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Molecular Analysis of Resistance to Streptogramin A Compounds Conferred by the Vga Proteins of Staphylococci

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The Vga and Msr resistance determinants, encoded by mobile genetic elements in various staphylococcal strains, belong to a family of ATP-binding cassette (ABC) proteins whose functions and structures are ill defined. Their amino acid sequences are similar to those of proteins involved in the immunity of streptomycetes to the macrolide-lincosamide-streptogramin antibiotics that they produce. Sequence analysis of the genomes of the gram-positive bacteria with low G+C contents revealed that Lmo0919 from *Listeria monocytogenes* is more closely related to Vga variants than to Msr variants. In the present study we compared the antibiotic resistance profiles conferred by the Vga-like proteins in two staphylococcal hosts. It was shown that Vga(A), the Vga(A) variant [Vga(A)v], and Lmo0919 can confer resistance to lincosamides and streptogramin A compounds, while only Vga(B) is able to increase the level of resistance to pristinamycin, a mixture of streptogramin A and streptogramin B compounds. By using polyclonal antibodies, we found that the Vga(A) protein colocalized with the β subunit of the F_1-F_0 ATPase in the membrane fractions of staphylococcal cells. In order to identify functional units in these atypical ABC proteins, such as regions that might be involved in substrate specificity and/or membrane targeting, we analyzed the resistance phenotypes conferred by various plasmids carrying parts or modified versions of the *vga*(A) gene and we determined the subcellular localization of the gene products. Only polypeptides composed of two ABC domains were detected in the cell membranes. No region of drug specificity was identified. Resistance properties were dependent on the integrities of both Walker B motifs.

ATP-binding cassette (ABC) systems share a highly conserved ATP-binding and -hydrolyzing domain or protein. More than 95% of the ABC systems are transporters made up of two hydrophobic transmembrane domains associated with two hydrophilic ATP-binding domains (18). Antibiotic resistance mediated by ABC systems is usually attributable to efflux pumps containing these four core domains (19, 21, 23, 28, 31, 41). By contrast, a growing number of proteins that confer resistance to macrolide, lincosamide, and streptogramin (MLS) antibiotics consist of a single polypeptide chain carrying two fused hydrophilic ABC domains (for a review, see reference 33). They form a distinct subfamily named ARE (for antibiotic resistance) in the phylogenetic and functional classification of the ABC proteins (6).

Many members of the ARE subfamily have been found in MLS producers, such as Car(A) in *Streptomyces thermotolerans*, Srm(B) in *Streptomyces ambofaciens*, Tlr(C) in *Streptomyces fradiae*, Ole(B) in *Streptomyces antibioticus*, and Lmr(C) in *Streptomyces lincolnensis* (for a review, see reference 28). Others have been described in pathogens, mainly gram-positive bacteria, where they are either encoded by transposable elements, such as Mel or Orf5 in streptococci (10, 13, 37), or by the chromosome, such as Lsa recently characterized in *Enterococcus faecalis* (38) and Msr(C) in *Enterococcus faecium* (32, 39).

The assumption that ABC proteins of the ARE subfamily are parts of efflux pumps mediating resistance is based on transport experiments with radioactively labeled derivatives of the antibiotics. After an initial uptake phase, a decrease in the cell-associated radioactivity is observed, and this decrease is strongly inhibited by respiratory chain and proton motive force inhibitors (30, 34). However, since the ribosomes provide the main driving force for MLS uptake, it cannot be ruled out that the observed efflux is a consequence of an ABC-mediated change of the affinity of the ribosomes toward drugs. Moreover, and despite the efforts of several groups, the putative permeases that are expected to interact with such ABC proteins in order to form a canonical ABC transporter have not been identified so far (33, 35).

The Vga proteins encoded by plasmids or transposons in staphylococci belong to the ARE subfamily of ABC systems. Vga(A), the Vga(A) variant [Vga(A)v], and Vga(B) were initially reported to be determinants of resistance to streptogramin A (SGA) compounds (1, 3, 4, 11, 15). Msr(A) is responsible for inducible resistance to erythromycin (ERY) and type B streptogramins (SGBs) but not to lincosamides or 16-membered-ring macrolides (26, 27, 34, 35). The Vga(A) and Msr(A) proteins exhibit structural similarities but have distinct drug specificities. To study the basis of these specificities and the sequence-function relationships in this group of proteins, we analyzed the properties of Vga(A) and of other Vga(A)-related proteins: Vga(A)v, Vga(B), and a protein-coding open reading frame (ORF), Lmo0919, from *Listeria monocytogenes*. The role of the ABC domains of Vga(A) was investigated by generating clones for further analysis. The relevant phenotypes and subcellular localization of the proteins are reported and provide prospects for future investigations that might be helpful in understanding the mechanism of resistance.

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TABLE 1. Oligodeoxynucleotides used as primers and plasmids used as templates in the PCR experiments

