-3' and subcloned in pAT28 (30). The recombinant plasmid was constructed in *E. coli* DH5 $\alpha$  and subsequently transformed into strains *cylAde11* and *cylAde12*.

For expression of *cylA* as a C-terminal histidine-tagged protein, *cylA* was amplified with the primers 5'-GGCGGCATATGGAATTAACCTCAAAAATATTGG-3' and 5'-GGCGGCCTCGAGAACATTGTTTCTCTTTCTTTTCTCTC-3' and subcloned into the vector pET21a in *E. coli* strain DH5 $\alpha$ . For protein expression, the plasmid was transferred into *E. coli* strain BL21(DE3).

**Detection of MDR substrate export.** Transport of the polyketide doxorubicin from *S. agalactiae* cells was measured by a method adapted from Nishino and Yamaguchi (19). To load the cells with the polyketides, *S. agalactiae* wild-type strain O90R and the *cylAde11* mutant strain were grown to equal optical densities at late exponential phase in medium supplemented with 0.5 mg/liter of doxorubicin, harvested by centrifugation, and washed in phosphate-buffered saline (PBS). Cells of a 30-ml culture were resuspended in 2 ml of PBS containing 100 mM glucose as an energy source. To measure release of the polyketides into the supernatant, aliquots of the suspension were removed at different time points (0, 2, 5, and 10 min) and subjected to centrifugation, and fluorescence of the supernatant was quantified in a fluorescence counter (Cytofluor II; Perseptive Biosystems, Inc.). Doxorubicin fluorescence was detected with an excitation at 485 and an emission at 590 nm.

**Expression of recombinant CylA.** For the expression of histidine-tagged CylA protein, *E. coli* BL21(DE3) harboring the respective pET21a plasmid was grown to an OD<sub>600</sub> of 0.6 at 30°C in Luria-Bertani broth. Protein expression was induced by the addition of 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside for 3 h at

30°C. Bacterial cells were harvested by centrifugation, resuspended in lysis buffer B (8 M urea, 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, 0.01 M Tris Cl [pH 8.0]), placed on ice for 30 min, and disrupted by sonication. Recombinant CylA was purified from the cell lysate by passage over a commercial nickel affinity matrix (Ni-NTA Spin kit; QIAGEN) and eluted under denaturing conditions.

**Western immunoblot analysis.** Polyclonal rabbit antibodies directed against CylA were generated by Eurogentec (Brussels, Belgium). Bacterial cells were grown to late logarithmic phase in THB supplemented with 1% proteose peptone 3 (Beckton Dickinson, Heidelberg, Germany) and 5% fetal calf serum. Subcellular fractions were prepared from strains O90R, *cylAde11*, and *cylAde12* as previously described (14). Samples of cell wall fractions, cytoplasmic membrane fractions, and cytoplasm containing 40  $\mu$ g of protein each were separated by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 12% gel and transferred to an Immobilon P polyvinylidene difluoride membrane (Millipore, Eschborn, Germany). The blots were probed with the polyclonal anti-CylA antibody at a dilution of 1:1,000. Controls were probed with preimmune rabbit serum at a dilution of 1:1,000. An alkaline phosphatase-labeled anti-rabbit immunoglobulin G antibody was used as secondary antibody at a dilution of 1:5,000. Bound secondary antibody was visualized by the chemiluminescent CSPD (Roche, Mannheim, Germany).

**RNA preparation and analysis.** Total RNA was prepared from strains O90R and *cylAde11* grown to mid-logarithmic phase in THY broth and treated with RNAProtect bacteria reagent (QIAGEN) according to the manufacturer's instructions. Bacterial cells were lysed in a cell disrupter (Fastprep; QBiogene, Heidelberg, Germany) in the presence of RNeasy lysis buffer (QIAGEN). Subsequent purification of RNA was carried out with an RNeasy minikit (QIAGEN). For reverse transcription-PCR (RT-PCR), experiments 50 ng of DNase-treated RNA was used as a template in an RT reaction. RT-PCR experiments were carried out with the QIAGEN One Step RT-PCR kit (QIAGEN). The primer 5'-CAGTTTTAACCCGAACCC-3' was used as the RT primer, and the primer combination 5'-CTTAGAAGATTATCTGAAGTGG-3' and 5'-CAGTTTTAACCCGAACCC-3' was used for the subsequent PCR.

**Nucleotide sequence of *cylE*.** The nucleotide sequence of *cylE* was amplified from genomic DNA of strain *cylAde11* with the primers 5'-GTCTTAATGAAGGGTTCTATTGC-3' and 5'-GTAGCCACAAGTATC TGTATCTGC-3'. In addition to the amplification primers, the primers 5'-CTTAGAAGATTATCTGAAGTGG-3', 5'-AATGGCTTCAAAGAGTATG-3', 5'-CCTAGCCTAACTTTAAGTTCG-3', and 5'-ATCATGACATTTACAAGTGACG-3' were used to sequence the purified PCR product.

