ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 2005, p. 32–39 Vol. 49, No. 1
0066-4804/05/$08.00+0 doi:10.1128/AAC.49.1.32–39.2005

Differences in the *Enterococcus faecalis lsa* Locus That Influence Susceptibility to Quinupristin-Dalfopristin and Clindamycin

Kavindra V. Singh[1,2] and Barbara E. Murray[1,2,3]*

Center for the Study of Emerging and Reemerging Pathogens,[1] Department of Internal Medicine, Division of Infectious Diseases,[2] and Department of Microbiology and Molecular Genetics,[3] The University of Texas Medical School at Houston, Houston, Texas

Received 2 June 2004/Returned for modification 19 July 2004/Accepted 18 September 2004

We have previously shown that the *Enterococcus faecalis lsa* gene, encoding the putative ABC protein Lsa, influences resistance to quinupristin-dalfopristin (Q-D) and clindamycin (CLI). We have now found that, while cloned *lsa* from *E. faecalis* strain V583 (lsa_V) fully restored resistance to Q-D, CLI, and dalfopristin (DAL) lost by the OG1 *lsa* disruption mutant TX5332 and also caused increased MICs for *Lactococcus lactis* LM2301, cloned *lsa* from OG1 (lsa_{OG}) did not cause any increase in MICs for either species. Sequencing of ca. 2 kb of these two *lsa* alleles found differences between lsa_{OG} and lsa_V in the upstream region as well as in the 5′ and 3′ halves of the *lsa* gene. To investigate the reason for the phenotypic differences expressed by the two cloned loci, 5′ half plus 3′ half hybrid constructs were created. When introduced into both TX5332 and *L. lactis*, cloned $lsa_{V5'OG3'}$ conferred increases in MICs of Q-D, CLI, and DAL similar to those of cloned lsa_V while cloned $lsa_{OG5'V3'}$ showed a moderate increase in MICs relative to those of lsa_{OG}, indicating that both halves of the locus can influence resistance expression. After site-directed mutagenesis of the cloned *lsa* alleles at positions −131 and −133 (relative to the putative Lsa start codon ATG), which converted two A's of lsa_V to the G and T of lsa_{OG} and vice versa, MIC testing showed that mutagenized lsa_{OG} ($lsa_{OG\text{-}M}$) was strongly influenced by these changes in terms of conferring increased MICs of Q-D, CLI, and DAL relative to lsa_{OG} while the phenotype of mutagenized lsa_V ($lsa_{V\text{-}M}$) was less influenced, with moderately decreased MICs, primarily to CLI, relative to lsa_V. In conclusion, this study found that changes in different regions of the *E. faecalis lsa* locus influence the ability of cloned *lsa* to confer resistance to Q-D, CLI, and DAL.

Quinupristin-dalfopristin (Q-D) is a mixture of streptogramin B and streptogramin A, respectively, and studies have shown that the species *Enterococcus faecalis* has intrinsic resistance to this compound (2, 8, 19, 25). Our previous study of Lsa (which stands for lincosamide and streptogramin A resistance), a predicted ABC protein homologue, implicated it as being the cause of the intrinsic resistance of *E. faecalis* to clindamycin (CLI), dalfopristin (DAL), and Q-D (25). Specifically, TX5332, a mutant of OG1RF (henceforth referred to as OG1) with a disruption in *lsa* (formerly *abc23*) (6) showed a marked increase in susceptibility to Q-D, CLI, and DAL, and complementation of TX5332 with an intact *lsa* gene (lsa_V) from the sequenced *E. faecalis* strain V583 (17) restored resistance to Q-D, CLI, and DAL (25). Consistent with this finding, a subsequent study by Dina et al. (7) found that the *lsa* alleles of two clinical isolates of *E. faecalis* susceptible to lincosamides and dalfopristin contained mutations within *lsa* that produced premature termination codons, further supporting the role of *lsa* in Q-D resistance.

In the present study, we attempted complementation of the mutant TX5332 with a recombinant plasmid carrying an intact *lsa* allele from wild-type *E. faecalis* strain OG1 (lsa_{OG}) and found that this strain's gene did not restore resistance, unlike the equivalent fragment from strain V583 in our previous study (25). We compared the *lsa* sequences derived from both *E. faecalis* V583 and OG1, constructed hybrid fragments by combining different regions of *lsa* from each strain, generated site-directed mutagenized DNA fragments of *lsa*, and then tested the impact of these cloned elements on the susceptibility of TX5332 to Q-D, CLI, quinupristin, and DAL. We also tested these constructs in another gram-positive host, *Lactococcus lactis* LM2301 (27), and PCR amplified, sequenced, and studied *lsa* and upstream sequences from six Q-D- and CLI-susceptible *E. faecalis* isolates of different origins.

MATERIALS AND METHODS

Bacterial strains. The strains and plasmids used in the study are listed in Table 1. These include *E. faecalis* strains OG1RF (14) (referred to here as OG1) and V583 (17, 22), *L. lactis* LM2301 (27), four Q-D- and CLI-susceptible *E. faecalis* isolates TX4107 to TX4110 from animal feed (provided courtesy of S. Simjee, U.S. Food and Drug Administration, Bethesda, Md.), and two Q-D-susceptible clinical *E. faecalis* isolates, TX0263 and TX0271, isolated from the Houston area. Mutant strains of *E. faecalis* used as controls to determine the effect of kanamycin (KAN; used for selection of the OG1 *lsa* disruption mutant) on MICs of test compounds include TX5076 (30), TX5243 (18), and TX5248 (18), which were constructed by using cloned intragenic gene fragments in the suicide vector pTEX4577 containing *aph*(3′)-*llla* (24), the method used for the construction of the OG1 *lsa* disruption mutant, TX5332 (6, 25).

MIC studies. MICs were determined by the broth microdilution method (15, 16) or by E-test (PDM Epsilometer test; AB BIODISK North America, Inc., Piscataway, N.J.) by using Mueller-Hinton II broth (MHB) or Mueller-Hinton II agar (MHA) (Becton Dickinson Company, Sparks, Md.) as test media following the manufacturer's instruction. KAN, CLI, and chloramphenicol (CHL) were purchased from Sigma Chemical Co., St. Louis, Mo., and quinupristin, DAL, and Q-D were provided by Aventis Pharma S.A., Vitry-sur-Seine Cedex, France. For the *lsa* disruption mutant TX5332 and for TX5332 derivatives containing the shuttle vector pWM401 (29), KAN (2,000 μg/ml) and KAN (2,000 μg/ml) plus

* Corresponding author. Mailing address: Center for the Study of Emerging and Reemerging Pathogens, University of Texas Medical School—Houston, 6431 Fannin, 2.112MSB, Houston TX 77030. Phone: (713) 500-6745. Fax: (713) 500-5495. E-mail: bem.asst@uth.tmc.edu.