Primer pair	Nucleotide sequence (5′ to 3′)[a]	Template[b]	Purpose
VG1-F	GATT**AGGATCC**TTTTATTGTCTTC	pIP1617	Cloning of *vga*(A) gene
VG2-R	ATCAA**GAATTC**AATAAAAAGACAAC		
XbBaMut1-F	CCTGCAGGTCGACTCTAGCCTTTTATTGTCTTCAATTAC	pIP1707	Site-directed mutagenesis
XbBaMut2-R	GTAATTGAAGACAATAAAAGGCTAGAGTCGACCTGCAGG		
EcoMut1-F	TTATAAGAGGATGAGAATTCATGAAAATAATGTTAGAGG	pIP1708	Site-directed mutagenesis
EcoMut2-R	CCTCTAACATTATTTTCATGAATTCTCATCCTCTTATAA		
VG3-F	TTA**GAATTC**ATGAAAATAAAAGGAGC	pIP1617	Cloning of the second half of *vga*(A) gene
VG2-R	ATCAA**GAATTC**AATAAAAAGACAAC		
Kp-F	GCAAGCTTG**GGTACC**TTAAGGTTGACTCTAGCC	pIP1837	Construction of pIP1846 from pIP1835
Kp-R	CGCAGTGCAGCG**GGTACC**AATAAAAAGACAAC		
MSR1-F	GGAGTGG**GAATTC**ATGGAACAATATAC	IPF69	Cloning of first half of *msr*(A) gene
MSR2-R	AA**GAATTC**TAGATAACTTTGTGGTTTTTCAAC		
MSR3-F	GTTA**TCTAGA**ATTCAATTTTCCAC	IPF69	Cloning of second half of *msr*(A) gene
MSR4-R	T**TCTAGA**TCGGTTATGGTACTATTG		
WB1Mut1-F	ACTGCTATTAGCAAAGGAACCAACAACTAAC	pIP1845	Mutagenesis of first Walker B motif of Vga(A)
WB1Mut2-R	GTTAGTTGTTGGTTCCTTTGCTAATAGCAGT		
WB2Mut1-F	AATACGTTGGTACTAAAGGAACCAACAAAC	pIP1845	Mutagenesis of second Walker B motif of Vga(A)
WB2Mut2-R	GTTTGTTGGTTCCTTTAGTACCAACGTATT		
ListA-F	CAGGAGGTTA**GAATTC**ATGTCTACAATCGA	CLIP72401	Cloning of *lmo0919* from *L. monocytogenes*
ListA-R	CTATT**GAATTC**TAAGCCTATCTTTTGCAG		

[a] The sequences in boldface letters correspond to the restriction sites used to clone the amplified DNA fragments.
[b] Plasmids from *E. coli* strains or cellular DNA from *S. epidermidis* IPF69 and *L. monocytogenes* CLIP72401.

cago, Ill., 14 to 17 September 2003 [O. Chesneau, H. Ligeret, N. Hosan-Aghaie, E. Dassa, and N. El Solh, Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. C1-2071, 2003].)

MATERIALS AND METHODS

Strains and growth conditions. *Escherichia coli* strains XL-2 Blue (Stratagene, Cedar Creek, Tex.) and PM9, which is a derivative of strain JM109 carrying a deletion of the *malE* gene (9), were used for the large-scale preparation of plasmids and expression of the MalE-fusion protein, respectively. *Staphylococcus aureus* RN4220 (22) and *Staphylococcus epidermidis* BM3302 (12), both of which are susceptible to MLS antibiotics, were used as recipient strains in the transformation experiments. Three clinical isolates were investigated: *S. aureus* BM3327 and *S. epidermidis* BM10385, which are resistant to SGA by the presence of the *vga*(A)*v* and *vga*(A) genes, respectively (16, 25), and *S. epidermidis* IPF69, which is resistant to ERY and SGB by the presence of the *msr*(A) gene.

E. coli strains and staphylococci were grown in Luria medium and brain heart infusion (BHI) broth or agar (Becton Dickinson, le Pont de Claix, France), respectively, supplemented as necessary with antibiotics. Bacterial growth was monitored by measuring the optical density of the cell suspension at 600 nm. Transformation of the staphylococcal strains was performed as described previously (2, 7).

Chemicals and reagents. Ampicillin, kanamycin, and chloramphenicol were purchased from Sigma (Lisle d'Abeau, Chesnes, France). The other antibiotics were kindly provided by their manufacturers: lincomycin (LC) and clindamycin (CLI) were provided by Pharmacia & Upjohn (Kalamazoo, Mich.); and the two components of pristinamycin (PT), with PTI as the SGB compound and PTII as the SGA compound, were provided by Aventis-Pharma (Vitry-sur Seine, France). Antibiotic disks came from Bio-Rad (Marnes-la-Coquette, France) or Eurobio (les Ulis, France). Disks of the SGA and SGB compounds, which contained 20 μg of PTII and 40 μg of PTI, respectively, were homemade. Isopropyl-β-D-thiogalactopyranoside (IPTG) was from Euromedex (Mundolsheim, France).

Restriction enzymes, T4 DNA ligase, alkaline phosphatase, and antiprotease cocktail were from Roche Diagnostics (Mannheim, Germany). Amylose resin was supplied by New England BioLabs (Beverly, Mass.), and enhanced chemiluminescence detection products, including polyvinylidene difluoride membranes, were from Amersham Biosciences (Little Chalfont, England). Size markers were a 1-kb DNA ladder (BioLabs) for agarose gel electrophoresis and Rainbow (Amersham) for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentrations were determined with a bicinchoninic acid kit (Pierce, Rockford, Ill.) with bovine serum albumin (Sigma) as the standard. Lysostaphin was from Ambi UK (Trowbridge, England). Lysozyme and all the other chemical reagents were from Sigma.

DNA manipulations. All plasmids were constructed by common cloning techniques (36). Extraction of the DNA fragments separated by agarose gel electrophoresis was carried out with a Gene-Clean II kit (Bio 101, La Jolla, Calif.). Sequencing reactions and synthesis of the oligodeoxynucleotides (Table 1) were performed by Genome-Express (Meylan, France). The mutagenic primers were designed and used according to the guidelines supplied with the reagents of the QuikChange site-directed mutagenesis kit (Stratagene). High-fidelity DNA synthesis was done with *Pfu* DNA polymerase (Stratagene). The PCR assays were run on a Crocodile III apparatus (Appligène, Illkirch, France) with thermocycling conditions that varied according to the length of the DNA fragment amplified. Amplicons of the expected size were cloned into pCR4 (Invitrogen, Carlsbad, Calif.) and were sequenced to verify the integrity of the gene constructs. Inserts were subcloned into vectors of the pRB series, which enabled propagation of the plasmid constructs in staphylococci (7). Because transformation of *S. epidermidis* yielded more plasmid-less small-colony variants on kanamycin plates than that of *S. aureus*, pRB474 (with the *cat* gene as a resistance marker) was used instead of pRB374 (with the *aadD* gene as a resistance marker) throughout the study (Table 2).

