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An *Enterococcus faecalis* ABC Homologue (Lsa) Is Required for the Resistance of This Species to Clindamycin and Quinupristin-Dalfopristin

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Enterococcus faecalis **isolates are resistant to clindamycin (CLI) and quinupristin-dalfopristin (Q-D), and this is thought to be a species characteristic. Disruption of a gene (*abc-23*, now designated *lsa*, for "lincosamide and streptogramin A resistance") of *E. faecalis* was associated with a ≥40-fold decrease in MICs of Q-D (to 0.75 μg/ml), CLI (to 0.12 to 0.5 μg/ml), and dalfopristin (DAL) (to 4 to 8 μg/ml) for the wild-type *E. faecalis* parental strain (Q-D MIC, 32 μg/ml; CLI MIC, 32 to 48 μg/ml; DAL MIC, 512 μg/ml). Complementation of the disruption mutant with *lsa* on a shuttle plasmid resulted in restoration of the MICs of CLI, Q-D, and DAL to wild-type levels. Under high-stringency conditions, *lsa* was found in 180 of 180 isolates of *E. faecalis* but in none of 189 other enterococci. Among 19 *erm*(B)-lacking *Enterococcus faecium* strains, 9 (47%) were highly susceptible to CLI (MIC, 0.06 to 0.25 μg/ml) and had DAL MICs of 4 to 16 μg/ml; for the remaining *erm*(B)-lacking *E. faecium* strains, the CLI and DAL MICs were 4 to >256 and 2 to >128 μg/ml, respectively. In contrast, none of 32 *erm*(B)-lacking *E. faecalis* strains were susceptible (CLI MIC range, 16 to 32 μg/ml; DAL MIC range, ≥32 μg/ml). When *lsa* was introduced into an *E. faecium* strain initially susceptible to CLI, the MICs of CLI and DAL increased ≥60-fold and that of Q-D increased 6-fold (to 3 to 6 μg/ml). Introduction of *lsa* into two DAL-resistant (MICs, >128 μg/ml), Q-D-susceptible (MICs, 0.5 and 1.5 μg/ml) *E. faecium* strains (CLI MICs, 12 and >256 μg/ml) resulted in an increase in the Q-D MICs from 3- to 10-fold (to 8 and >32 μg/ml), respectively. Although efflux was not studied, the similarity (41 to 64%) of the predicted Lsa protein to ABC proteins such as Vga(A), Vga(B), and Msr(A) of *Staphylococcus aureus* and YjcA of *Lactococcus lactis* and the presence of Walker A and B ATP-binding motifs suggest that this resistance may be related to efflux of these antibiotics. In conclusion, *lsa* appears to be an intrinsic gene of *E. faecalis* that explains the characteristic resistance of this species to CLI and Q-D.**

Over the past few years, enterococci have emerged as important bacterial pathogens in nosocomial infections (12, 25–27, 40). These organisms have acquired and/or intrinsic resistance to many different antibiotics (18, 19), which poses a serious problem for the treatment of patients infected with these organisms. Studies have shown that *Enterococcus faecalis*, unlike *E. faecium*, is usually resistant to quinupristin-dalfopristin (Q-D), with MICs of 4 to ≥32 μg/ml (3, 10, 12, 35), and that both species are typically resistant to clindamycin (CLI) (12). Acquired resistance to Q-D in *E. faecium* has also been described, and contributing mechanisms include drug inactivation by enzymes, structural or conformational alterations in ribosomal target binding sites, and efflux of antibiotic out of cells (3, 10). In *E. faecalis*, however, the mechanism of resistance to Q-D has not been well studied.

We recently investigated the presence of putative transporters in *E. faecalis*, identified 34 possible transporter homologs, and made disruption mutants of 31 of these (8). Among these mutants were ones with increased susceptibility to novobiocin, pentamidine, daunorubicin, and norfloxacin and one, whose disrupted gene was originally designated *abc-23*, with reduced susceptibility to Q-D and CLI; the MICs of ~20 other compounds were similar to those for wild-type OG1RF (8). In the present study, we have further studied this gene and its effect by comparing it with known ABC transporters, by complementing the disruption mutant and introducing the gene into *E. faecium* strains on a shuttle vector, and by determining its distribution among *Enterococcus* spp. Based on these results, we have renamed this gene *lsa* in recognition of its apparent role in the intrinsic resistance of *E. faecalis* to lincosamides (CLI) and streptogramin A (dalfopristin [DAL]).