**Hemolysin assay.** Bacterial cells from a fresh overnight culture were inoculated into 15 ml of fresh culture medium and grown at 37°C for 5 h. Cells were harvested by centrifugation and washed in PBS. The OD of each sample was



measured at 600 nm and adjusted to equal values in order to compensate for minimal differences in growth kinetics between the samples. Hemolytic activity was extracted from bacterial cells by a modification of the method described by Marchlewicz and Duncan (17). After centrifugation, the bacterial pellet was resuspended in 600  $\mu$ l of hemolysin extraction buffer (PBS, 1% starch, 1% glucose, 3% Tween 80) and incubated for 5 min at 37°C. After centrifugation, the supernatant was transferred to a new tube and stored on ice. For the determination of hemolytic activity, 15 to 100  $\mu$ l of the extract was added to a 1% erythrocyte suspension in 1 ml of PBS and incubated for 10 min at 37°C. Hemolysis was detected by release of hemoglobin from erythrocytes. Hemoglobin was measured by the OD<sub>540</sub> after centrifugation of the sample.

## RESULTS

**Transporter gene deletion mutants.** In our previous work we described several mutants of *cylA* and *cylB* that result in a nonhemolytic phenotype (27, 28). These mutants were targeted pGhost5 insertion mutants as well as naturally occurring nonhemolytic strains harboring insertion elements in *cylA* or *cylB*. To exclude the possibility of downstream polar effects on hemolysin expression, two different deletion mutants of *cylA* were constructed by insertion of the vector pGhost5 into *cylA* and subsequent mobilization of the integrated plasmid. One mutation comprises the Walker A motif generating a strain (*cylAdel1*) with a deletion of amino acids 35 to 42 of CylA. In the second strain (*cylAdel2*), amino acids 24 to 289 of the 309 residues of CylA are deleted (Fig. 1B). Both strains have a nonhemolytic and nonpigmented phenotype (Fig. 2).

***cylE* transcription and nucleotide sequence in the transporter deletion mutants.** *cylE*, the gene immediately downstream of *cylB*, has been proposed to encode the structural hemolysin of *S. agalactiae* (21). To ensure that a deletion of *cylA* does not impair the transcription of *cylE*, we performed RT-PCR experiments with strain *cylAdel1*. The transcription of *cylE* was unaltered compared to the wild-type strain (Fig. 1C). In addition, nonspecific mutations of *cylE* in the *cylA* mutant strain were excluded by sequencing of *cylE*.

**Complementation studies.** To investigate whether the *cylA* deletion mutants can be complemented in *trans*, the coding region for CylA was amplified and subcloned into the plasmid pAT28. Transfer of this construct into the strains *cylAdel1* and *cylAdel2* resulted in a weak reconstitution of hemolysis for both strains (Fig. 2A). Further complementation studies were conducted with a recombinant pAT28 vector harboring the genes *cylA* as well as *cylB* in *trans*, resulting in a clearly visible hemolysis of both mutant strains (Fig. 2B). As a control, the strains were transformed with a plasmid harboring *cylE*, which failed to have any effect on the nonhemolytic phenotype (Fig. 2A). These results confirm the requirement of CylAB for  $\beta$ -hemolysis of *S. agalactiae*.

**Hemolytic activity following cell lysis.** In *E. coli* intracellular hemolytic activity in nonhemolytic transporter mutants can be released by sonication (16). To investigate if the *cylA* mutant strains harbor intracellular hemolytic activity, strain *cylAdel1* was grown to late logarithmic phase. Bacterial cells were disrupted with glass beads and subsequently subjected to hemolysin extraction. Hemolysin extracts of cellular fragments as well as hemolysin extracts from nondisrupted *cylAdel1* cells were, however, nonhemolytic (data not shown).

**Susceptibility to classical MDR substrates.** Analysis of the deduced protein sequence of *cylA* with the conserved domain search at the NCBI website reveals that it contains the sequence COG1131. COG1131 is a motif identified in the clus-