The wild-type *vga*(A) gene was amplified from plasmid pIP1617 (4). The resulting BamHI-EcoRI-digested product was inserted into pRB374, leading to

TABLE 2. Plasmids and MICs of lincosamides and streptogramins[a]

Plasmid	Relevant characteristics	MIC[b] (μg/ml)				
		SGA	SGB	PT	LC	CLI
pRB374	Shuttle vector, Km^r, polylinker, *vegII* promoter	1 (NT)	8 (NT)	0.25 (NT)	1 (NT)	0.125 (NT)
pIP1746	pRB374Ω*vga*(A) expressing WT Vga(A)	32 (NT)	8 (NT)	0.5 (NT)	4 (NT)	0.25 (NT)
pRB474	Shuttle vector, Cm^r, polylinker, *vegII* promoter	1	8	0.25 (0.125)	1 (0.5)	0.125
pIP1845	pRB474Ω*vga*(A) expressing WT Vga(A)	32	8	0.5	4 (8)	0.25 (1)
pIP1863	pRB474Ω*vga*(A) online expressing WT Vga(A)$_v$	32	8	0.5	8	0.25 (1)
pIP1861	pRB474Ω*vga*(B) expressing WT Vga(B)	4	8	2	2	0.25 (0.5)
pIP1867	pRB474Ω*lmo0919* expressing WT Lmo0919	8	8	0.5	2 (4)	0.25 (0.5)
pIP1877	pRB474Ω*msr*(A)α-*vga*(A)β coding for chimera Msr(A)-Vga(A)	1	8	0.25 (0.125)	1 (0.5)	0.125
pIP1879	pRB474Ω*vga*(A)α-*msr*(A)β coding for chimera Vga(A)-Msr(A)	1	8	0.25 (0.125)	1 (0.5)	0.125
pIP1835	pRB474Ω*vga*(A)α coding for N-terminal half of WT Vga(A)	1	8	0.25 (0.125)	1 (0.5)	0.125
pIP1837	pRB474Ω*vga*(A)β coding for C-terminal half of WT Vga(A)	1	8	0.25 (0.125)	1 (0.5)	0.125
pIP1846	pRB474Ω*vga*(A)α/*vga*(A)β coding for both halves of WT Vga(A)	1[c]	8	0.25	1 (0.5)	0.125 (0.25)
pIP1887	pRB474Ω*vga*(A)$_{A311G313}$ coding for Vga(A)$_{K104}$[d]	1	8	0.5 (0.25)	1 (0.5)	0.125
pIP1838	pRB474Ω*vga*(A)$_{A1226G1228}$ coding for Vga(A)$_{K409}$[d]	1	8	0.25 (0.125)	1 (0.5)	0.125
pIP1880	pRB474Ω*msr*(A)$_{T843A844}$ coding for Msr(A)$_{L281}$[d]	1	128 (256)	0.25	1 (0.5)	0.125

[a] Abbreviations: Cm, chloramphenicol; Km, kanamycin; NT, not tested; WT, wild type. Symbols: α and β, the 5′ and 3′ parts of the genes, respectively, which can be fused in frame (hyphens) or expressed separately (slashes).
[b] MICs are for *S. aureus* RN4220 transformants, and those in parentheses are for *S. epidermidis* BM3302 transformants when the MICs for the two strains are different.
[c] Few colonies grew at higher concentrations, but they harbored a full-length *vga*(A) gene restored by an in-frame fusion of the two separate halves.
[d] Bases relative to the ATG start codon of the corresponding gene and modified by site-directed mutagenesis.

pIP1707 (Fig. 1A). Two rounds of site-directed mutagenesis were then performed to remove undesirable XbaI and BamHI sites and to add an EcoRI site between the ribosome-binding site (RBS) and the translation start of *vga*(A), leading to pIP1748 (Fig. 1B). Plasmid pIP1835, obtained by subcloning the HindIII-XbaI fragment of pIP1748 into pRB474, was the basis of all subsequent modifications. This plasmid codes for the N-terminal half of Vga(A) (Fig. 1C). Plasmids pIP1845, pIP1837, and pIP1867, which express the wild-type Vga(A) protein, the C-terminal half of Vga(A), and the Lmo0919 protein, respectively, resulted from the replacement of the EcoRI fragment of pIP1835 by the corresponding EcoRI-digested PCR products. Plasmid pIP1846, which directs the independent expression of the two halves of Vga(A), was obtained by subcloning a KpnI-digested PCR product encompassing the RBS and the distal half of *vga*(A) from pIP1837 into the unique KpnI site of pIP1835. Plasmids that encode chimeric proteins were constructed by using the internal XbaI site of the *vga*(A) gene as a frontier for domain shuffling (4). The proximal half of the *msr*(A) gene, which contains an additional XbaI site at the 3′ end of the sequence, was amplified and digested with EcoRI to replace the EcoRI fragment of pIP1835, leading to pIP1873 (Fig. 1D). XbaI-digested PCR products of the distal half of *msr*(A) were inserted into the unique XbaI sites of pIP1835 and pIP1873. The resulting plasmids, pIP1879 and pIP1880, respectively, encode the N-terminal ABC domain of Vga(A) fused to the C-terminal domain of Msr(A) and a full-length Msr(A) protein in which histidine 281 is replaced by leucine (H281L), respectively. This change in the amino acid sequence of Msr(A) was due to the creation of the XbaI site within the nucleotide sequence of the gene. The 900-bp XbaI fragment from plasmid pIP1739 (see below), which encodes the C-terminal half of Vga(A), was inserted into the unique XbaI site of pIP1873 to generate the fusion of the N-terminal ABC domain of Msr(A) with the C-terminal domain of Vga(A). Plasmids pIP1861 and pIP1863, which carry the *vga*(B) and *vga*(A)*v* genes, respectively, were constructed by subcloning HindIII-KpnI and BamHI-KpnI fragments from pIP1717 (3) and pIP1810 (15), respectively, into pRB474. All the gene constructs were therefore under the control of the *Bacillus subtilis vegII* promoter (7), and all but the last two constructions were placed 9 nucleotides downstream of the RBS of *vga*(A) (Fig. 1).

