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 F1-F0 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 integrity 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 ambifaciens, 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, 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

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Nucleotide sequence (5' to 3')</th>
<th>Template</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>VG1-F</td>
<td>GATTAGGATCCCTTTTATTGCTTCT</td>
<td>pIP1617</td>
<td>Cloning of vga(A) gene</td>
</tr>
<tr>
<td>VG2-R</td>
<td>ATCAAGAATCTCAATAAAACACAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XbBaMut1-F</td>
<td>CTCGCAGGATGACTCTAGCCCTTTTTATTTGCTTCAATTAC</td>
<td>pIP1707</td>
<td>Site-directed mutagenesis</td>
</tr>
<tr>
<td>XbBaMut2-R</td>
<td>GTAATTGAGAACAATAAAAAGCCTAGTACCTCGACCTGACG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EcoMut1-F</td>
<td>TTATAAGAGGGACTAGTTAGAATATATTGAGG</td>
<td>pIP1708</td>
<td>Site-directed mutagenesis</td>
</tr>
<tr>
<td>EcoMut2-R</td>
<td>CTTCTTTTATTGTCTTCAATTAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VG3-F</td>
<td>TTAGAACCATTGAAATTAAGGAGC</td>
<td>pIP1617</td>
<td>Cloning of the second half of vga(A) gene</td>
</tr>
<tr>
<td>VG2-R</td>
<td>ATCAAGAATCTCAATAAAACACAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kp-F</td>
<td>GCAAGCTGTTGATAGCTATCTGACC</td>
<td>pIP1837</td>
<td>Construction of pIP1846 from pIP1835</td>
</tr>
<tr>
<td>Kp-R</td>
<td>GCAGTGCAGTGGCCCTTTTATTGTCTTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSR1-F</td>
<td>GGATGAGGGAATCTAATGGAACAAATATAC</td>
<td>IPF69</td>
<td>Cloning of first half of msr(A) gene</td>
</tr>
<tr>
<td>MSR2-R</td>
<td>AAAGAATCTCACTAGATAACCTTGGTTTTTCACCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSR3-F</td>
<td>GTTATCTAGAATTTCAATTTTCCAC</td>
<td>IPF69</td>
<td>Cloning of second half of msr(A) gene</td>
</tr>
<tr>
<td>MSR4-R</td>
<td>TCTATAGCTGTTATGGCTACTATTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WB1Mut1-F</td>
<td>AATCCTTGGAGAATGTTATCAACACACACACACAC</td>
<td>pIP1845</td>
<td>Mutagenesis of first Walker B motif of Vga(A)</td>
</tr>
<tr>
<td>WB1Mut2-R</td>
<td>GTTATCGTTGATGGTCCTATTAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WB2Mut1-F</td>
<td>AACCCTTGGAGAATGTTATCAACACACACACACACACACAC</td>
<td>pIP1845</td>
<td>Mutagenesis of second Walker B motif of Vga(A)</td>
</tr>
<tr>
<td>WB2Mut2-R</td>
<td>GTTATCGTTGATGGTCCTATTAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ListA-F</td>
<td>CAGGAGCTGATATTCAATCTCAACATCGA</td>
<td>CLIP72401</td>
<td>Cloning of lmo0919 from L. monocytogenes</td>
</tr>
<tr>
<td>ListA-R</td>
<td>CTATCGGTATCTCGCTCAGCATTCGCG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*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.

**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 (*m*) was used for the large-scale preparation of plasmids and expression of the MalE-fusion protein, respectively. *Staphylococcus aureus* strain 8325 (ATCC 12201) and *S. epidermidis* strain IPF69 (obtained from Dr. K. Duggan, Institute of Ophthalmology, London, England) were used as recipients in the site-directed mutagenesis experiments. Three clinical isolates were investigated: *S. aureus* BM10385, which is resistant to SGA by the presence of PTII (a 3.7-kb HindIII fragment containing the entire gene) and *S. epidermidis* strains RN4220 (22) and PM9, which is a derivative of strain JM109 carrying a deletion of the *malE* gene (*m*), respectively (16, 25), and *S. aureus* BM3302 (12), both of which contained 20 Eurobio (les Ulis, France). Disks of the SGA and SGB compounds, which were provided by Aventis-Pharma (Vitry-sur Seine, France), were compared with the SGA compound, were purchased from Sigma (Lisle d’Abeau, Chesnes, France). Other antibiotics 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-Cotquette, France) or Eurobio (les Ulis, France). Disks of the SGA and SGB compounds, which contained 20 μg of PTI and 40 μg of PTII, respectively, were homod. 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 (Beaver, 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 bicinechonic 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 QuickChange site-directed mutagenesis kit (Stratagene). High-fidelity DNA synthesis was performed with Pfu DNA polymerase (Stratagene). The PCR assays were run on a Crocodile III apparatus (Appligene, Illkirch, France) with thermocycling conditions that varied according to the length of the DNA fragment amplified. Amplifications 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*, pIP4734 (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