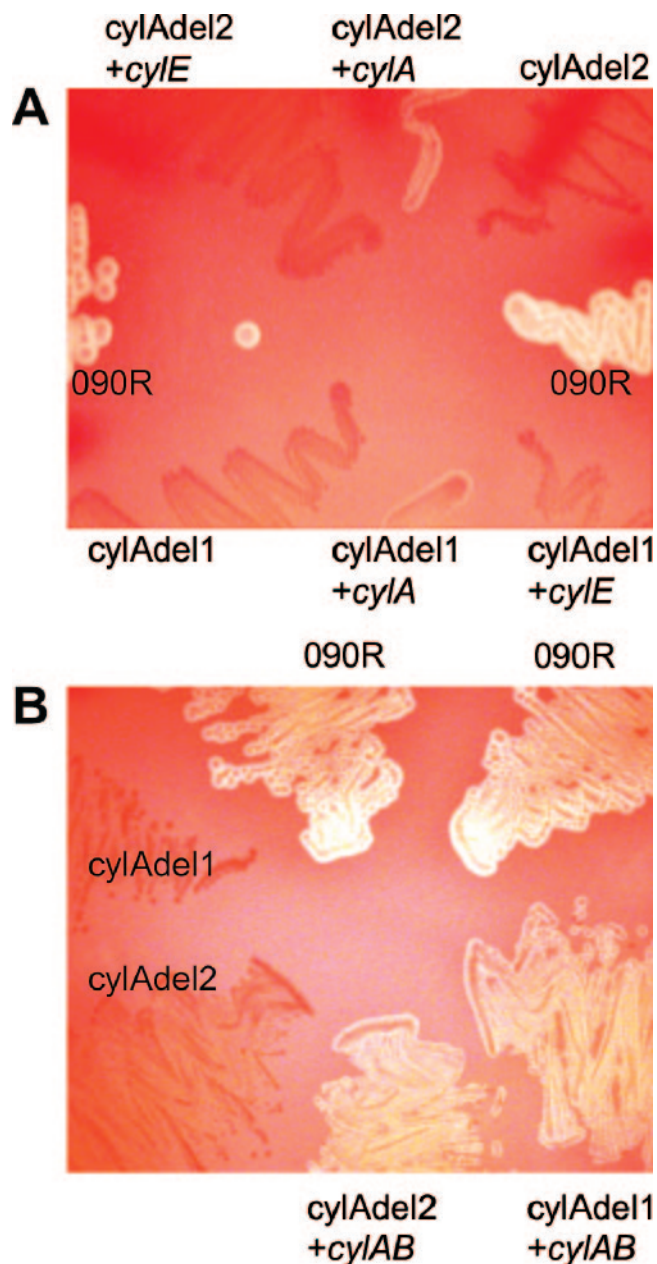


FIG. 2. Phenotype of *cylA* deletion mutants and complemented strains on sheep blood agar plates. (A) Complementation studies with a plasmid harboring *cylA* or *cylE* in *trans* as indicated. (B) Strains complemented with a plasmid harboring *cylAB* in *trans*. Strain designations are indicated: 090R, wild-type strain; *cylAdel1*, *cylA* mutant with deleted Walker A motif; *cylAdel2*, *cylA* deletion as depicted in Fig. 1B.

ters of orthologous groups (COG) database (29). It is specific for the ATPase component of ABC-type multidrug transport systems. To investigate if classical MDR substrates can be transported via CylAB, we measured the growth of the wild-type strain 090R and the *cylAdel1* mutant in the presence of daunorubicin, doxorubicin, rhodamine 6G, and benzalkonium chloride. Significant effects on the growth kinetics were observed for cultures containing daunorubicin, doxorubicin, and