Expression and purification of the MalE fusion protein. The MalE fusion pMal-c0 expression vector was a modified version of pMal-c2 (New England Biolabs). In pMal-c0, the polylinker at the 5′ end of the *malE* gene was replaced by the authentic *malE* sequence (J.-M. Clément, unpublished data). The EcoRI fragment of pIP1746 carrying the *vga*(A) gene was inserted into pMal-c0 at the EcoRI site. The orientation of the insert, in frame with the 3′ end of the *malE* gene, was confirmed by restriction mapping. The resulting plasmid, pIP1739, was transformed into *E. coli* PM9. Cultures of a transformant were induced with 0.2 mM IPTG, and the MalE-Vga(A) fusion protein was observed at 100 kDa in Coomassie blue-stained gels (data not shown). Cells were broken in a French press at 16,000 lb/in^2, and the fusion protein was purified from the lysate on amylose resin by standard protocols. A total of 6 mg of protein, which eluted from the column as a single peak, was obtained from 2 liters of culture. The purity of the eluate was checked by SDS-PAGE. The size of the purified protein, which reacted with an antibody elicited against MalE, was consistent with that expected for the hybrid protein. The final preparation was dialyzed against phosphate-buffered saline, concentrated to 1.5 ml, and injected into rabbits to raise antisera.

Production of antibodies directed against MalE-Vga(A). Two New Zealand rabbits were immunized by intradermal injection of 200 μg of antigen preparation in Freund's complete adjuvant. The immune response was boosted with the same dose of proteins in Freund's incomplete adjuvant given subcutaneously every 2 weeks for 1.5 months. Serum samples were then taken twice weekly. The animals were bled 3 weeks after the last immunization. Antibodies were purified on a protein A-Sepharose column.

Susceptibility tests. The MICs of lincosamides and streptogramins were determined in triplicate by the twofold dilution method with Mueller-Hinton agar and 10^4 CFU per spot after 24 h of incubation at 37°C. The concentrations of antibiotics ranged from 0.064 to 512 μg/ml. Disk diffusion assays carried out with various antibiotics were used in parallel for each recombinant strain to check the purity of the bacterial culture.

Preparation and analysis of cell fractions. Cultures of staphylococci were grown in brain heart infusion medium at 37°C with vigorous shaking until the absorbance at 600 nm reached 0.7 units. Cells from 50-ml cultures were harvested by centrifugation at 5,000 × *g* for 10 min at 4°C, washed once in 50 mM Tris (pH 7.5), and resuspended in 1/10 of the same buffer with 30% (wt/vol) sucrose. Protoplasts were prepared by using 1 mg of lysostaphin per liter and 5

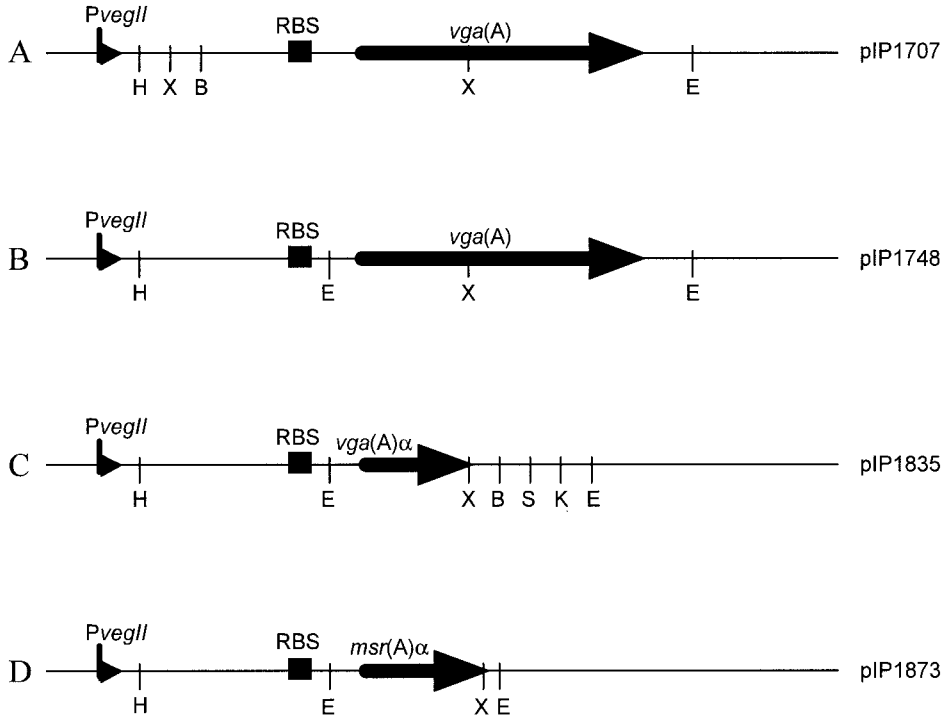

FIG. 1. Progeny of the plasmids used. Abbreviations: P*vegII*, constitutive promoter; RBS, ribosome-binding site of the *vga*(A) gene; B, BamHI; E, EcoRI; X, XbaI; H, HindIII, S, SmaI; K, KpnI.