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant characteristics</th>
<th>SGA (µg/ml)</th>
<th>SGB (µg/ml)</th>
<th>PT (µg/ml)</th>
<th>LC (µg/ml)</th>
<th>CLI (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRB374</td>
<td>Shuttle vector, Km&lt;sup&gt;r&lt;/sup&gt;, polylinker, vgal promoter expressing WT Vga(A)</td>
<td>1 (NT)</td>
<td>8 (NT)</td>
<td>0.25 (NT)</td>
<td>1 (NT)</td>
<td>0.125 (NT)</td>
</tr>
<tr>
<td>pPI1746</td>
<td>pRB374vlvga(A) expressing WT Vga(A)</td>
<td>32 (NT)</td>
<td>5 (NT)</td>
<td>0.5 (NT)</td>
<td>4 (NT)</td>
<td>0.25 (NT)</td>
</tr>
<tr>
<td>pRB474</td>
<td>Shuttle vector, Km&lt;sup&gt;r&lt;/sup&gt;, polylinker, vgal promoter expressing WT Vga(A)</td>
<td>1</td>
<td>8</td>
<td>0.25 (0.125)</td>
<td>1 (0.5)</td>
<td>0.125</td>
</tr>
<tr>
<td>pPI1845</td>
<td>pRB474vlvga(A) online expressing WT Vga(A), pIP1810 (15), respectively</td>
<td>32</td>
<td>8</td>
<td>0.5</td>
<td>8</td>
<td>0.25 (1)</td>
</tr>
<tr>
<td>pPI1863</td>
<td>pRB474vlvga(B) expressing WT Vga(B)</td>
<td>4</td>
<td>8</td>
<td>2</td>
<td>2</td>
<td>0.25 (0.5)</td>
</tr>
<tr>
<td>pPI1867</td>
<td>pRB474vlvga(B) online expressing WT Vga(B)</td>
<td>8</td>
<td>8</td>
<td>0.5</td>
<td>2 (4)</td>
<td>0.25 (0.5)</td>
</tr>
<tr>
<td>pPI1877</td>
<td>pRB474vmsr(A)α-vgal(A)β coding for chimera Msr(A)-Vga(A)</td>
<td>1</td>
<td>8</td>
<td>0.25 (0.125)</td>
<td>1 (0.5)</td>
<td>0.125</td>
</tr>
<tr>
<td>pPI1879</td>
<td>pRB474vmsr(A)α-msr(A)β coding for chimera Vga(A)-Msr(A)</td>
<td>1</td>
<td>8</td>
<td>0.25 (0.125)</td>
<td>1 (0.5)</td>
<td>0.125</td>
</tr>
<tr>
<td>pPI1835</td>
<td>pRB474vlvga(A)α coding for N-terminal half of WT Vga(A)</td>
<td>1</td>
<td>8</td>
<td>0.25 (0.125)</td>
<td>1 (0.5)</td>
<td>0.125</td>
</tr>
<tr>
<td>pPI1837</td>
<td>pRB474vlvga(B)β coding for C-terminal half of WT Vga(A)</td>
<td>1</td>
<td>8</td>
<td>0.25 (0.125)</td>
<td>1 (0.5)</td>
<td>0.125</td>
</tr>
<tr>
<td>pPI1846</td>
<td>pRB474vlvga(B)α/vgal(A)β coding for both halves of WT Vga(A)</td>
<td>1</td>
<td>8</td>
<td>0.25 (0.125)</td>
<td>1 (0.5)</td>
<td>0.125</td>
</tr>
<tr>
<td>pPI1887</td>
<td>pRB474vlvga(A)α/glh131 coding for Vga(A)K409&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1</td>
<td>8</td>
<td>0.5 (0.25)</td>
<td>1 (0.5)</td>
<td>0.125</td>
</tr>
<tr>
<td>pPI1838</td>
<td>pRB474vlvga(A)α/glh220 coding for Vga(A)K409&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1</td>
<td>8</td>
<td>0.25 (0.125)</td>
<td>1 (0.5)</td>
<td>0.125</td>
</tr>
<tr>
<td>pPI1880</td>
<td>pRB474vlvga(A)κ283A44 coding for Msr(A)2&lt;sup&gt;201&lt;/sup&gt;</td>
<td>1</td>
<td>128 (256)</td>
<td>0.25</td>
<td>1 (0.5)</td>
<td>0.125</td>
</tr>
</tbody>
</table>

<sup>a</sup> 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).