TABLE 1. Bacterial strains and plasmids used in the study

Strains or plasmid	Purpose and relevant characteristic(s)	Reference(s) or source
E. faecalis strain(s)		
OG1RF	Used for *lsa* insertional mutagenesis and to amplify *lsa* for complementation experiments; Rif^r, Fus^r	14
V583	TIGR-sequenced strain; used to amplify *lsa* for complementation experiments	17, 22
TX4107–TX4110	Q-D- and CLI-susceptible isolates from animal feed	This study
TX0263, TX0271	Q-D- and CLI-susceptible clinical isolates	This study
TX5076	Antigen gene disruption mutant generated with pTEX4577 containing *aph*(3′)-*llla;* Kan^r	30
TX5243	*sprE* gene disruption mutant [OG1RF *sprE*::pTEX4577 containing *aph*(3′)-*llla*]; Kan^r	18
TX5248	*orf1* gene disruption mutant [OG1RF *orf1*::pTEX4577 containing *aph*(3′)-*llla*]; Kan^r	18
TX5332	*lsa* gene disruption mutant [OG1RF *lsa*::pTEX4577 containing *aph*(3′)-*llla*]; Kan^r	25
	TX5332(pTEX5333.04) (contains lsa_V); Kan^r, Chl^r	25
	TX5332(pTEX5333.03) (contains lsa_{OG}); Kan^r, Chl^r	This study
	TX5332(pTEX5333.08) (contains $lsa_{OG5'V3'}$); Kan^r, Chl^r	This study
	TX5332(pTEX5333.09) (contains $lsa_{V5'OG3'}$); Kan^r, Chl^r	This study
	TX5332(pTEX5333.12) (contains a mutagenized gene, $lsa_{V\text{-}M}$); Kan^r, Chl^r	This study
	TX5332(pTEX5333.13) (contains a mutagenized gene, $lsa_{OG\text{-}M}$); Kan^r, Chl^r	This study
L. lactis strains		
LM2301	Used as a gram-positive host for *E. faecalis lsa*	27
	LM2301(pTEX5333.04) (contains lsa_{V5}); Chl^r	This study
	LM2301(pTEX5333.03) (contains lsa_{OG}); Chl^r	This study
	LM2301(pTEX5333.08) (contains $lsa_{OG5'V3'}$); Chl^r	This study
	LM2301(pTEX5333.09) (contains $lsa_{V5'OG3'}$); Chl^r	This study
	LM2301(pTEX5333.12) (contains $lsa_{V\text{-}M}$); Chl^r	This study
	LM2301(pTEX5333.13) (contains $lsa_{OG\text{-}M}$); Chl^r	This study
TX5392	LM2301(pWM401); Chl^r	This study
Plasmids		
pTEX4577	pBluescript SK(−) with *aph*(3′)-*IIIa* inserted into the ScaI site; Kan^r, used for insertional mutagenesis	24
pWM401	Shuttle vector; Chl^r, Tet^r	29
pCR2.1 vector	PCR product cloning vector	Invitrogen
pTEX5333.03	pWM401::lsa_{OG}, contains a ca. 2-kb *lsa* fragment from OG1RF, used for complementation of TX5332 and transformation of *L. lactis* LM2301; Chl^r	This study
pTEX5333.04	pWM401::*lsa*v, contains a ca. 2-kb *lsa* fragment from V583, used for complementation of TX5332 and transformation of *L. lactis* LM2301; Chl^r	25
pTEX5333.08	pWM401::$lsa_{OG5'V3'}$, contains a hybrid gene with the 5′ half of lsa_{OG} and 3′ half of lsa_V, used for complementation of TX5332 and transformation of *L. lactis* LM2301; Chl^r	This study
pTEX5333.09	pWM401::$lsa_{V5'OG3'}$, contains a hybrid gene with the 5′ half of lsa_V and 3′ half of lsa_{OG}, used for complementation of TX5332 and transformation of *L. lactis* LM2301; Chl^r	This study
pTEX5333.12	pWM401::$lsa_{V\text{-}M}$, contains ca. 2-kb lsa_V fragment mutagenized in its upstream region at positions −131 and −133 with A's replaced with a G and a T, used for complementation of TX5332 and transformation of *L. lactis* LM2301; Chl^r	This study
pTEX5333.13	pWM401::$lsa_{OG\text{-}M}$, contains ca. 2-kb lsa_{OG} fragment mutagenized in its upstream region at positions −131 and −133 with G and T replaced by A's, four additional nucleotide changes were found at −109, +1248 (silent), +1572 (downstream), and +1517 (downstream), used for complementation of TX5332 and transformation of *L. lactis* LM2301; Chl^r	This study

CHL (8 μg/ml), respectively, were added to the MIC test media; for LM2301 derivatives with pWM401, CHL (8 μg/ml) was added to the test media. For wild-type strains, only test compounds were added to the media.

DNA extraction, PCR, sequencing, and cloning. DNA extraction was done by using previously published methods (12, 28). PCRs were performed by using the optimized buffer B kit (Invitrogen, San Diego, Calif.). PCR was generally performed in volumes of 50 μl, with an initial denaturation at 94°C for 2 min followed by 25 to 30 cycles consisting of 1 min of denaturation at 94°C, 2 min of annealing at 55°C, 3 min of extension at 72°C, and a 10-min final extension at 72°C. Primers used for amplification and sequencing are listed in Table 2 and were initially designed by using the V583 genome database (www.tigr.org). PCR products were analyzed by automated DNA sequencing at the Microbiology and