mg of lysozyme per liter at 37°C for 20 min. The protoplast pellets were lysed in 2 ml of buffer consisting of 50 mM Tris (pH 7.5), 150 mM NaCl, 10 mM $MgCl_2$, and 20 mM dithiothreitol, hereafter called the sample buffer, in the presence of 15 μg of protease inhibitors per ml and with the help of ultrasonic disruption for 3 min in 30-s pulses at 4°C. Unbroken cells and debris were removed by centrifugation at 16,000 × *g* for 4 min, and the lysates were ultracentrifuged in a Beckman TL-100 instrument. Membranes were isolated by centrifugation at 30,000 × *g* for 30 min, and ribosomes were separated from the cytoplasm by centrifugation at 150,000 × *g* for 1 h. Because the membranes and ribosomes were resuspended in 200 μl of sample buffer, the cytosolic fractions were 10 times as dilute as both particulate fractions. For SDS-PAGE analysis, each sample was mixed with an identical volume of 2× Laemmli buffer and boiled for 5 min. Gels (10% [wt/vol] acrylamide) in Tris-glycine buffer were loaded with 1 volume of membranes and ribosomes and 10 volumes of the cytosolic fractions. Runs were performed at room temperature under a constant current of 40 mA. Proteins were either stained with Coomassie brilliant blue R-250 (Sigma) or transferred to a polyvinylidene difluoride membrane (Amersham).

Immunoblotting was carried out with antibodies directed against MalE-Vga(A) and with two antiserum samples used as controls for cellular fractionation. The first was raised against the β subunit of the F1 protein of *E. coli* ATP synthase, and the second was raised against the L24 protein from *B. subtilis*. The working dilutions of these antibodies were 1/400 for those reacting against MalE and Vga(A), 1/15,000 for those reacting against anti-F1β, and 1/1,000 for those reacting against anti-L24. Anti-rabbit or anti-mouse immunoglobulin G–peroxidase conjugates were diluted 10,000-fold. Bound peroxidase activity was revealed with enhanced chemiluminescence substrates on Fuji HR-E30 films.

RESULTS AND DISCUSSION

Phenotypes conferred by wild-type genes. The MICs of lincosamides (LC and CLI) and streptogramins (SGA, SGB, and PT) were determined for two staphylococcal hosts, *S. aureus* RN4220 and *S. epidermidis* BM3302, carrying *vga*(A)-related genes (Table 2). The *vga*(A) gene conferred high-level resistance to SGA (MIC = 32 μg/ml) in *S. aureus*, whatever the vector used. The *vga*(A)*v* gene, which is more prevalent than *vga*(A) among clinical isolates of this species (17), was not more efficient at conferring resistance (MIC = 32 μg/ml). These findings were described earlier (4, 15). The MICs of SGA due to the presence of *vga*(A) or *vga*(A)*v* were the same for *S. epidermidis*. Interestingly, we observed that *vga*(A) or *vga*(A)*v* was able to confer low-level resistance to LC (MICs = 4 and 8 μg/ml for *S. aureus* and *S. epidermidis*, respectively). This resistance-conferring trait of *vga*(A) was not described in previous analyses because of the presence of a constitutively expressed *erm*(C) gene in the vector pOX300 (4). Each gene, *vga*(A) or *vga*(A)*v*, conferred a slight increase in the basal level of resistance to CLI (MIC from 0.125 to 1 μg/ml for *S. epidermidis* and from 0.125 to 0.25 μg/ml for *S. aureus*). This phenotype is similar to that conferred by the *lsa* gene in *Enterococcus faecalis*, although the levels of resistance to lincosamides were much lower for staphylococci with the *vga*(A) and the *vga*(A)*v* genes (38). We suggest, therefore, that *vga*(A) or its variant, *vga*(A)*v*, is involved in the LSA phenotype occasionally found among staphylococcal isolates (14, 17, 24).

Neither *vga*(A) nor *vga*(A)*v* was able to confer resistance to PT, which is a mixture of 70% SGA and 30% SGB. Conversely, we found that *vga*(B) determines a large increase in the level of resistance to PT (MIC from 0.125 to 2 μg/ml for *S. epidermidis*), while it confers low-level resistance to SGA (MIC = 4 μg/ml). To the best of our knowledge, this has never been described. The detection of this gene in clinical strains might therefore be of importance for prediction of the in vivo activity of quinupristin-dalfopristin (2, 11, 17, 24).