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MATERIALS AND METHODS

Bacterial strains and MIC studies. The bacteria used in this study were obtained from the collection of our laboratory, which was compiled over the past 20 years. The recipient strains and plasmids used in the study are listed in Table 1. These include *E. faecalis* strain OG1RF (29) and *E. faecium* isolates SE34 (TX1330; recovered from feces of a healthy community volunteer [7]), TX2466 (a clinical isolate [23]), and D344-S (36); the *E. faecium* strains were chosen because of their differing susceptibilities to CLI (MICs of 8 to 16, <0.25, and >256 μg/ml, respectively). A total of 492 isolates of enterococci, including 257 of *E. faecalis*, 216 of *E. faecium*, 6 of *E. hirae*, 5 of *E. durans*, 2 of *E. casseliflavus*, 2 of *E. mundtii*, 1 of *E. gallinarum*, 2 of *E. solitarius*, and 1 of *E. raffinosus* (12, 44), were used for *lsa* probing and/or susceptibility testing. MICs were determined by agar dilution (30, 31) or by the E test (PDM Epsilometer test; AB BIODISK North America, Inc., Piscataway, N.J.) in accordance with the manufacturer's instructions. Erythromycin (ERY), CLI, kanamycin (KAN), and chloramphenicol (CHL) were purchased from Sigma Chemical Co., St. Louis, Mo., and

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Purpose and relevant characteristic(s)	Reference(s) or source
Strains		
E. faecalis OG1RF	Rif^r Fus^r; used for *lsa* insertional mutagenesis	29
E. faecalis V583	TIGR sequenced strain; used to amplify *lsa* for disruption and complementation experiments	40
E. faecium TX2466	Used as recipient strain; CLI MIC, 0.19 μg/ml	23
E. faecium TX1330	Used as recipient strain; CLI MIC, 12–24 μg/ml	7
E. faecium D344-S	Used as recipient strain; CLI MIC, >256 μg/ml; *erm*(B)$^+$	36
TX5332	*lsa* gene disruption mutant (OG1RF *lsa*::pTEX4577); Kan^r	8
TX5333	Complemented *lsa* gene disruption mutant [TX5332(pWM401::*lsa*)]; Kan^r Chl^r	This study
Plasmids		
pTEX4577	pBluescript SK (−) with *aph*(*3*')-*IIIa* inserted into *Sca*I site; Kan^r; used for *lsa* insertion mutagenesis	13, 43
pWM401	Shuttle vector; Chl^r Tet^r	47
pCR2.1 vector	PCR product cloning vector	Invitrogen
pTEX5333	pWM401::*lsa*; Chl^r; used for complementation of TX5332	This study

quinupristin, DAL, and Q-D were provided by Aventis Pharma S.A., Vitry-sur-Seine Cedex, France.

DNA extraction, PCR, sequencing, and cloning. DNA extraction (46) and PCRs were performed with the PCR Optimizer kit (Invitrogen, San Diego, Calif.); PCR products were analyzed by automated DNA sequencing at the Microbiology and Molecular Genetics core facility, University of Texas Medical School, Houston, Tex. Sequence analysis was done by using the BLAST network service of the National Center for Biotechnology Information. The Genetics Computer Group software package (Genetics Computer Group, Madison, Wis.) was used to compare similarities among other sequences. ClustalW, at the Baylor College of Medicine website, was used, and the GeneDoc software was used for editing and shading of sequences. Cloning was done with standard methods (42).

Disruption mutation in *lsa* (*abc-23*) of *E. faecalis*. The disruption mutation in strain OG1RF was created previously (8). Briefly, the disruption mutant was created by using a PCR-amplified ~700-bp intragenic DNA fragment from *E. faecalis* strain V583 inserted into previously described pBluescript derivative pTEX4577 containing *aph*(*3′*)-*IIIa* (13, 43), resulting in pTEX5332, which was electroporated into competent cells of OG1RF and selected with KAN at 2,000 μg/ml. The resulting mutant was previously shown by PCR to have the targeted insertion of the plasmid (8). In the present study, the insertion was also confirmed by hybridizing *Eco*RI digests of genomic DNAs of wild-type *E. faecalis* OG1RF and the *lsa* disruption mutant with an intragenic *lsa* DNA probe under high-stringency conditions. In addition, the susceptibility of this mutant to quinupristin, DAL, ampicillin, tetracycline, and ERY was determined by the E test. Recombinant colonies of TX5332 (*lsa* disruption mutant) were further analyzed by pulsed-field gel electrophoresis (28) of *Sma*I-digested genomic DNA by comparison with wild-type OG1RF to confirm the host background.