TABLE 2. Primers used in the study

Primer	Position[a]	Sequence (5′–3′)[b]
abc2F[c]	−309, −288	+GGCAATCGCTTGTGTTTTAGCG
abc2R	+1735, +1713	−GTGAATCCCATGATGTTGATACC
abc3F[d]	+108, +129	+GATTGGCCGCAATGGCCGTGGG
abc3R	+1123, +1101	−GGTGAGCCAAAGTGGCTTCGCC
abc3F2	+1349, +1372	+GATGAACCCCTTAATTACTTGG
abc7F	−203, −182	+GACGAAAAGAGGGATTCGTGCG
abc7R	−11, −32	−CCATAAAGCAAAATTGATGCAG
abc2F[e]	−309, −288	+GGCAATCGCTTGTGTTTTAGCG
lsa mut	−105, −85	−GAATTCTTTAAGCTAATTTC
Mega primer F (OG1) (double stranded)[f]	−309, −85	GGCAATCGCTTGTGTTTTAGCGAATAATTTTTAATCAGAATGAAAGCAAGGGGCCTTCGTTGACAAATCAGTGAAGAG ATGCTAAATTCTAGATAATAAAAGACGAAAAGAGGGATTCGTGCGTTGAAAATTTAAGTTAATTTTTTAGAGAAACTGTATA ACTTTGCCTGATTGTAACGGGTGGAGTGTGCGGATTTTCGGAAATTAGCTTAAAGAATTC
Mega primer F (V583) (double stranded)[g]	−309, −85	GGCAATCGCTTGTGTTTTAGCGAATAATTTTTAAATTTTTATCAGAATGAAAGCAAGGGGCCTTCGTTGACAAATCAGTGAAGAG ATGCTAAATTCTAGATAATAAAAGACGAAAAGAGGGATTCGTGCGTTGAAAATTTAAGTTTAATTTTTTAGAGAAACTGTATA ACTTTGCCTGAATATAACGGGTGGAGTGTGCGGATTTTCGGAAATTAGCTTAAAGAATTC

[a] Bases relative to ATG.
[b] +, sense primer; −, antisense primer.
[c] Primers abc2F and abc2R were used to amplify intact *lsa* on a 2-kb fragment.
[d] Primers abc3F to abc7R are intragenic primers used to confirm the *lsa* sequence.
[e] Primers abc2F and lsa mut were used for site-directed mutagenesis (to generate mega primer).
[f] Mega primer F(OG1) and abc2R were used to generate $lsa_{V\text{-}M}$. Boldfaced T and G were targeted in site-directed mutagenesis.
[g] Mega primer F(V583) and abc2R were used to generate $lsa_{OG\text{-}M}$. Boldfaced A and A were targeted in site-directed mutagenesis.

Molecular Genetics core facility, University of Texas Medical School, Houston. Sequence analysis was done by using the BLAST network service of the National Center for Biotechnology Information, and DNASTAR software (Madison, Wis.) was used to compare similarities among other sequences. Cloning was done by using the pCR2.1 vector of the TA cloning kit following the manufacturer's instructions or with standard methods (23) into the shuttle vector pWM401 (29).

Generation of OG1 *lsa* and hybrid *lsa* fragments. A ca. 2-kb OG1 fragment consisting of intact *lsa* (lsa_{OG}) equivalent to the previously published ca. 2-kb V583 *lsa* fragment (lsa_V) (25) was PCR amplified with primers abc2F and abc2R from wild-type *E. faecalis* strain OG1, cloned into the pCR2.1 vector, and designated pTEX5333.01. The lsa_{OG} fragment was then excised from pTEX5333.01 by digestions with XbaI and BamHI and was recloned into the shuttle vector pWM401, resulting in pTEX5333.03. The ca. 2-kb V583 *lsa* fragment (lsa_V) previously cloned into pCR2.1 was designated pTEX5333.02 and, in pWM401, pTEX5333.04 (Table 1).

Using the single KpnI site located in pCR2.1 and a SmaI site located in approximately the middle of the *lsa* gene of both OG1 and V583, we exchanged the two halves (including the upstream region with the 5′ coding half). The resulting plasmid constructs in pCR2.1 were designated pTEX5333.06, containing the upstream plus 5′ half (nucleotides −309 to +1102 relative to the predicted start codon) of OG1 *lsa* plus the 3′ half (nucleotides +1103 to +2044) of *lsa* of V583 ($lsa_{OG5'V3'}$), and pTEX5333.07, containing the upstream plus 5′ half (nucleotides −309 to +1102) of V583 *lsa* plus the 3′ half (nucleotides +1103 to +2044) of *lsa* of OG1 ($lsa_{V5'OG3'}$). The cloned fragments were excised from the pCR2.1 vector by digestion with BamHI and EcoRV and recloned into the shuttle vector pWM401. The recombinant shuttle plasmids containing hybrid *lsa* genes were designated pTEX5333.08 (containing $lsa_{OG5'V3'}$) and pTEX5333.09 (containing $lsa_{V5'OG3'}$) (Table 1). In cloning experiments, we noticed many deletions by restriction enzyme digestions; therefore, all of the cloned *lsa* derivatives in the shuttle vector were resequenced in both directions before use for complementation experiments.

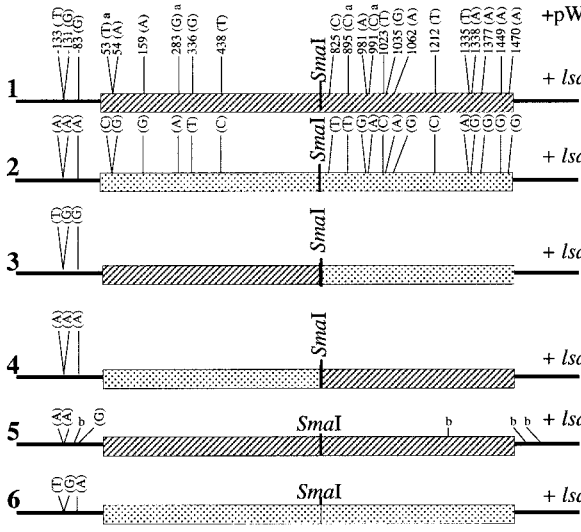
Site-directed mutagenesis of *lsa*. Two nucleotides in the regions immediately upstream of V583 and of OG1 *lsa* were targeted for mutagenesis by using the previously published mega primer method (5). The primers used for mutagenesis experiments are listed in Table 2. The A's at positions −131 and −133 (bases relative to the predicted ATG start codon) in the upstream region of the ca. 2-kb lsa_V fragment were replaced with a G and a T, respectively. Similarly, the same nucleotide positions were targeted in the ca. 2-kb lsa_{OG} fragment for changing the G and T at −131 and −133, respectively, to A's. Mutagenized *lsa* fragments were generated by PCR. Both mutagenized *lsa* fragments were cloned into pCR2.1 and then into pWM401. The resulting recombinant shuttle plasmids were designated pTEX5333.12 ($lsa_{V\text{-}M}$) and pTEX5333.13 ($lsa_{OG\text{-}M}$), respectively (Table 1), and their sequences were reconfirmed by sequencing.