A search for protein sequences similar to the sequences of the Vga proteins led us to identify a *L. monocytogenes* protein-

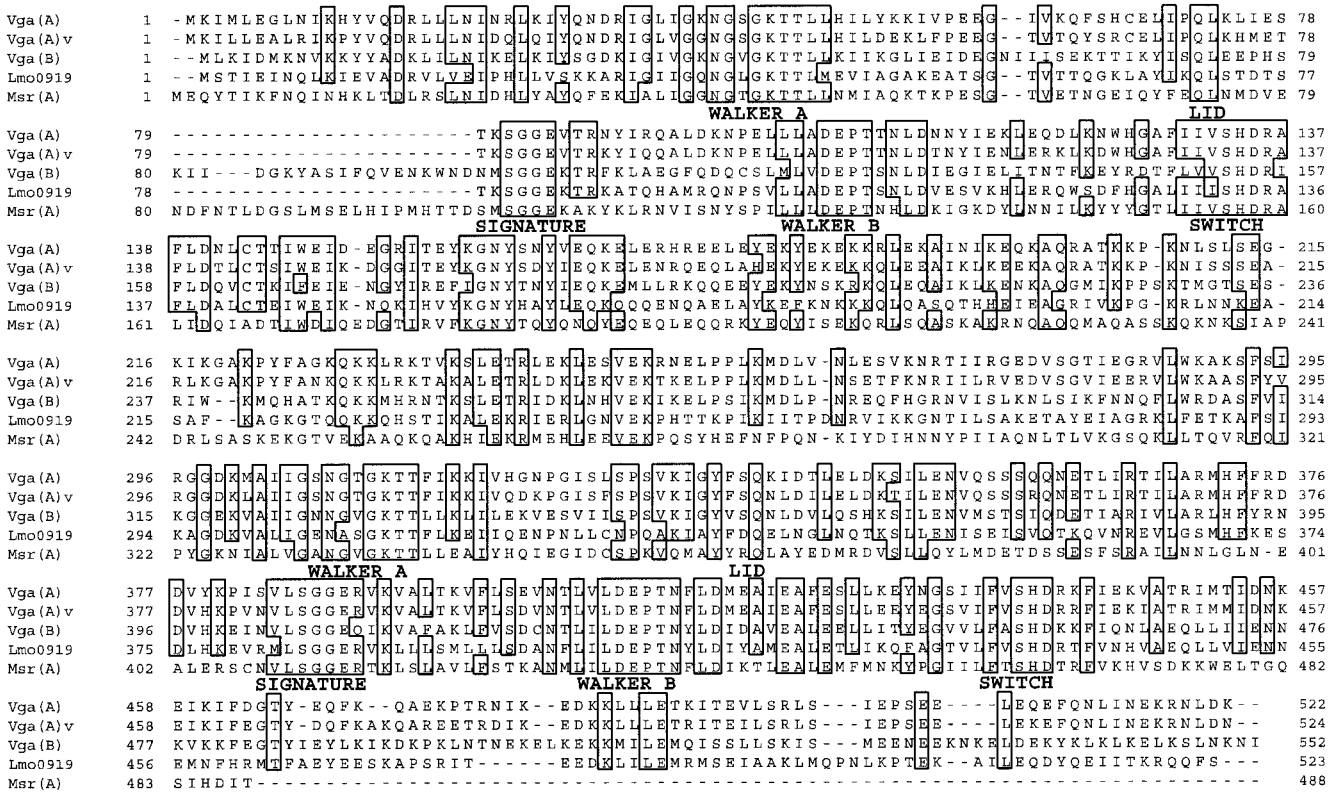

FIG. 2. Alignment of the proteins studied. Identical residues are boxed. Highly conserved functional motifs of ABC proteins (WALKER A, LID, SIGNATURE, WALKER B, and SWITCH) are indicated. Vga(A), Vga(A)v, and Lmo0919 are characterized by shorter helical domains encompassing residues between the LID and the SIGNATURE motifs of the N-terminal ABC domain.

coding ORF, Lmo0919, which displays 41% amino acid identity with Vga(A) (Fig. 2). This ORF is chromosomally borne, and no gene encoding transmembrane proteins was found in its vicinity. When *lmo0919* was cloned on pRB474, it was able to confer resistance to SGA (MIC = 8 μg/ml for both hosts) and LC (MIC = 4 μg/ml for *S. epidermidis*). This phenotype is similar to that conferred by *vga*(A) and *vga*(A)*v* in the same hosts, although the levels of resistance are lower. The slight difference might be due to the less efficient expression of *lmo0919* in the staphylococcal background. A less efficient interaction with a putative partner encoded by the host chromosome could be another explanation. Nevertheless, we postulate that Lmo0919 contributes to the intrinsic resistance of *Listeria* strains to SGA compounds and lincosamide antibiotics (5, 40). We are attempting to inactivate the *lmo0919* gene in *L. monocytogenes* CLIP72401 to further document the antibiotic resistance-conferring properties of this chromosomal gene.

The amino acid sequences of the five proteins analyzed in the present study exhibited from 45 to 85% similarity in pairwise comparisons. Conserved regions found universally in ABC proteins were present, such as the Walker A and B motifs and the signature motif involved in ATP binding and hydrolysis; the switch motif, which could also be involved in ATP hydrolysis; and the LID motif, which has been shown to be essential for the interaction of the MalK ABC ATPase with its membrane partners, MalF and MalG, in the *E. coli* maltose transporter (29). Except for Msr(A), which is 488 amino acids (aa) in length, all the other Vga(A)-related proteins had a lysine- and aspartate-rich C-terminal region. One of the common traits of Vga(A), Vga(A)v, and Lmo0919 (522, 524, and 523 aa, respectively) is the 22-aa deletion found in a region between the LID motif and the signature motifs of the N-terminal ABC domain. Vga(B) and Msr(A) do not present such deletions (Fig. 2). Despite this, it is rather difficult to correlate the sequence-structure traits of these proteins with their specificities for MLS antibiotics. We suspect, however, that the two halves of the Vga(A)-related proteins are involved in separate functions, given their structural dissimilarities.

Immune detection and subcellular localization of Vga(A) protein. Since many ABC systems are known to be involved in the import or export of a wide variety of substances, the Vga(A) protein and similar systems have been thought to be part of classical transporters that expel the MLS antibiotics from bacteria (11, 34). However, this assumption has not been confirmed by establishment of the membrane localization of these proteins or by characterization of any partner that might anchor the ABC proteins in the bacterial membranes. We thus analyzed the subcellular localization of Vga(A) by using the antiserum raised against MalE-Vga(A).