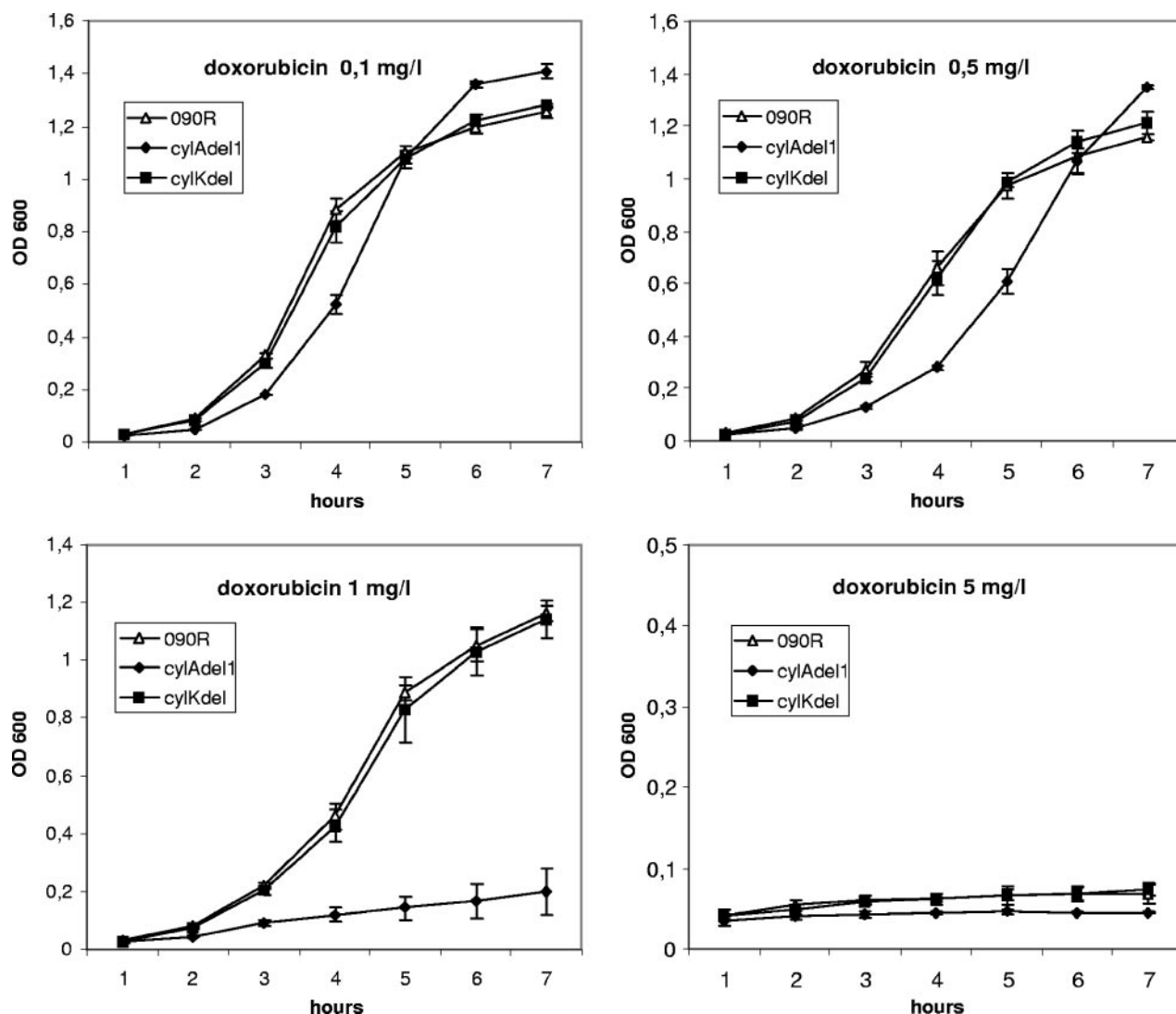


FIG. 3. Growth curves of the 090R wild-type strain as well as the nonhemolytic strains *cylAdel1* and *cylKdel* in the presence of the MDR substrate doxorubicin. Bacteria were inoculated into THB from a fresh overnight culture, and growth was determined by the measurement of the OD<sub>600</sub> for 7 h. Depicted are mean values and standard deviations of three independent experiments.

rhodamine 6G. In comparison to the 090R wild-type strain, growth of the mutant strain *cylAdel1* was significantly impaired in the presence of 1 mg/liter doxorubicin (Fig. 3), 0.5 mg/liter and 1 mg/liter daunorubicin (Fig. 4), and 10 mg/liter rhodamine 6G (data not shown). The increased susceptibility of the strain was limited to specific MDR substrates. For benzalkonium chloride, no increased sensitivity of *cylAdel1* could be observed at concentrations of 1, 3, and 5 mg/liter (data not shown). Growth of the wild-type and mutant strains was unaffected at 1 mg/liter and completely inhibited at 5 mg/liter benzalkonium chloride.

To exclude the possibility that the effects of MDR substrates on strain *cylAdel1* are caused by the nonhemolytic phenotype of the strain, a *cylK* deletion strain (*cylKdel*) served as a control. The strain is nonhemolytic due to a deletion of amino acids 22 to 115 of CylK. The gene is located more than 6.3 kb downstream of the stop codon of *cylB*, and the deduced protein consists of 178 amino acids with no significant homology

to proteins of known function in the GenBank database. Growth of *cylKdel* was indistinguishable from the parent strain 090R (Fig. 3 and 4) for all substrates and concentrations tested. Our data indicate that CylAB can function as a transporter for the MDR substrates daunorubicin, doxorubicin, and rhodamine 6G.

**Export of doxorubicin.** To further investigate the hypothesis that mutation of the transporter CylAB results in an inability to export toxic MDR substrates, strains 090R and *cylAdel1* were loaded with subinhibitory concentrations of doxorubicin. Bacterial cells were resuspended in buffer containing glucose as an energy source, and the kinetics of doxorubicin export from bacterial cells into the supernatant was measured fluorometrically. Compared to the wild-type strain, the mutant strain displayed a markedly reduced capacity to export doxorubicin (Fig. 5).