Complementation of TX5332 (*lsa* disruption mutant). An ~2-kb fragment (the 1,497-bp *lsa* ORF, ~300 bp upstream and ~200 bp downstream obtained from the V583 *E. faecalis* TIGR database) was PCR amplified from wild-type *E. faecalis* V583 and was first cloned into the pCR2.1 vector of the TA cloning kit. This PCR fragment was excised from vector pCR2.1 by digestion with restriction enzymes *Xba*I and *Bam*HI and then recloned into shuttle vector pWM401 (47), resulting in pTEX5333. Plasmid pTEX5333 DNA was electroporated into competent cells of TX5332 (*lsa* disruption mutant), and selection was made on Todd-Hewitt agar (Becton Dickinson, Cockeysville, Md.) supplemented with 0.25 M sucrose, KAN at 2,000 μg/ml and CHL at 8 μg/ml. The resulting colonies were restreaked on KAN-CHL plates and analyzed for resistance to CLI, Q-D, and DAL by the E-test or agar dilution method.

Determination of the stability of the components of TX5333. Growth curves comparing wild-type OG1RF, TX5332 (the *lsa* disruption mutant), and TX5333 (the complemented *lsa* disruption mutant) were determined with and without antibiotics selective for the chromosomal disruption and/or the shuttle plasmid. Observations were made by measuring optical density at 600 nm hourly, and CFU determinations at 24 h were made on brain heart infusion (BHI) agar (Difco Laboratories, Detroit, Mich.) with and without antibiotics. BHI broth was used to grow wild-type OG1RF; BHI and BHI-KAN were used to grow TX5332; and BHI, BHI-KAN, and BHI-KAN-CHL were used to grow TX5333.

Effect of *lsa* on *E. faecium* antibiotic resistance . Electrocompetent cells of *E. faecium* strains TX1330, TX2466, and D344-S were prepared as previously described (14, 20). Following electroporation of pTEX5333, selection was made on Todd-Hewitt agar supplemented with 0.25 M sucrose and CHL at 8 μg/ml. The resulting colonies were restreaked onto BHI agar-CHL plates and tested for susceptibility to CLI, Q-D, DAL, and quinupristin by the E-test or agar dilution method and also by pulsed-field gel electrophoresis (28) of *Sma*I-digested genomic DNA and compared to each parental *E. faecium* strain.

Distribution of *lsa* among *Enterococcus* spp. Three hundred sixty-nine enterococcal isolates were tested for the presence of *lsa* by colony lysate hybridization under high-stringency conditions with an *lsa* intragenic DNA probe as previously described (43). The *erm*(B) DNA gene probe was PCR amplified as previously described (44) and used for hybridization under high-stringency conditions. The DNA gene probes and hybridization conditions for $efaA_{fs}$ and $aac(6')\text{-}Ii_{fm}$ were the same as those used in a previous study (12).

RESULTS AND DISCUSSION

Determination of stability of components of the *E. faecalis lsa* disruption mutant by complementation and susceptibility testing. Testing of the complemented *lsa* disruption mutant (TX5333) showed that when TX5333 was grown in BHI broth

TABLE 2. MICs for *E. faecalis* OG1RF, TX5332 (*lsa* disruption mutant), and TX5333 (complemented mutant)

Organism	MIC (μg/ml)				
	CLI[a]	Q-D[a]	DAL[b]	Quinupristin[b]	ERY[a]
OG1RF	32–48	32	512	16	1
TX5332 (OG1RF *lsa*::pTEX4577)[c]	0.12–0.5	0.75	4–8	16	1
TX5333 [TX5332(pWM401::*lsa*)][d]	32–48	32	512	16	1

[a] E-test MICs (determined multiple times, resulting in a range of concentrations).
[b] Agar dilution MICs.
[c] Determined in the presence of KAN at 2,000 μg/ml in Mueller-Hinton agar to maintain the chromosomal disruption.
[d] Determined in the presence of KAN at 2,000 μg/ml and CHL at 8 μg/ml in Mueller-Hinton agar to maintain the chromosomal disruption and the shuttle plasmid.