Complementation of the OG1 *lsa* disruption mutant, TX5332, and transformation of *L. lactis* LM2301. Electrocompetent cells of TX5332 and LM2301 were prepared by following the previously published method (9), and the electroporation conditions used were the same as those previously published (10). Recombinant plasmids pTEX5333.03 (lsa_{OG}), pTEX5333.04 (lsa_V), pTEX5333.08 ($lsa_{OG5'V3'}$), pTEX5333.09 ($lsa_{V5'OG3'}$), pTEX5333.12 ($lsa_{V\text{-}M}$), and pTEX5333.13 ($lsa_{OG\text{-}M}$) were electroporated into competent cells of TX5332 and LM2301. Complemented colonies were recovered on Todd-Hewitt agar (Difco Laboratories, Sparks, Md.) plus KAN (2,000 μg/ml) plus CHL (8 μg/ml) and Todd-Hewitt agar plus CHL (8 μg/ml) for TX5332 and LM2301, respectively. The presence of *lsa* and the identity of the host were confirmed by pulsed-field gel electrophoresis (PFGE) (13) and hybridization (14) to an intragenic *lsa* DNA probe. The resulting complemented colonies in TX5332 and LM2301 were designated as described in Table 1.

Q-D- and CLI-susceptible *E. faecalis* isolates. For Q-D- and CLI-susceptible isolates (TX4107 to TX4110, TX0263, and TX0271), PCR-amplified *lsa*-containing fragments were sequenced in both directions, and the sequences were aligned with each other as well as with *lsa* from OG1 and V583 (The Institute for Genomic Research [TIGR] genome database [www.tigr.org] and our own results). The relatedness of the isolates was examined by PFGE (14, 26).

RESULTS

Sequencing of lsa_{OG} and lsa_V and use for complementation of TX5332 and LM2301. Our previously published study showed that the intact lsa_V gene on a ca. 2-kb DNA fragment cloned into a shuttle vector (pTEX5333.04) was able to fully restore the MIC decreases seen with TX5332 (Fig. 1A) for Q-D (from 0.75 to 32 μg/ml), CLI (from 0.12 to 0.5 μg/ml to 32



A

E. faecalis strains	MICs (μg/ml) Q-D	CLI	DAL
OG1RF	32	32-48	≥512
TX5332	0.75	0.12-0.5	16
+pWM401	0.75	0.12-0.5	16
+ lsa_{OG}	0.75	0.12	16
+ lsa_{V}	32	32	≥512
+ $lsa_{OG5'V3'}$	8-12	8-12	≥512
+ $lsa_{V5'OG3'}$	32	32	≥512
+ $lsa_{OG\text{-}M}$	32	16-24	≥512
+ $lsa_{V\text{-}M}$	32	16-24	≥512

B

L. lactis strains	MICs (μg/ml) Q-D	CLI	DAL
LM2301	0.75-1	0.09-0.12	32
+pWM401	0.75-1	0.09-0.12	16
+ lsa_{OG}	1	0.09-0.12	16
+ lsa_{V}	3	3-4	128
+ $lsa_{OG5'V3'}$	1-1.5	0.38	32
+ $lsa_{V5'OG3'}$	3-4	3-4	64
+ $lsa_{OG\text{-}M}$	2-3	1.5	128
+ $lsa_{V\text{-}M}$	2	0.5-0.75	64

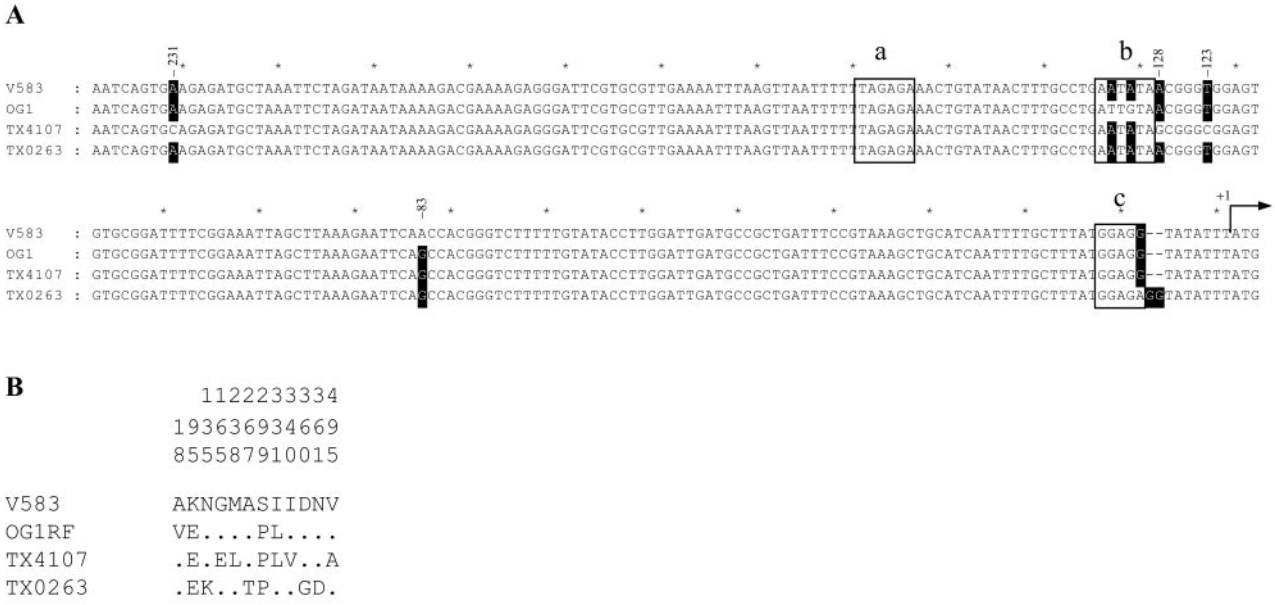
a: These n.t. differences result in amino acid changes;
b: The four additional (unintended) n.t. changes at −109, +1248 (silent) and at +1517 and +1578 (downstream of predicted stop codon).

FIG. 1. Susceptibility of *E. faecalis* and *L. lactis* derivatives to Q-D, DAL, and CLI. Rows 1 to 6 show the *lsa* gene constructs and nucleotide differences of lsa_{OG} (ca. 2-kb *lsa* from OG1RF), lsa_{V} (ca. 2-kb *lsa* from V583), $lsa_{OG5'V3'}$ (hybrid *lsa* with 5′ half of OG1RF and 3′ half of V583), $lsa_{V5'OG3'}$ (hybrid *lsa* with 5′ half of V583 and 3′ half of OG1RF), $lsa_{OG\text{-}M}$ (mutagenized lsa_{OG} at positions −131 and −133 where G and T were replaced with A's [and four unintended changes were also found]), and $lsa_{V\text{-}M}$ (mutagenized lsa_{V} at positions −131 and −133 where A's were replaced with G and T, respectively) cloned into pWM401 and their influence on susceptibility of TX5332 (*E. faecalis* OG1 *lsa* disruption mutant) (A) and *L. lactis* LM2301 (B) to Q-D, DAL, and CLI. n.t., nucleotide.