**Hemolysis in the presence of reserpine.** Based on the detection of the ATP-type MDR transporter motif COG1131 in the

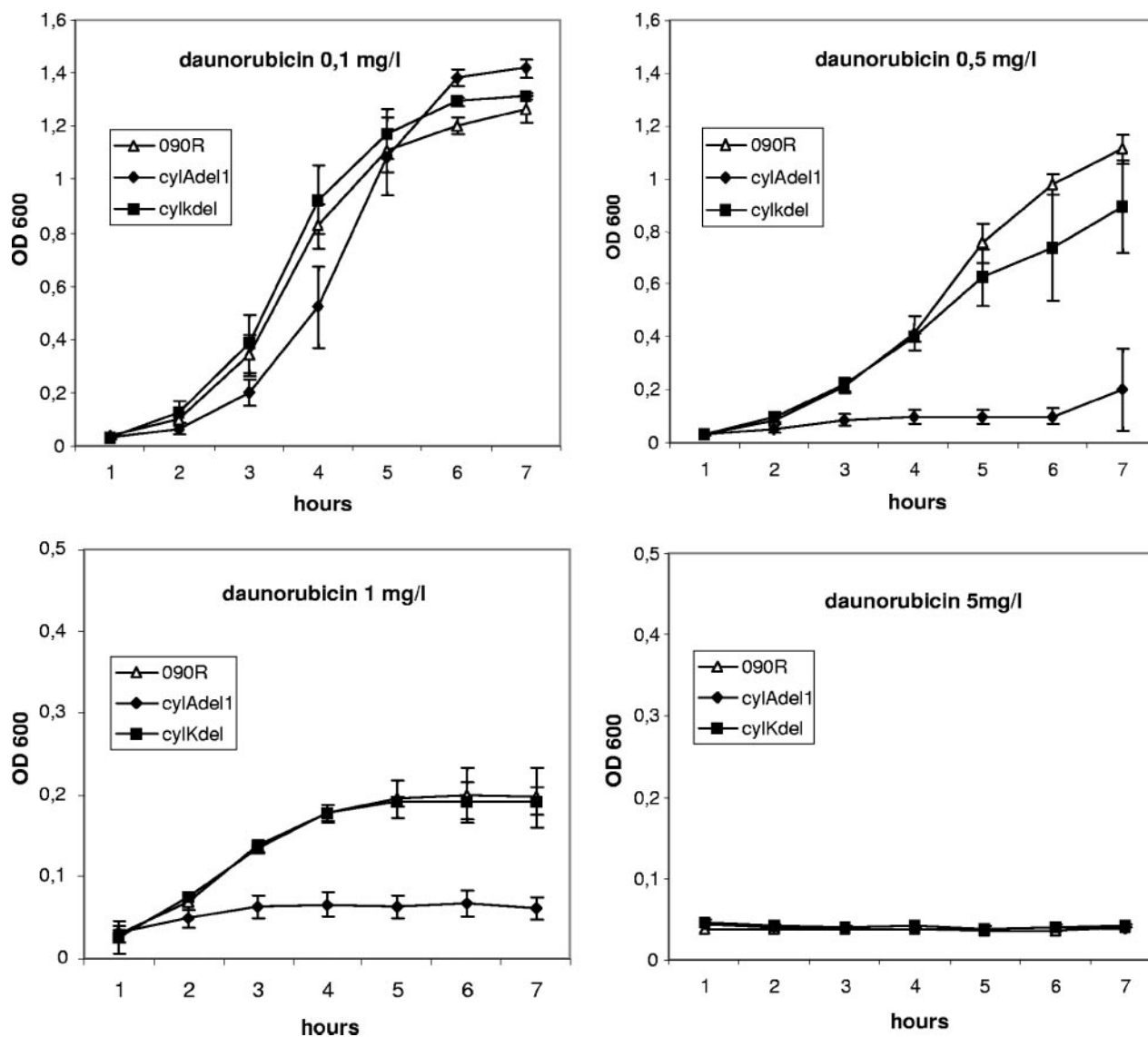


FIG. 4. Growth curves of the 090R wild-type strain as well as the nonhemolytic strains *cylAdel1* and *cylKdel* in the presence of the MDR substrate daunorubicin. Bacteria were inoculated into THB from a fresh overnight culture, and growth was determined by the measurement of the OD<sub>600</sub> for 7 h. Depicted are mean values and standard deviations of three independent experiments.

amino acid sequence of CylA, the increased sensitivity of the *cylAdel1* mutant to MDR substrates, and the reduced transport of doxorubicin in *cylAdel1*, we hypothesized that *cylA* and *cylB* code for a transporter resembling ABC-type MDR transporters. To further substantiate our hypothesis, we analyzed the hemolytic activity of hemolysin extracts after growth of the wild-type strain AC475 in the presence of 0 to 25 mg/liter reserpine. Reserpine is a well-known inhibitor of MDR transporters and growth in the presence of reserpine resulted in a dose-dependent decrease of extractable hemolytic activity (Fig. 6).

**Subcellular localization of the transporter.** Antibodies against recombinant CylA were used to analyze subcellular fractions of *S. agalactiae* strain 090R and the deletion mutants *cylAdel1* and *cylAdel2* by Western blotting. A positive reaction with the antibody could be observed for the membrane fraction

of the wild-type strain and the *cylAdel1* deletion mutant (Fig. 1D), which carries a *cylA* gene lacking the Walker A motif. No CylA protein was detectable in the membrane fraction of *cylAdel2* (Fig. 1D). Analysis of the cytoplasm fraction, the cell wall fraction, and the culture supernatant did not reveal the presence of any CylA-specific bands in wild-type and mutant strains (data not shown).

## DISCUSSION

Based on amino acid homologies, CylAB encodes the ATP binding and the transmembrane component of an ABC transporter. The genes are located in the *cyl* gene cluster that is required for  $\beta$ -hemolysin expression in *S. agalactiae*. Involvement of both genes in the production of  $\beta$ -hemolysin has been suggested by the analysis of various nonhemolytic insertion

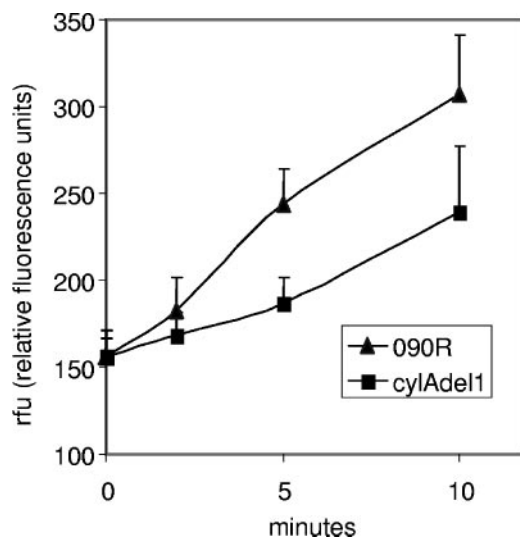


FIG. 5. Export studies of doxorubicin from *S. agalactiae* wild-type strain 090R and *cylA* mutant strain cylAdel1. Bacterial cells were grown in the presence of doxorubicin (0.5 mg/liter). Export of doxorubicin into the supernatant was determined fluorometrically as detailed in Materials and Methods. Mean values and the standard deviation of eight independent experiments are shown.