Lysates obtained from *S. aureus* RN4220 and BM3327 and *S. epidermidis* BM3302, BM10385, and IPF69 were probed with the antibodies. Because of smearing problems due to protein A, only the *S. epidermidis* strains were used for Western blot analyses. There were few cross-reactive bands in *S. epidermidis* BM3302 (Fig. 3, lane 2). Lysates of strain BM3302 transformed by *vga*(A) gave, as expected, a band of 58 kDa that reacted with

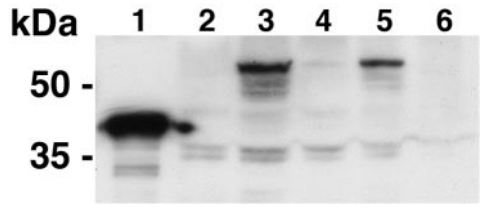

FIG. 3. Analysis of lysates extracted from *S. epidermidis* BM3302 transformants and probed with antibodies raised against MalE-Vga(A). Lanes: 1, 10 ng of purified MalE protein; 2, strain BM3302 transformed by pRB474; 3, strain BM3302 transformed by pIP1845 expressing Vga(A); 4, strain BM3302 transformed by pIP1861 expressing Vga(B); 5, strain BM3302 transformed by pIP1863 expressing Vga(A)v; 6, strain BM3302 transformed by pIP1867 expressing Lmo0919.

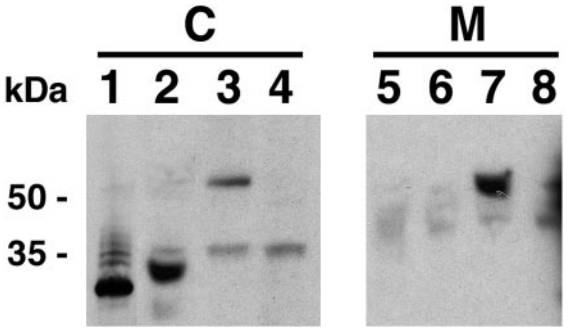

FIG. 5. Analysis of the cytoplasmic (C) and membrane (M) fractions from *S. epidermidis* BM3302 transformants probed with antibodies raised against MalE-Vga(A). Lanes: 1 and 5, strain BM3302 transformed by pIP1835 expressing the first half of Vga(A); 2 and 6, strain BM3302 transformed by pIP1837 expressing the second half of Vga(A); 3 and 7, strain BM3302 transformed by pIP1845 expressing Vga(A); 4 and 8, strain BM3302 transformed by pRB474, used as a control.

the antibodies (Fig. 3, lane 3). This band was probably the Vga(A) protein, since its size corresponded to that predicted. It was observed that Vga(A)v strongly cross-reacted with the polyclonal antiserum (Fig. 3, lane 5), whereas Vga(B) and Lmo0919 did not (Fig. 3, lanes 4 and 6).

Fractionation of cells from clinical isolates BM10385 and IPF69, which express the *vga*(A) and the *msr*(A) genes, respectively, from their native promoters, showed that Vga(A) colocalized with the β subunit of the F_1-F_0 ATPase in the membrane fraction (Fig. 4). No trace of Vga(A) was detectable in the cytosolic or the ribosomal fractions analyzed. To the best of our knowledge, there are no previous reports of the membrane localization of Vga(A)-related systems in bacteria of medical interest. Since Msr(A) does not cross-react with the antibodies raised against Vga(A), we cannot draw a conclusion about the subcellular localization of Msr(A) in staphylococci.

A similar experiment was performed with laboratory strain BM3302 transformed with pIP1845, which expresses *vga*(A) under the control of the constitutive *vegII* promoter. Most of the antibody-reacting material of this strain was detected in the membrane fraction (Fig. 5). However, it is worth mentioning that approximately 10 to 20% of the Vga(A) protein was also detected in the cytosolic fraction (Fig. 5, lanes 3 and 7). This result was probably due to overexpression of the *vga*(A) gene from *vegII* and might suggest that the putative membrane proteins, which were expected to form a canonical ABC transporter with Vga(A), are present in limiting amounts. Olano et al. (30) showed that Ole(B) partitions equally between the membrane and the cytosolic fractions in *Streptomyces*. Their observation and our findings give credence to the hypothesis that Vga(A) and, probably, Msr(A) are able to interact with the membranes of staphylococci.

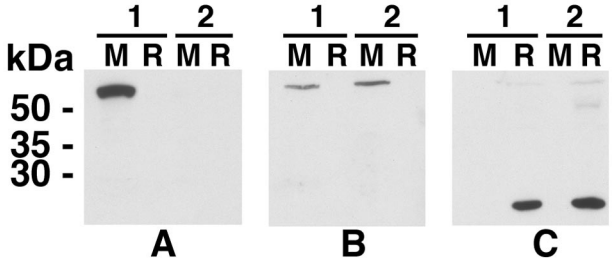

FIG. 4. Analysis of membranes (M) and ribosomes (R) extracted from two *S. epidermidis* clinical isolates and probed with various immune sera. The first part (lanes 1) of each panel corresponds to strain BM10385 carrying *vga*(A), and the second part (lanes 2) corresponds to strain IPF69 carrying *msr*(A). (A) Immunoblot probed with antibodies raised against MalE-Vga(A); (B) immunoblot probed with antibodies raised against the β subunit of the F_1-F_0 ATPase; (C) immunoblot probed with antibodies raised against the L24 protein.