TABLE 3. Susceptibility of *E. faecalis* and *E. faecium* strains to CLI and streptogramins

Organism (no. of isolates)	MIC (μg/ml) range (agar dilution)			
	CLI	Q-D	DAL	Quinupristin
E. faecium (68)				
erm(B)$^+$ (49)	>256	1–16	8–≥32[a]	≥64[a]
erm(B) lacking (19)				
Cli^{rb} (10)	4–>256	1–16	2–>128	8–>32
Cli^s (9)	0.06–0.25	0.5–1	4–16	8–32
E. faecalis (95)				
erm(B)$^+$ (63)	128–>256	2–64	≥32[c]	≥64[c]
erm(B) lacking (32)	16–32	2–64	≥32[d]	8–≥64[d]

[a] Only 18 isolates were tested.
[b] Determined by using NCCLS breakpoints for *S. aureus* (susceptible, ≤0.5 μg/ml; intermediate, 1–2 μg/ml, resistant, ≥4 μg/ml).
[c] Only nine isolates were tested.
[d] Only 10 isolates were tested.

or in BHI with CHL at 8 μg/ml, there was an ~3-log reduction in the number of CFU of Kan^r colonies per ml versus when this strain was grown in the presence of KAN at 2,000 μg/ml–CHL at 8 μg/ml. Because of the apparent instability of the chromosomal disruption (Kan^r), the mutant was subsequently tested for susceptibility in the presence of KAN at 2,000 μg/ml in Mueller-Hinton II agar (Becton Dickinson) for TX5332 to maintain the chromosomal disruption and in Mueller-Hinton II agar with KAN at 2,000 μg/ml and CHL at 8 μg/ml to maintain the chromosomal disruption and shuttle plasmid.

The MICs determined for wild-type *E. faecalis* OG1RF, TX5332, and TX5333 are presented in Table 2; the MICs of ampicillin, tetracycline (data not shown), norfloxacin, ciprofloxacin, ethidium bromide, and other compounds previously tested (8) showed no difference among these strains. The *lsa* mutant was tested on multiple occasions and showed a >40-fold decrease in the MIC of Q-D (0.75 μg/ml) and a ≥64-fold decrease in the MICs of CLI (0.12 to 0.5 μg/ml) and DAL (4 to 8 μg/ml) versus the wild-type *E. faecalis* parental strain (Q-D MIC, 32 μg/ml; CLI MIC, 32 to 48 μg/ml; DAL MIC, 512 μg/ml). This indicates that *lsa* or some downstream function is necessary for resistance to CLI and DAL in *E. faecalis*. Complementation of the disruption mutant with *lsa* (and ~300 bp of the upstream sequence and ~200 bp of the downstream sequence) on a shuttle plasmid resulted in restoration of the MICs of CLI (from 0.12 to 0.5 μg/ml to 32 to 48 μg/ml), Q-D (from 0.75 μg/ml to 32 μg/ml), and DAL (from 4 to 8 μg/ml to 512 μg/ml) (Table 2). This confirms the importance of *lsa*, as opposed to a possibly cotranscribed downstream gene. The MIC of quinupristin was 16 μg/ml for OG1RF and the two derivatives, and the ERY MIC was 1 μg/ml. The increase in the MICs of CLI (a lincosamide) and DAL (streptogramin A) and the lack of a change in the MICs of macrolides (ERY) or quinupristin (streptogramin B) correspond to the LS_A phenotype (5), and the data presented here clearly indicate the involvement of *lsa* in this phenotype in *E. faecalis*.