mutants of *cylA* and *cylB* (27, 28). To further investigate the role of these genes for the expression of  $\beta$ -hemolysis and to rule out polar effects we constructed two nonpolar deletion mutants of the putative transporter. The mutants display a nonhemolytic phenotype which can be restored by a plasmid harboring *cylAB* in *trans*. Partial reconstitution of the beta-hemolytic phenotype was observed after complementation with *cylA* (Fig. 2). The partial reconstitution that was observed for complementation experiments with *cylA* alone may be explained by translational coupling of the genes *cylA* and *cylB*. Polar effects on genes further downstream could be ruled out by a plasmid harboring *cylE* which failed to complement the nonhemolytic mutant strains. Furthermore, RT-PCR analysis and sequencing of *cylE* did not give any indication that other genes of the *cyl* cluster are responsible for the observed effects. However, conflicting results have been obtained regarding the phenotype of insertion mutants of *cyl* transporter genes by other groups. Pritzlaff et al. reported that *cylB* insertion mutants display a regular hemolytic phenotype. This observation seems to contradict our finding that naturally occurring *cylB* insertion mutants (27) as well as a targeted disruption of the *cylB* gene (28) result in a loss of hemolytic activity. The hemolytic phenotype of the transporter mutant Pritzlaff described might be explained by the fact that it harbors an insertion at codon 265 of the 292-amino-acid-long CylB protein (21). It is possible that the expression of a slightly truncated CylB protein does not interfere with the function of the transporter.

The presence of a conserved protein motif for an ABC-type MDR transporter (COG1131) in the deduced protein sequence of *cylA* and the homology of CylB to the daunorubicin transporter of *Streptomyces peuceticus* prompted us to investigate the effect of a CylAB transporter mutation on the extrusion of classical MDR substrates. MDR proteins transport multiple, structurally dissimilar compounds that are character-

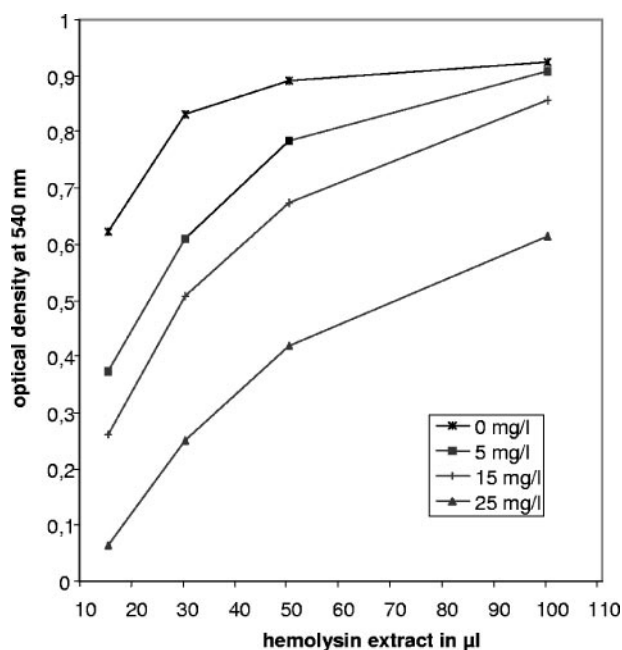


FIG. 6. Effect of reserpine on the hemolytic activity of the wild-type strain AC475. Bacteria were grown in the presence of 0 to 25 mg/liter reserpine to late logarithmic phase and subjected to hemolysin extraction as detailed in Materials and Methods. Hemolysin extracts were incubated with a 1% erythrocyte suspension in PBS for 15 min at 37°C. To detect hemolysis, hemoglobin release was measured as the OD<sub>540</sub> of the supernatant. Depicted are mean values of five independent experiments.

ized by common physicochemical characteristics such as high hydrophobicity, an amphiphilic nature, and a positive or neutral charge. Deletion of the Walker A motif in *cylA* resulted in an increased sensitivity of the strain to the MDR substrates daunorubicin, doxorubicin, and rhodamine 6G (Fig. 3 and 4). The increased sensitivity to MDR substrates is probably due to a failure of the mutant strain to export the toxic compounds to the same extent as the wild-type strain. Further experiments showed a decreased export of doxorubicin from the bacterial cells and support this interpretation of our results (Fig. 5). Genome sequencing projects of various streptococcal species demonstrated multiple putative MDR transporters in each streptococcal genome, a fact that may explain why the mutation of one transporter does not lead to a complete loss of MDR substrate transport. To test our hypothesis that CylAB closely resembles MDR transporters, we investigated if the hemolytic activity of an *S. agalactiae* wild-type strain can be inhibited by reserpine and found a dose-dependent decrease of extractable hemolytic activity (Fig. 6). Reserpine is a well-known inhibitor of bacterial MDR transporters. It inhibits the MDR transporter Bmr of *Bacillus subtilis* (1) and NorA of *Staphylococcus aureus* (18) at concentrations we tested in our hemolysin assay. Based on these data, we conclude that the hemolysin transporter of *S. agalactiae* is capable of transporting MDR substrates.

Although MDR transporters extrude a wide variety of structurally dissimilar compounds and CylE of *S. agalactiae* has been proposed to encode the structural hemolysin (21), the transport of a 78-kDa protein by this type of transporter seems



unlikely. However, the hypothesis that CylE represents the hemolysin was only one of the explanations offered in the paper by Pritzlaff et al. The failure to induce antibodies against the *S. agalactiae* hemolysin in repeated attempts (4) would also argue against a large protein. Moreover, the presence of CylE has not been demonstrated in either hemolytic *S. agalactiae* extracts or the membrane of erythrocytes, nor does the deduced protein itself display any similarities to other characterized hemolysin molecules. Taken together, the data that were generated by the different groups could better be explained by a model in which *cylE* participates in the synthesis of the hemolysin rather than representing the hemolysin itself. Based on the fact that the transporter displays characteristic features of MDR transporters, it is tempting to speculate that the natural substrate of CylAB possesses certain structural similarities with classical MDR substrates. While the genes present in the *cyl* gene cluster do not represent a classical polyketide synthesis cluster, many genes resemble fatty acid synthesis genes that are also essential for polyketide synthesis. The data of the different *S. agalactiae* genome projects show that *S. agalactiae* has regular fatty acid synthesis genes apart from the *cyl* genes. This might explain why a mutation of *cyl* genes is not lethal for *S. agalactiae* and supports the theory that these genes participate in another biosynthetic pathway.