to 48 μg/ml) and DAL (from 16 to 512 μg/ml) (25) (Fig. 1A, row 2). In the present study, cloned lsa_{OG} on the equivalent ca. 2-kb fragment (pTEX5333.03) did not increase the resistance of TX5332 to Q-D, CLI, or DAL (Fig. 1A, row 1). We also determined the effect of lsa_{OG} and lsa_{V} on MICs of Q-D, CLI, and DAL in another gram-positive host LM2301 (Fig. 1B, rows 1 and 2), and again, no increase in MICs was conferred by lsa_{OG} versus wild-type LM2301 or LM2301(pWM401) while lsa_{V} conferred an increase in MICs of Q-D (from 0.75 to 1 μg/ml to 3 μg/ml), CLI (from 0.094 to 0.125 μg/ml to 3 to 4 μg/ml) and of DAL (from 32 to 128 μg/ml), consistent with the results with the *E. faecalis lsa* disruption mutant, TX5332. MICs of quinupristin were the same for LM2301 and LM2301 containing the recombinant plasmids, as was also the case with TX5332 (data not shown).

In an effort to understand these differences, we determined the nucleotide sequence of lsa_{OG} and lsa_{V}, which showed that the ca. 2-kb lsa_{OG} (accession no. AY587982) fragment differed by 22 nucleotides from the lsa_{V} sequence (Fig. 1, rows 1 and 2, and 2A). In the upstream region, we found differences at positions −131 and −133 (relative to the predicted ATG start codon), a possible strong promoter region for lsa_{V}, where the A's present in lsa_{V} were replaced with a G and a T in lsa_{OG} (Fig. 2A), and at −83 where A in lsa_{V} was replaced with a G in lsa_{OG}. The peptide sequence of Lsa_{OG} also showed 4 amino acid changes versus Lsa_{V}; two of those are in the N-terminal half (V18 and E95 in OG1 versus A18 and K95 in V583) while the other two amino acid differences are in the C-terminal half (P299 and L331 in OG1 versus S299 and I331 in V583) (Fig. 2B).

In an effort to determine the influence of the difference in these three regions of lsa_{OG} versus lsa_{V}, we first exchanged the two halves of *lsa*. The hybrid *lsa* fragment of pTEX5333.09 (containing $lsa_{V5'OG3'}$) (Fig. 1A, row 4) fully restored the MICs of Q-D (from 0.75 to ≥32 μg/ml), CLI (from 0.125 to 0.5 μg/ml to 32 μg/ml), and DAL (from 16 μg/ml to 256 to >512 μg/ml) to levels equivalent to those of lsa_{V} (Fig. 1, row 2). Similarly, this plasmid in LM2301 also conferred an increase in MICs of Q-D similar to the MIC levels of these drugs against LM2301 containing cloned lsa_{V} (Fig. 1B, row 4), with slightly less increase for DAL (64 versus 128 μg/ml for lsa_{V}). This suggests that the presence of A's at the −131, −133, and −83 positions of lsa_{V} and the two amino acid changes present in the 5′ half are sufficient to fully complement the *lsa* disruption in TX5332 as well as cause the increased MICs of Q-D and CLI in LM2301 (Fig. 1, row 4).



B

	1122233334 193636934669 855587910015
V583	AKNGMASIIDNV
OG1RF	VE....PL....
TX4107	.E.EL.PLV..A
TX0263	.EK..TP..GD.

FIG. 2. Sequence differences among four strains of *E. faecalis*. (A) Region upstream (from −239 to +1) of *lsa* (including putative start codon) derived from *E. faecalis* strains V583, OG1RF, TX4107 (representative of feed clone), and TX0263 (a human clinical isolate). The nucleotide differences are highlighted (black with white letters). The nucleotide sequence from −309 to −240 is not shown, as it was identical among all strains. Predicted (from V583) −35 (a), −10 (b), and ribosomal binding sites (c) of V583 are marked by boxes. (B) Amino acid differences encoded by the *lsa* alleles. The amino acids that are shared by all of the strains are not shown. The amino acids present in *E. faecalis* V583 at each of the variable sites are shown (B). The position of each variable amino acid within the sequenced fragment is shown in the numbers above the amino acids, read vertically. The sequence of *lsa* from TX4107 is identical to that of TX4108, TX4109, and TX4110, and the sequences of TX0263 and TX0271 are also identical.

TX5332 complemented with a second hybrid fragment (pTEX5333.08 containing $lsa_{OG5'V3'}$) showed a moderate increase in MICs of Q-D and CLI (from 0.75 and 0.125 μg/ml, respectively, to 8 to 12 μg/ml for both drugs) (Fig. 1A, row 3) and higher MICs of DAL (from 16 to >512 μg/ml). This indicates that the amino acid changes in the 3′ half of *lsa* can also contribute to resistance. LM2301 containing cloned $lsa_{OG5'V3'}$ showed little to modest increases in MICs relative to lsa_{OG} of Q-D (1 versus 1 to 1.5 μg/ml), DAL (16 versus 32 μg/ml), and CLI (0.09 to 0.12 versus 0.38 μg/ml) (Fig. 1B, row 3), implying that the host background can also influence the effect of the *lsa* gene.

To explore the independent contribution of the nucleotide changes at the −131 and −133 positions (which could generate a strong promoter in V583) versus the changes in the coding region, we mutagenized the lsa_{OG} and lsa_{V} fragments with primers directed toward this region. The *lsa* nucleotide sequences of two mutagenized fragments, pTEX5333.12 containing $lsa_{V\text{-}M}$, and pTEX5333.13, containing $lsa_{OG\text{-}M}$, were reconfirmed in both directions. No nucleotide changes were found in $lsa_{V\text{-}M}$ except the ones targeted at nucleotide positions −131 and −133 (Fig. 1, row 6). However, in $lsa_{OG\text{-}M}$ (Fig. 1, row 5), four unintended nucleotide changes were found (one upstream of *lsa* at nucleotide position −109, one at +1248 [silent] in the *lsa* coding region, and two downstream of the putative stop codon at positions +1517 and +1572). We did not evaluate the specific effect of each unintended nucleotide change.

The *lsa* disruption mutant, TX5332, complemented separately with $lsa_{V\text{-}M}$ and $lsa_{OG\text{-}M}$, showed full restoration of MICs of Q-D (from 0.75 to ≥32 μg/ml) and DAL (from 16 to ≥512 μg/ml) (Fig. 1A), similar to MICs of Q-D and DAL for wild-type *E. faecalis* OG1 and the *lsa* disruption mutant complemented with lsa_{V}. Both $lsa_{V\text{-}M}$ and $lsa_{OG\text{-}M}$ also conferred an increase in MICs of CLI (from 0.12 to 0.5 μg/ml to 16 to 24 μg/ml); however, this increase was less than that seen with the cloned lsa_{V} (from 0.12 to 0.5 μg/ml to 32 μg/ml) (Fig. 1A). These results show that the changes in the coding region, as well as those in the upstream region, can independently influence resistance to Q-D, DAL, and/or CLI.