Effect of *lsa* on the antibiotic resistance of *E. faecium*. To determine if the 2-kb *lsa* region could function in a heterologous host, we selected strains of *E. faecium* to serve as recipients of the *lsa* gene by evaluating 68 *E. faecium* strains for susceptibility to CLI and for the presence of *erm*(B), since the latter influences CLI susceptibility (Table 3). While most of the isolates were *erm*(B)$^+$, an interesting observation was the bimodal distribution of CLI MICs among the *erm*(B)-lacking *E. faecium* isolates. Of 19 *erm*(B)-lacking *E. faecium* isolates, 10 showed high CLI MICs (MIC, 4 to >256 μg/ml) and 9 showed low CLI MICs (MIC, 0.06 to 0.25 μg/ml). In contrast, none of the 32 *erm*(B)-lacking *E. faecalis* isolates showed low CLI MICs (MIC, 16 to 32 μg/ml). We chose three *E. faecium* strains (Tables 1 and 4) with different CLI and DAL susceptibilities [one of which is *erm*(B)$^+$] and introduced the *lsa* gene on a shuttle plasmid. For the most susceptible strain (TX2466 [CLI MIC, 0.19 μg/ml; DAL MIC, 2 μg/ml; Q-D MIC, 0.5 μg/ml]), there was a marked increase in the MICs of CLI (12 μg/ml) and DAL (>128 μg/ml) and an increase to 3 to 6 μg/ml in the Q-D MIC. The ranges of the MICs of Q-D and the other agents were derived by testing on three or more different occasions. For the highly DAL-resistant (MIC, >128 μg/ml) and moderately CLI-resistant (MIC, 12 to 24 μg/ml) recipient TX1330, the MICs of DAL and CLI changed very little, if at all, after *lsa* was introduced but the Q-D MIC increased from

TABLE 4. MICs for *E. faecium* strains with and without *lsa*

Strain	MICs (μg/ml)[a]				
	CLI[b]	Q-D[b]	DAL[c]	Quinupristin[c]	ERY[b]
TX2466	0.19	0.5	2	8–16	1.5–2
TX2466(pWM401)	0.064	0.5	4	16	0.75
TX2466(pWM401::*lsa*)	12	3–6	>128	16	1–1.5
TX1330	12–24	0.5	>128	16	0.25
TX1330(pWM401)	12	1.5	>128	16	0.125
TX1330(pWM401::*lsa*)	16–24	6–8	>128	16	0.125–0.25
D344-S [*erm*(B$^+$)][d]	>256	1.5–3	>128	16	>256
D344-S(pWM401)	>256	3	>128	16	>256
D344-S(pWM401::*lsa*)	>256	≥32	>128	16	>256

[a] MICs for *E. faecium* containing shuttle vector pWM401 or the shuttle vector with *lsa* were determined in the presence of CHL at 8 μg/ml in the medium.
[b] E-test MICs.
[c] Agar dilution MICs.
[d] Slow-growing strain. MIC data were obtained on BHI agar.