The *S. agalactiae* hemolysin is associated with the bacterial cell surface, from where it can be extracted by mild detergents. It is not released into the culture supernatant under regular growth conditions (17). To further investigate the transporter function of CylAB, we tried to extract hemolytic activity from the transporter mutants *cylAdel1* and *cylAdel2* after cellular disruption. In *E. coli*, sonication of nonhemolytic transporter mutants leading to a disruption of bacterial cells results in the recovery of intracellular hemolytic activity (16). Cell disruption of our transporter mutants, however, did not result in the detection of any extractable hemolytic activity. A possible explanation is provided by the finding that MDR transporters mediate the extrusion of lipophilic compounds from the inner leaflet of the plasma membrane to the outside of the cell rather than just representing an outlet for cytosolic compounds to the extracellular environment. For *LmrA* of *L. lactis*, it has been shown that hydrolysis of ATP results in the transfer of the MDR substrate from a high-affinity drug binding site at the inner membrane surface to a low-affinity drug binding site on the outer membrane surface (31). We think that a simple destruction of the cellular integrity of *S. agalactiae* cannot substitute for this complex extrusion mechanism. Another possible explanation could be that modifications of the hemolysin molecule are made during or after export to the bacterial cell surface and that no functional hemolysin molecule is present in the cytosolic compartment.

*CylA* deletion mutants of *S. agalactiae* are nonhemolytic and can be complemented by the transporter genes. Mutations of the transporter lead to an increased sensitivity to typical MDR substrates and a decreased export of doxorubicin. Furthermore, hemolytic activity of *S. agalactiae* is decreased in the presence of a classical MDR-transport inhibitor. Based on these data, we conclude that CylAB closely resembles ABC-type MDR transporters and propose that the *S. agalactiae* hemolysin molecule represents a natural substrate of the transporter.

## ACKNOWLEDGMENTS

The work of B.S. and M.K. was supported by DFG grant Sp511/4-1. We thank Simone Martin and Claudia Reichardt for expert technical assistance.