<sup>b</sup> MICs are for <i>S. aureus</i> RN4220 transformants, and those in parentheses are for <i>S. epidermidis</i> BM3302 transformants when the MICs for the two strains are different.

<sup>c</sup> Few colonies grew at higher concentrations, but they harbored a full-length vgal(A) gene restored by an in-frame fusion of the two separate halves.

<sup>d</sup> Bases relative to the ATG start codon of the corresponding gene and modified by site-directed mutagenesis.

pPI1707 (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 vgal(A), leading to pPI1748 (Fig. 1B). Plasmid pPI1835, obtained by subcloning the HindIII-XbaI fragment of pPI1748 into pRB474, was the basis of all subsequent modifications. This plasmid codes for the N-terminal half of Vga(A) (Fig. 1C). Plasmids pPI1845, pPI1837, and pPI1867, which express the wild-type Vga(A) protein, the C-terminal half of Vga(A), and the Lmo0919 protein, respectively, were constructed by subcloning an XbaI-digested PCR product encompassing the RBS and the distal half of vgal(A) from pRB374 into the unique XbaI site of pPI1835. Plasmids that encode chimeric proteins were constructed by using the internal XbaI site of the vgal(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 pPI1835, leading to pPI1873 (Fig. 1D). XbaI-digested PCR products of the distal half of mstr(A) were inserted into the unique XbaI sites of pPI1835 and pPI1873. The resulting plasmids, pPI1879 and pPI1880, 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 pPI1739 (see below), which encodes the C-terminal half of Vga(A), was inserted into the unique XbaI site of pPI1873 to generate the fusion of the N-terminal ABC domain of Msr(A) with the C-terminal domain of Vga(A). Plasmids pPI1861 and pPI1863, which carry the vgal(A) and vgal(B) genes, respectively, were constructed by subcloning HindIII-KpnI and BamHI-KpnI fragments from pPI1717 (3) and pPI1810 (15), respectively, into pRB474. All the gene constructs were therefore under the control of the Bacillus subtilis vagl promoter (7), and all the last two constructions were placed 9 nucleotides downstream of the RBS of vgal(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 polynucleotides encoding the MalE part of the MalE fusion gene were replaced by the authentic malE sequence (J.-M. Clément, unpublished data). The EcoRI fragment of pPI1746 carrying the vgal(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, pPI1739, 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<sup>2</sup>, 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 observed at 100 kDa in SDS-PAGE. 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 White 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<sup>4</sup> 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 <i>S. aureus</i> RN4220 transformants and those in parentheses are for <i>S. epidermidis</i> BM3302 transformants when the MICs for the two strains are different.
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 MgCl₂, 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 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 antisera 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-FIβ, 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)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)w were the same for S. epidermidis. Interestingly, we observed that vga(A) or vga(A)w 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)w, 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)w genes (38). We suggest, therefore, that vga(A) or its variant, vga(A)w, is involved in the LSA phenotype occasionally found among staphylococcal isolates (14, 17, 24).

Neither vga(A) nor vga(A)w 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-
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 \( \text{lmo0919} \) was cloned on pRB474, it was able to confer resistance to SGA (MIC \( \text{H11005} \) g/ml for both hosts) and LC (MIC \( \text{H9262} \) g/ml for \( \text{S. epidermidis} \)). This phenotype is similar to that conferred by \( \text{vga} \) and \( \text{vga} \) in the same hosts, although the levels of resistance are lower. The slight difference might be due to the less efficient expression of \( \text{lmo0919} \) 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 \( \text{Listeria} \) strains to SGA compounds and lincosamide antibiotics (5, 40). We are attempting to inactivate the \( \text{lmo0919} \) gene in \( \text{L. monocytogenes} \) 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 encompassing residues between the LID and the SIGNATURE motifs of the N-terminal ABC domain.

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 \( \text{S. aureus} \) RN4220 and BM3327 and \( \text{S. epidermidis} \) BM3302, BM10385, and IPF69 were probed with the antibodies. Because of smearing problems due to protein A, only the \( \text{S. epidermidis} \) strains were used for Western blot analyses. There were few cross-reactive bands in \( \text{S. epidermidis} \) strains (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.

**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.
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) strongly cross-reacted with the polyclonal antiserum (Fig. 3, lane 5), whereas Vga(B) and Lmo919 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 F1-F0 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 protein, 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.

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 of Vga(A) 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)
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 lincomamide 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 lincomamide 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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REFERENCES