In *L. lactis*, both $lsa_{OG\text{-}M}$ and $lsa_{V\text{-}M}$ increased the MICs of Q-D (from 0.75 to 1 μg/ml to 2 to 3 μg/ml), DAL (from 32 μg/ml to 64 to 128 μg/ml), and CLI (from 0.09 to 0.12 μg/ml to 0.5 to 1.5 μg/ml) (Fig. 1B); the latter are somewhat lower than those conferred by lsa_{V} or $lsa_{V5'OG3'}$, consistent with the effect of these plasmids on CLI MICs when present in TX5332. Preliminary reverse transcription (RT)-PCR results (unpublished data) implied much lower expression of *lsa* in $lsa_{V\text{-}M}$ than $lsa_{OG\text{-}M}$, consistent with the increased levels of drug resistance of $lsa_{OG\text{-}M}$ in *L. lactis*. However it is also possible that some of the unintended nucleotide changes may influence RNA stability.

Even though MIC comparisons were done to derivatives also grown in KAN with and without CHL, we also tested other *E. faecalis* OG1-derived strains, TX5076 (30), TX5243 (18), and TX5248 (18), which have genes inactivated with the help of suicide vector pTEX4577 containing *aph*(3′)-*llla* (conferring KAN resistance), the same vector used in construction of TX5332 (6, 25). All three mutants showed MICs of Q-D and CLI on MHA plus KAN (2,000 μg/ml) plates similar to those determined with wild-type *E. faecalis* OG1 on MHA, indicating that the presence of KAN in the test media did not decrease

the Q-D and CLI MICs (data not shown) for KAN-resistant mutants. Similarly, both LM2301(pWM401) in MHB plus CHL (8 μg/ml) and LM2301 in MHB showed equivalent MICs of test drugs (Fig. 1B), suggesting that the presence of the shuttle vector or CHL (8 μg/ml) in test media did not affect the MICs of all of the drugs tested, except for a slight decrease in MICs of DAL with pWM401.

Q-D- and CLI-susceptible *E. faecalis* isolates. To investigate the Q-D and CLI susceptibility noted among several other *E. faecalis* isolates, their *lsa* sequences were analyzed. Nucleotide sequences of *lsa* from a group of animal feed isolates TX4107 (accession no. AY737525) to TX4110 (MICs: Q-D, 0.5 μg/ml; CLI, 0.38 to 0.5 μg/ml; DAL, 8 μg/ml; quinupristin, 8 to 16 μg/ml) were found to be identical with each other; by PFGE, TX4107 and TX4108 appeared clearly related (a three-band difference) while TX4109 and TX4110 are possibly related to TX4107 and TX4108 (four- to five-band differences). The two clinical isolates TX0263 (accession no. AY737526) and TX0271 (MICs for both: Q-D, 1 μg/ml; CLI, 2 to 4 μg/ml; DAL, 128 to 256 μg/ml; quinupristin, 8 to 16 μg/ml) had identical nucleotide sequences with each other; however, they were different (a >7-band difference) from each other and from the animal feed isolate by PFGE. In the *lsa* allele of the feed isolates (e.g., TX4107) (Fig. 2A), four and five nucleotide changes were found versus the upstream sequence of the *lsa* alleles of V583 and OG1, respectively. Like lsa_V, the feed allele has −131A and −133A and, like lsa_{OG}, −83G; at the other upstream positions, where the feed *lsa* allele differs, lsa_V and lsa_{OG} are the same (Fig. 2A). In the coding region of the feed isolates, Lsa differs from Lsa_V by 7 amino acids (three in the N-terminal half and four in the C-terminal half) (Fig. 2B); three of these amino acids (at positions 95, 299, and 331) are also found in Lsa_{OG}. There were 23 silent nucleotide changes in lsa_{TX4107} versus lsa_V.

The *lsa* sequence of the susceptible human isolate (TX0263), in comparison to lsa_V, had −83G (like lsa_{OG}) and one nucleotide difference in the putative ribosomal binding site, followed immediately by two additional nucleotides (Fig. 2A). In the Lsa coding region, there were five amino acid differences versus Lsa_V (two in the N-terminal half and three in the C-terminal half) with K95E (like Lsa_{OG} and the feed isolate), S299P (like Lsa_{OG} and the feed isolate), D360G, and N361D (Fig. 2B). In lsa_{TX0263}, there were 15 silent nucleotide changes versus lsa_V. We did not find any frameshift mutations in *lsa* of TX4107 or TX0263, as has been reported previously as the cause of susceptibility of *E. faecalis* isolates to lincosamide and streptogramin A compounds (7). While we note the consistent changes in all susceptible strains at −83 and residues 95 and 299, these changes have not been further evaluated.

DISCUSSION

We previously identified 34 putative transporter system components in the *E. faecalis* V583 sequence at TIGR and made mutants of 31 of these in strain OG1, some of which showed increased susceptibility to various compounds including novobiocin, pentamidine, daunorubicin, norfloxacin, Q-D, and CLI (6). One of these mutants, *abc23*, with reduced susceptibility to Q-D, DAL, and CLI, was renamed *lsa* when we showed, with an *E. faecalis* OG1 disruption mutant as well as with complementation, that this gene influenced resistance to lincosamide (e.g., CLI) and streptogramin A compounds (e.g., DAL) (25). We also described the presence of conserved elements of ABC proteins, including the Walker A and B motifs, which are involved in the binding and hydrolysis of ATP, as well as an ABC signature sequence thought to be involved in energy transduction (11, 25). Lsa does not have any obvious transmembrane domains (7, 25), and it appears that the predicted two ATP-binding regions of Lsa represent a fused single protein, as has been previously described for Msr(A) (21). In the literature, both Msr(A) and Lsa, along with Vga(B), Vga(A), Orf5, and MsrC, have been classified into the class 2 type of ABC proteins which have duplicated fused ABC domains and lack identifiable transmembrane domains, and it has been suggested that these systems may be involved in cellular processes other than transport (4, 20).