## REFERENCES

- Ahmed, M., C. M. Borsch, A. A. Neyfakh, and S. Schuldiner. 1993. Mutants of the *Bacillus subtilis* multidrug transporter Bmr with altered sensitivity to the antihypertensive alkaloid reserpine. *J. Biol. Chem.* **268**:11086–11089.
- Biswas, I., A. Gruss, D. Ehrlich, and E. Maguin. 1993. High-efficiency gene inactivation and replacement system for gram-positive bacteria. *J. Bacteriol.* **175**:3628–3635.
- Bolhuis, H., D. Molenaar, G. Poelarends, H. W. van Veen, B. Poolman, A. J. Driessen, and W. N. Konings. 1994. Proton motive force-driven and ATP-dependent drug extrusion systems in multidrug-resistant *Lactococcus lactis*. *J. Bacteriol.* **176**:6957–6964.
- Dal, M. C., and H. Monteil. 1983. Hemolysin produced by group B *Streptococcus agalactiae*. *FEMS Microbiol. Lett.* **16**:89–94.
- Davis, D. R., J. B. McAlpine, C. J. Pazoles, M. K. Talbot, E. A. Alder, C. White, B. M. Jonas, B. E. Murray, G. M. Weinstock, and B. L. Rogers. 2001. *Enterococcus faecalis* multi-drug resistance transporters: application for antibiotic discovery. *J. Mol. Microbiol. Biotechnol.* **3**:179–184.
- De la Rosa, M., R. Villareal, D. Vega, C. Miranda, and A. Martinzbocral. 1983. Granada medium for the detection and identification of group B streptococci. *J. Clin. Microbiol.* **18**:779–785.
- Doran, K. S., J. C. Chang, V. M. Benoit, L. Eckmann, and V. Nizet. 2002. Group B streptococcal beta-hemolysin/cytolysin promotes invasion of human lung epithelial cells and the release of interleukin-8. *J. Infect. Dis.* **185**:196–203.
- Doran, K. S., G. Y. Liu, and V. Nizet. 2003. Group B streptococcal beta-hemolysin/cytolysin activates neutrophil signaling pathways in brain endothelium and contributes to development of meningitis. *J. Clin. Investig.* **112**:736–744.
- Farley, M. 1995. Group B streptococcal infection in older patients. Spectrum of disease and management strategies. *Drugs Aging* **6**:293–300.
- Franken, C., G. Haase, C. Brandt, J. Weber-Heynemann, S. Martin, C. Lammler, A. Podbielski, R. Luttkicken, and B. Spellerberg. 2001. Horizontal gene transfer and host specificity of beta-haemolytic streptococci: the role of a putative composite transposon containing *scpB* and *lmb*. *Mol. Microbiol.* **41**:925–935.
- Guilfoile, P. G., and C. R. Hutchinson. 1991. A bacterial analog of the *mdr* gene of mammalian tumor cells is present in *Streptomyces peucetius*, the producer of daunorubicin and doxorubicin. *Proc. Natl. Acad. Sci. USA* **88**:8553–8557.
- Holland, B., S. Cole, K. Kuchler, and C. Higgins. 2003. ABC proteins from bacteria to man. Academic Press, San Diego, Calif.
- Karow, M., and C. Georgopoulos. 1993. The essential *Escherichia coli* *msbA* gene, a multicopy suppressor of null mutations in the *htrB* gene, is related to the universally conserved family of ATP-dependent translocators. *Mol. Microbiol.* **7**:69–79.
- Kling, D. E., L. C. Madoff, and J. L. Michel. 1999. Subcellular fractionation of group B *Streptococcus*. *BioTechniques* **27**:24–6:28.
- Luck, S., M. Tornay, K. d'Agapeyeff, A. Pitt, P. Heath, A. Breathnach, and A. B. Russell. 2003. Estimated early-onset group B streptococcal neonatal disease. *Lancet* **361**:1953–1954.
- Mackman, N., and I. B. Holland. 1984. Functional characterization of a cloned haemolysin determinant from *E. coli* of human origin, encoding information for the secretion of a 107K polypeptide. *Mol. Gen. Genet.* **196**:129–134.
- Marchlewicz, B. A., and J. L. Duncan. 1980. Properties of a hemolysin produced by group B streptococci. *Infect. Immun.* **30**:805–813.
- Neyfakh, A. A., C. M. Borsch, and G. W. Kaatz. 1993. Fluoroquinolone resistance protein *NorA* of *Staphylococcus aureus* is a multidrug efflux transporter. *Antimicrob. Agents Chemother.* **37**:128–129.
- Nishino, K., and A. Yamaguchi. 2001. Overexpression of the response regulator *evgA* of the two-component signal transduction system modulates multidrug resistance conferred by multidrug resistance transporters. *J. Bacteriol.* **183**:1455–1458.
- Pospiech, A., and B. Neumann. 1995. A versatile quick-prep of genomic DNA from gram-positive bacteria. *Trends Genet.* **11**:217–218.
- Pritzlaff, C. A., J. C. Chang, S. P. Kuo, G. S. Tamura, C. E. Rubens, and V. Nizet. 2001. Genetic basis for the beta-haemolytic/cytolytic activity of group B *Streptococcus*. *Mol. Microbiol.* **39**:236–247.
- Putman, M., H. W. van Veen, and W. N. Konings. 2000. Molecular properties of bacterial multidrug transporters. *Microbiol. Mol. Biol. Rev.* **64**:672–693.
- Reuter, G., T. Janvilisri, H. Venter, S. Shahi, L. Balakrishnan, and H. W. van Veen. 2003. The ATP binding cassette multidrug transporter *LmrA* and lipid transporter *MsbA* have overlapping substrate specificities. *J. Biol. Chem.* **278**:35193–35198.



24. Ricci, M. L., R. Manganelli, C. Berneri, G. Orefici, and G. Pozzi. 1994. Electrotransformation of *Streptococcus agalactiae* with plasmid DNA. FEMS Microbiol. Lett. **119**:47–52.
25. Sakamoto, K., A. Margolles, H. W. van Veen, and W. N. Konings. 2001. Hop resistance in the beer spoilage bacterium *Lactobacillus brevis* is mediated by the ATP-binding cassette multidrug transporter HorA. J. Bacteriol. **183**:5371–5375.
26. Spellerberg, B., S. Martin, C. Brandt, and R. Lütticken. 2000. The *cyl* genes of *Streptococcus agalactiae* are involved in the production of pigment. FEMS Microbiol. Lett. **188**:125–128.
27. Spellerberg, B., S. Martin, C. Franken, R. Berner, and R. Lütticken. 2000. Identification of a novel insertion sequence element in *Streptococcus agalactiae*. Gene **241**:51–56.
28. Spellerberg, B., B. Pohl, G. Haase, S. Martin, J. Weber-Heynemann, and R. Lütticken. 1999. Identification of genetic determinants for the hemolytic activity of *Streptococcus agalactiae* by ISS1 transposition. J. Bacteriol. **181**:3212–3219.
29. Tatusov, R. L., R. V. Koonin, and D. J. Lipman. 1997. A genomic perspective on protein families. Science **278**:631–637.
30. Trieu-Cuot, P., C. Carlier, C. Poyart-Salmeron, and P. Courvalin. 1990. A pair of mobilizable shuttle vectors conferring resistance to spectinomycin for molecular cloning in *Escherichia coli* and in gram-positive bacteria. Nucleic Acids Res. **18**:4296.
31. van Veen, H. W., A. Margolles, M. Muller, C. F. Higgins, and W. N. Konings. 2000. The homodimeric ATP-binding cassette transporter *LmrA* mediates multidrug transport by an alternating two-site (two-cylinder engine) mechanism. EMBO J. **19**:2503–2514.
32. Zangwill, K., A. Schuchat, and J. D. Wenger. 1992. Group B streptococcal disease in the United States, 1990: report from a multistate active surveillance system. Morb. Mortal. Wkly. Rep. CDC Surveill. Summ. **41**:25–32.