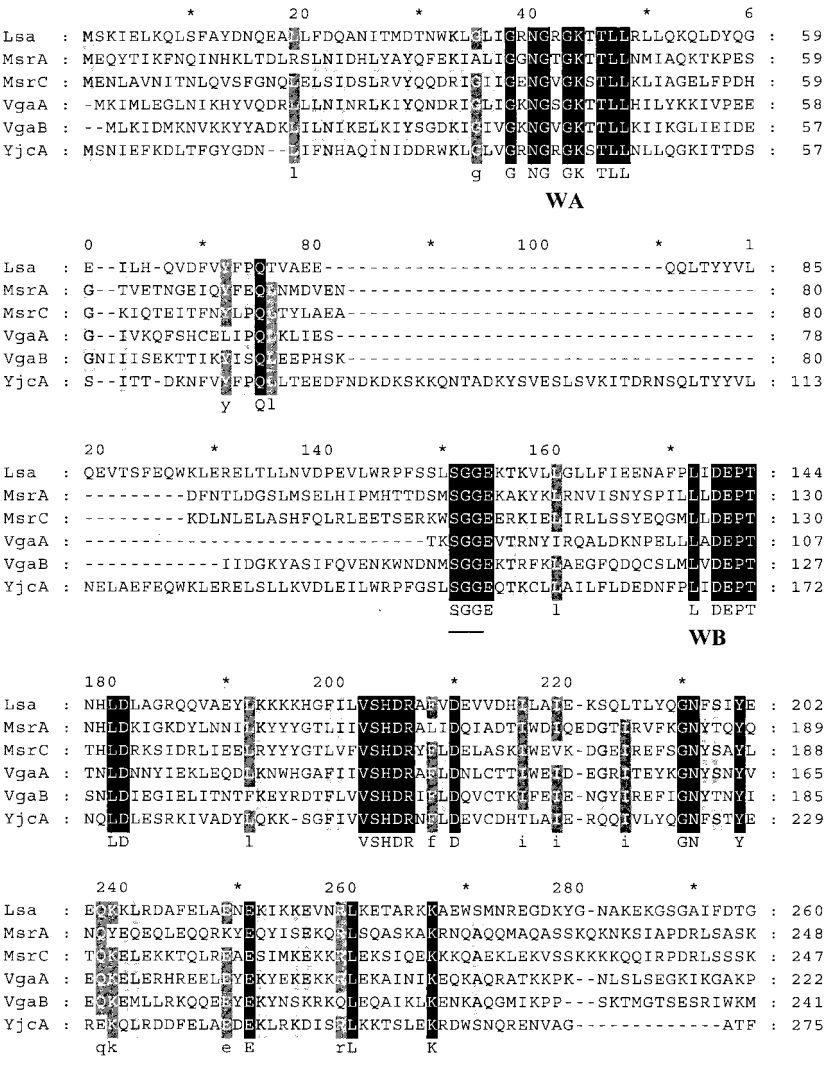

FIG. 1. Multiple-sequence alignment of Lsa, Msr(A), Msr(C), Vga(A), Vga(B), and YjcA. ClustalW, at the Baylor College of Medicine website, was used, and the GeneDoc software was used for editing and shading. Shown are the two ATP-binding domains, consisting of Walker A and B motifs (underlined) and an SGG sequence (underlined). The bottom line shows the consensus sequence. Regions in which the six proteins are 100% identical are marked in solid black, dark gray shows regions >80% identical, and light gray shows regions with >60% identical amino acids.

0.5 μg/ml to 6 to 8 μg/ml. The most pronounced increase in the Q-D MIC (from 3 to ≥32 μg/ml) was seen in strain D344 *erm*(B)$^+$, which was initially highly DAL resistant (MIC, >128 μg/ml) and CLI resistant (MIC, >256 μg/ml). These data show the interspecies function of *lsa* and show a marked increase in the MICs of CLI and DAL and a moderate increase in the MIC of Q-D when it is introduced into *E. faecium* strains susceptible to these antibiotics. Bozdogan and Leclercq (5) also noted the influence of an LS_A phenotype in *E. faecium* on Q-D MICs, where introduction of the *sat*(A) or *vgb* gene into a Q-D-susceptible *E. faecium* strain with the LS_A phenotype conferred resistance to Q-D while, in contrast, introduction of these genes into another *E. faecium* strain susceptible to lincosamide, streptogramin A, and streptogramin B resulted in a 1- or 2-dilution increase in the MIC of Q-D (5).

Distribution of *lsa* among *Enterococcus* spp. Under high-stringency conditions, hybridization of colony lysates of 369 enterococci showed that *lsa* was present in all 180 *E. faecalis* isolates but not in 189 other enterococcal isolates (data not shown). Although most of our isolates were of human origin and animal isolates may differ, these results suggest that *lsa* is species specific for *E. faecalis* and may be an intrinsic gene of this species.

Characterization of *lsa*. The 2-kb region used for complementation, consisting of the 1,497-bp ORF of *lsa*, ~300 bp upstream, and ~200 bp downstream, was analyzed. The predicted Lsa protein (498 amino acids [aa]) showed similarities to known or postulated ABC proteins of other gram-positive bacteria [64% similarity to YjcA (513 aa) of *Lactococcus lactis* (4), 42% similarity to MsrC (493 aa) of *E. faecium* (44), and ca. 41% similarity to Vga(A) (523 aa) (1), Vga(B) (2), and Msr(A) (38) of *Staphylococcus aureus* (Fig. 1)]. ABC transporters usually contain four single or joined components that are arranged into two homologous halves, each containing an ATP-binding