In the present study, we found that, in contrast to cloned *lsa* from V583, the cloned *lsa* allele of OG1 did not increase resistance to Q-D, CLI, and DAL when introduced into TX5332 or *L. lactis*. This is despite the fact that disruption of *lsa* eliminated resistance to these antibiotics in OG1. Sequencing found several differences between the OG1 and V583 *lsa* loci, including the N-terminal half of Lsa, where two amino acid changes were identified, the first of which (V18A) is located before the first conserved Walker A motif (amino acid positions 40 to 48) and the second of which (E95K) is located between the Walker A and Walker B motifs (amino acid positions 141 to 144) of the first ABC domain. The C-terminal Lsa half also contains two amino acid changes (P299S and L331I), both of which are located in the interdomain sequences before the Walker A (amino acid positions 347 to 351) motif of the second ABC domain. In considering other ABC homologues, we note that in a study on P-glycoprotein, the replacement of a small number of residues near the Walker B motif of nucleotide binding domain 1 by the corresponding nucleotide binding domain 2 residues caused profound alterations of the drug resistance profile both in mammalian and yeast cells, showing the importance of amino acids and their positions for proper functioning of nucleotide binding domain 1 (3). Also, in another published study (20), the functionality and importance of the N- and C-terminal domains of Msr(A), both of which are essential, were demonstrated when independently cloned domains did not function when introduced into *S. aureus* strain RN4220 separately and did not complement if expressed from separate plasmids, suggesting the essential structural or functional role of the Q-linker region that joins the two ATP-binding domains (1, 20). These observations suggested that the changes we found may indeed influence the function of Lsa.

While we did not interconvert specific residues, our experiments show that, in the face of what would be a poor −10 region (ATTGTA in OG1), the amino acid sequence encoded by *lsa* from OG1 did not cause an increase in MICs to the agents tested when cloned and inserted into either TX5332 or *L. lactis* LM2301. Preliminary RT-PCR results (unpublished data) also indicate considerably less *lsa* transcript from an OG1-like promoter region than a V583-like promoter region. However, even this predicted a poor −10 region which, together with the amino acids changes found in Lsa_V, can substantially restore resistance ($lsa_{V\text{-}M}$) (Fig. 1, row 6). The higher MICs with $lsa_{OG5'V3'}$ versus lsa_{OG} indicate that the 3′ amino acids can exert an effect independent of the 5′ half of the locus,

although the effect is less than that when both the 5′ and 3′ coding regions of V583 occur together. Conversely, when present together with the strong promoter-like region found in V583, the amino acids found in Lsa_{OG} restored MICs ($lsa_{OG\text{-}M}$) (Fig. 1, row 5) almost to the levels of wild-type lsa_V, and the presence of the V583 upstream sequence plus the V583 5′ half were sufficient to fully increase the MIC to those levels conferred by the entire lsa_V locus. A contribution of the N-terminal amino acid differences, independent of the different upstream nucleotides, is suggested by the higher MICs of $lsa_{V\text{-}M}$ (Fig. 1, row 6) versus $lsa_{OG5'V3'}$ (Fig. 1, row 3), which differ at residues 18 and 95, although these alleles also differ at the −83 position.

Although the cloned *lsa* locus from OG1 does not confer resistance, we know that disruption of *lsa* in OG1 causes loss of resistance. Since it appears very unlikely from sequence analysis that there is a polar effect, this suggests that another promoter or an upstream *cis*-acting element is functional in the natural setting in OG1, causing its resistance to Q-D, CLI, and DAL. Additional sequence analysis of *lsa* and preliminary mRNA and RT-PCR experiments also indicate that there is another potential promoter further upstream of *lsa*, which may drive expression of the native gene. Future studies, including mapping of the transcriptional start site(s), will assess these potential promoter regions and transcriptional differences in the wild-type strains versus recombinant genes.

We also analyzed other isolates of Q-D-susceptible *E. faecalis*. In a study by Dina et al. (7), it has been found previously with Q-D- and CLI-susceptible *E. faecalis* that frameshift mutations in *lsa* resulting in a premature termination codon were the cause of susceptibility in these isolates. In the present study of six Q-D- and CLI-susceptible *E. faecalis* isolates, among which we found two clonal types, we did not detect frameshift mutations or premature termination codons in our isolates. However, in addition to unique changes not shared with Lsa_{OG} or each other, there were common amino acids (95E and 299P) found among Lsa_{OG} and the representative types, Lsa_{TX4107} and Lsa_{TX0263}, which were different than that of Lsa_V and, additionally, 331L shared by Lsa_{OG} and Lsa_{TX4107}. These two *lsa* loci also share the −83G with lsa_{OG}, a position we did not study here. Whether these or other changes contribute to the lowered MICs for Q-D and CLI resistance in these isolates is also an area of further interest.

In conclusion, we demonstrated that changes in different regions of *lsa* (the upstream region of *lsa* which can create a strong promoter, the rest of the *lsa* 5′ half, and the *lsa* 3′ half) can influence the MICs for Q-D, CLI, and DAL. We have also shown that *lsa* is functional in the *L. lactis* LM2301 strain where changes in all three regions influenced the MICs of Q-D, CLI, and DAL to various degrees. Further studies of mRNA expression, start site mapping of lsa_{OG} and lsa_V, and efflux experiments to determine whether active transport occurs versus ribosomal protection, as suggested may occur with class 2 ABC systems (20), should help clarify the mechanisms by which these phenotypic differences occur.

ACKNOWLEDGMENTS

This work was supported in part by NIH grant R37 AI 47923 from the Division of Microbiology and Infectious Diseases, NIAID, to B.E.M.

We thank Fang Teng for helpful discussions, Sreedhar R. Nallapareddy for help with preparation of computer graphics, and S. Simjee (U.S. Food and Drug Administration) for providing some of the Q-D- and CLI-susceptible isolates.