Membrane targeting and expression of resistance. It has been reported that either one of the two ABC domains of Ole(B) is sufficient to confer resistance to oleandomycin (30). Moreover, it was demonstrated that the N-terminal ABC domain of Ole(B) is able to recognize oleandomycin (8). To test the possibility that each ABC domain of Vga(A) would behave similarly in conferring resistance to SGA, we constructed truncated versions of *vga*(A) by subcloning each half of the gene on separate plasmids. When the two plasmids were transformed into *S. aureus* and *S. epidermidis*, neither was able to confer resistance to any of the antibiotics tested (Table 2). In this respect, Vga(A) behaves like Msr(A), whose N-and C-terminal domains were found to be unable to promote erythromycin resistance (35). Moreover, each half of Vga(A) was expressed but was found exclusively in the cytosolic fraction of the cells (Fig. 5, lanes 1, 2, 5, and 6). These truncated proteins were unable to reach the membrane, probably because signals present in both moieties are needed to target the membrane. We also constructed plasmid pIP1846, in which the two halves were coexpressed in the same cells. This plasmid is also unable to confer resistance to the antibiotics tested, suggesting that the two halves of Vga(A) cannot interact together in order to reconstitute a functional system.

The Vga(A) protein consists of two ABC domains fused into a single polypeptide. In order to establish whether ATP hydrolysis is needed for resistance to SGA, we mutated an aspartate residue in either one of the two Walker B motifs of Vga(A). This quasi-invariant residue is in close contact with the γ-phosphate of ATP in crystallized ABC proteins, such as HisP (20). Vga(A) proteins carrying substitutions of D104 or D409 for lysine were unable to confer resistance to SGA (Table 2). This result demonstrates that the two ATP-binding sites of Vga(A)

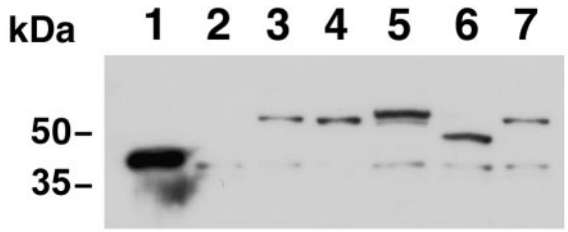

FIG. 6. Analysis of the membrane fractions from *S. epidermidis* BM3302 transformants probed with antibodies raised against MalE-Vga(A). Lanes: 1, 10 ng of MalE protein; 2, strain BM3302 transformed by pRB474; 3, strain BM3302 transformed by pIP1838 expressing Vga(A) mutated in the second Walker B motif; 4, strain BM3302 transformed by pIP1845 expressing a wild-type Vga(A) protein; 5, strain BM3302 transformed by pIP1877 expressing the chimera Msr(A)-Vga(A); 6, strain BM3302 transformed by pIP1879 expressing the chimera Vga(A)-Msr(A); 7, strain BM3302 transformed by pIP1887 expressing Vga(A) mutated in the first Walker B motif.

are needed for the protein to function. To further document this finding, we analyzed the subcellular localization of the plasmid-encoded proteins in *S. epidermidis* BM3302. Approximately 80% of the Vga(A) proteins with mutations in either one of the two Walker B motifs were found in the cell membrane fractions, as was also found for the wild-type protein (Fig. 6). Susceptibility to SGA and lincosamide antibiotics was therefore due to inactive rather than misexpressed or mislocated proteins.

Vga(A) and Msr(A) have been characterized as determinants of resistance to SGA and SGB, respectively. Since no transmembrane domains, which are known to be responsible for substrate specificity in ABC transporters, have been identified in these proteins, the substrate specificity might be determined by sequences found within the ABC proteins. To address this question, we constructed two chimeras fusing portions of the *vga*(A) and the *msr*(A) genes. DNA fragments encoding the N- and C-terminal halves of the Msr(A) protein was amplified from strain IPF69. These fragments were combined with similar fragments amplified from the *vga*(A) gene. As a control, the *msr*(A) fragments were fused in order to reconstitute a full-length *msr*(A) gene. The plasmid constructs were transformed into *S. aureus* RN4220 and *S. epidermidis* BM3302. The modified version of *msr*(A) promoted resistance to SGB (MICs = 128 and 256 μg/ml for *S. aureus* and *S. epidermidis*, respectively) at levels similar to that conferred by the *msr*(A) gene in clinical strains (26, 27, 34, 35). None of the two chimeras was able to confer resistance to any of the antibiotics tested (Table 2). A fractionation experiment performed with the *S. epidermidis* transformants showed that the two hybrid proteins were expressed, as they were recognized by the antibodies directed against Vga(A) (Fig. 6). The Vga(A)-Msr(A) chimera migrated faster than the other hybrid because of the shorter lengths of the Vga(A) N-terminal ABC domain and the Msr(A) C-terminal domain. The hybrid proteins displayed the same pattern of subcellular localization as the wild-type Vga(A).

In conclusion, the ABC domains of Vga(A), although dissimilar, must be present on the same polypeptide to confer resistance to SGA and lincosamide antibiotics. Only this mode of assembly, even between fused heterologous ABC domains from Vga(A) and Msr(A), can interact with the membranes of staphylococci. We do not know how such an interaction is mediated, but we propose that an as yet unidentified membrane protein encoded by the chromosome could act as an anchor for Vga(A). This hypothesis is in line with the observation that Lmo0919 from *Listeria* is less efficient than Vga(A) in conferring antibiotic resistance when it is expressed in *Staphylococcus.* Further analysis of the functional properties of this group of ABC proteins will be required in order to achieve a better understanding of their modes of functioning. We demonstrated here that modification of either one of the two Walker B motifs of Vga(A) suppresses resistance properties without altering the membrane localization of the protein. More work is needed, especially fundamental studies investigating the molecular basis of antibiotic(s) recognition.

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