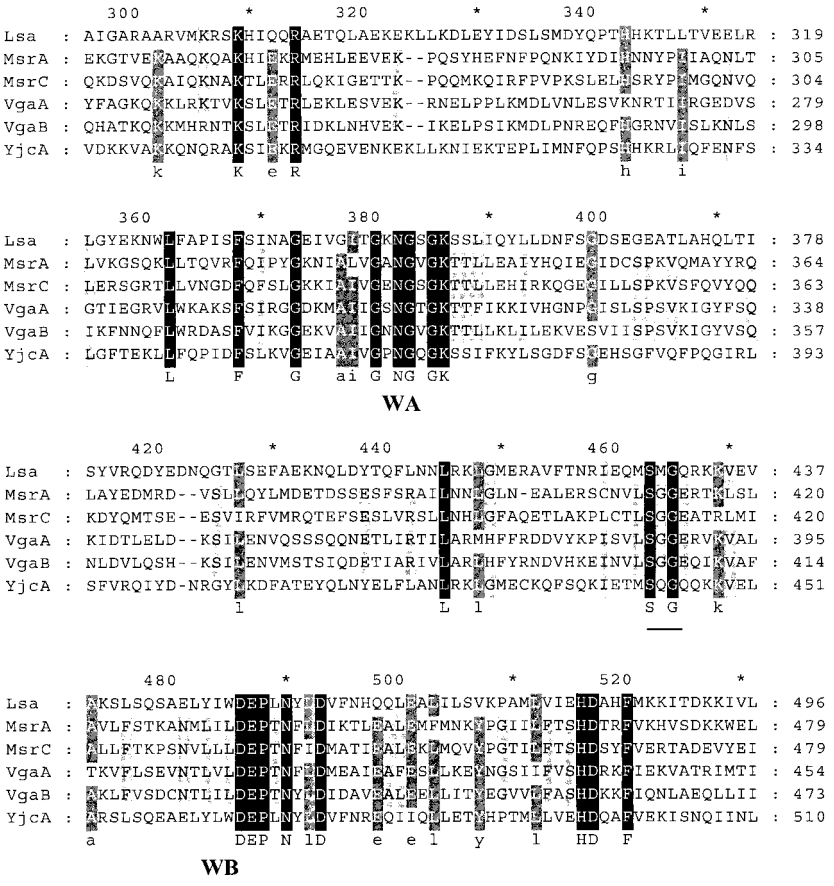

FIG. 1—*Continued.*

domain and a membrane-spanning domain composed of several (usually six) putative α-helical transmembrane segments (9, 16, 17, 41). In the case of Msr(A), the two ATP-binding regions are fused into a single protein with internally homologous domains while in other instances, the ATP-binding regions are monomeric and likely form dimers in vivo (9, 22, 24, 41). ABC-type ATPase characteristic features, including a putative ABC signature sequence and the Walker A and B motifs, as reported in the literature for Msr(A), Vga(A), and Vga(B) (1, 2, 37), were identified in the corresponding regions of Lsa (Fig. 1). Hydropathy analysis of Lsa with the TMAP and Tmpred website programs revealed no transmembrane helix or a single strong transmembrane helix, respectively. This is similar to Msr(A), which contains no hydrophobic stretches that might be potential membrane-spanning domains, and it remains unclear for Msr(A) whether it utilizes hydrophobic proteins encoded by the genes *smpA*, *smpB*, and *smpC* mapping on the staphylococcal chromosome (37). Genes encoding other ABC proteins that contain two ATP-binding domains but no hydrophobic domain in gram-positive organisms include *lmrC*, a lincomycin resistance gene from *Streptomyces lincolnensis* (33); *oleB*, an oleandomycin resistance gene from *S. antibioticus* (32); *srmB*, a spiramycin resistance gene from *S. ambofaciens* (15); and a tylosin resistance gene from *S. fradiae* (39). A 45-aa putative peptide was also identified preceding the *lsa* start codon; the presence of this sequence seems to be important for the expression of drug resistance, as attempts to complement the disruption mutant with only cloned *lsa* failed to restore resistance to CLI, Q-D, and DAL. The presence of leader peptide sequences for *msr*(A), *erm*(A), *erm*(B), and *erm*(C) has been reported or postulated to be involved in posttranscriptional regulation of the expression of these resistance genes (6, 11, 21, 24, 34, 38, 45).

In conclusion, we have shown the importance of the *lsa* gene of *E. faecalis* for the intrinsic LS_A phenotype (CLI and DAL resistance) and Q-D resistance of this species. The apparent species specificity of *lsa* also suggests that it may be useful for the identification of *E. faecalis* isolates. We did not study efflux, but Lsa showed sequence similarities to known and postulated ABC transporters, including Msr(A), Vga(A), and Vga(B), suggesting that the protection of *E. faecalis* against CLI and DAL may be related to ATP-energized efflux of these antibiotics. We have also shown that *lsa* is functional in isolates of *E. faecium* and increases the MICs of CLI, DAL, and/or Q-D to various degrees, depending on the initial host's level of susceptibility to these agents.

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