REFERENCES

1. **Allignet, J., and N. El Solh.** 1997. Characterization of a new staphylococcal gene, vgaB, encoding a putative ABC transporter conferring resistance to streptogramin A and related compounds. Gene **202:**133–138.
2. **Allington, D. R., and M. P. Rivey.** 2001. Quinupristin/dalfopristin: a therapeutic review. Clin. Ther. **23:**24–44.
3. **Beaudet, L., and P. Gros.** 1995. Functional dissection of P-glycoprotein nucleotide-binding domains in chimeric and mutant proteins. Modulation of drug resistance profiles. J. Biol. Chem. **270:**17159–17170.
4. **Dassa, E., and P. Bouige.** 2001. The ABC of ABCS: a phylogenetic and functional classification of ABC systems in living organisms. Res. Microbiol. **152:**211–229.
5. **Datta, A. K.** 1995. Efficient amplification using 'megaprimer' by asymmetric polymerase chain reaction. Nucleic Acids Res. **23:**4530–4531.
6. **Davis, D. R., J. B. McAlpine, C. J. Pazoles, M. K. Talbot, E. A. Alder, C. White, B. M. Jonas, B. E. Murray, G. M. Weinstock, and B. L. Rogers.** 2001. *Enterococcus faecalis* multi-drug resistance transporters: application for antibiotic discovery. J. Mol. Microbiol. Biotechnol. **3:**179–184.
7. **Dina, J., B. Malbruny, and R. Leclercq.** 2003. Nonsense mutations in the *lsa*-like gene in *Enterococcus faecalis* isolates susceptible to lincosamides and streptogramins A. Antimicrob. Agents Chemother. **47:**2307–2309.
8. **Duh, R. W., K. V. Singh, K. Malathum, and B. E. Murray.** 2001. In vitro activity of 19 antimicrobial agents against enterococci from healthy subjects and hospitalized patients and use of an ace gene probe from *Enterococcus faecalis* for species identification. Microb. Drug Resist. **7:**39–46.
9. **Friesenegger, A., S. Fiedler, L. A. Devriese, and R. Wirth.** 1991. Genetic transformation of various species of *Enterococcus* by electroporation. FEMS Microbiol. Lett. **63:**323–327.
10. **Li, X., G. M. Weinstock, and B. E. Murray.** 1995. Generation of auxotrophic mutants of *Enterococcus faecalis*. J. Bacteriol. **177:**6866–6873.
11. **Linton, K. J., and C. F. Higgins.** 1998. The *Escherichia coli* ATP-binding cassette (ABC) proteins. Mol. Microbiol. **28:**5–13.
12. **Malathum, K., K. V. Singh, G. M. Weinstock, and B. E. Murray.** 1998. Repetitive sequence-based PCR versus pulsed-field gel electrophoresis for typing of *Enterococcus faecalis* at the subspecies level. J. Clin. Microbiol. **36:**211–215.
13. **Murray, B. E., K. V. Singh, J. D. Heath, B. R. Sharma, and G. M. Weinstock.** 1990. Comparison of genomic DNAs of different enterococcal isolates using restriction endonucleases with infrequent recognition sites. J. Clin. Microbiol. **28:**2059–2063.
14. **Murray, B. E., K. V. Singh, R. P. Ross, J. D. Heath, G. M. Dunny, and G. M. Weinstock.** 1993. Generation of restriction map of *Enterococcus faecalis* OG1 and investigation of growth requirements and regions encoding biosynthetic function. J. Bacteriol. **175:**5216–5223.
15. **National Committee for Clinical Laboratory Standards.** 2003. Methods for dilution antimicrobial susceptibility tests for bacteria that grows aerobically; approved standard, 6th ed. NCCLS document M7-A6. National Committee for Clinical Laboratory Standards, Wayne, Pa.
16. **National Committee for Clinical Laboratory Standards.** 2003. MIC testing—supplemental tables. NCCLS document M100-S13 (M7). National Committee for Clinical Laboratory Standards, Wayne, Pa.
17. **Paulsen, I. T., L. Banerjei, G. S. Myers, K. E. Nelson, R. Seshadri, T. D. Read, D. E. Fouts, J. A. Eisen, S. R. Gill, J. F. Heidelberg, H. Tettelin, R. J. Dodson, L. Umayam, L. Brinkac, M. Beanan, S. Daugherty, R. T. DeBoy, S. Durkin, J. Kolonay, R. Madupu, W. Nelson, J. Vamathevan, B. Tran, J. Upton, T. Hansen, J. Shetty, H. Khouri, T. Utterback, D. Radune, K. A. Ketchum, B. A. Dougherty, and C. M. Fraser.** 2003. Role of mobile DNA in the evolution of vancomycin-resistant *Enterococcus faecalis*. Science **299:**2071–2074.
18. **Qin, X., K. V. Singh, G. M. Weinstock, and B. E. Murray.** 2000. Effects of *Enterococcus faecalis fsr* genes on production of gelatinase and a serine protease and virulence. Infect. Immun. **68:**2579–2586.
19. **Rende-Fournier, R., R. Leclercq, M. Galimand, J. Duval, and P. Courvalin.** 1993. Identification of the *satA* gene encoding a streptogramin A acetyltransferase in *Enterococcus faecium* BM4145. Antimicrob. Agents Chemother. **37:**2119–2125.
20. **Reynolds, E., J. I. Ross, and J. H. Cove.** 2003. Msr(A) and related macrolide/streptogramin resistance determinants: incomplete transporters? Int. J. Antimicrob. Agents **22:**228–236.
21. **Ross, J. I., E. A. Eady, J. H. Cove, and S. Baumberg.** 1995. Identification of a chromosomally encoded ABC-transport system with which the staphylococcal erythromycin exporter MsrA may interact. Gene **153:**93–98.
22. **Sahm, D. F., J. Kissinger, M. S. Gilmore, P. R. Murray, R. Mulder, J. Solliday, and B. Clarke.** 1989. In vitro susceptibility studies of vancomycin-resistant *Enterococcus faecalis*. Antimicrob. Agents Chemother. **33:**1588–1591.

23. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
24. **Singh, K. V., X. Qin, G. M. Weinstock, and B. E. Murray.** 1998. Generation and testing of mutants of *Enterococcus faecalis* in a mouse peritonitis model. J. Infect. Dis. **178:**1416–1420.
25. **Singh, K. V., G. M. Weinstock, and B. E. Murray.** 2002. An *Enterococcus faecalis* ABC homologue (Lsa) is required for the resistance of this species to clindamycin and quinupristin-dalfopristin. Antimicrob. Agents Chemother. **46:**1845–1850.
26. **Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan.** 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. **33:**2233–2239.
27. **Walsh, P. M., and L. L. McKay.** 1982. Restriction endonuclease analysis of the lactose plasmid in *Streptococcus lactis* ML3 and two recombinant lactose plasmids. Appl. Environ. Microbiol. **43:**1006–1010.
28. **Wilson, K.** 1994. Preparation of genomic DNA from bacteria, p. 2.4.1–2.4.2. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. M. David, J. G. Scidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. Green Publishing Associates, Brooklyn, N.Y.
29. **Wirth, R., F. An, and D. B. Clewell.** 1987. Highly efficient cloning system for *Streptococcus faecalis*: protoplast transformation, shuttle vectors, and applications, p. 25–27. *In* J. J. Ferretti and R. Curtiss III (ed.), Streptococcal genetics. American Society for Microbiology, Washington, D.C.
30. **Xu, Y., L. Jiang, B. E. Murray, and G. M. Weinstock.** 1997. *Enterococcus faecalis* antigens in human infections. Infect. Immun. **65:**4207–4215.