AAC
Journals.ASM.org

Genetic Basis for *In Vitro* and *In Vivo* Resistance to Lincosamides, Streptogramins A, and Pleuromutilins (LS_AP Phenotype) in *Enterococcus faecium*

Christophe Isnard,[a] Brigitte Malbruny,[a†] Roland Leclercq,[a,b] Vincent Cattoir[a,b]

CHU de Caen, Service de Microbiologie, Caen, France[a]; Université de Caen Basse-Normandie, EA4655 (Équipe Antibiorésistance), Faculté de Médecine, Caen, France[b]

As opposed to *Enterococcus faecalis*, which is intrinsically resistant to lincosamides, streptogramins A, and pleuromutilins (LS_AP phenotype) by production of the ABC protein Lsa(A), *Enterococcus faecium* is naturally susceptible. Since this phenotype may be selected for *in vivo* by quinupristin-dalfopristin (Q-D), the aim of this study was to investigate the molecular mechanism of acquired LS_AP resistance in *E. faecium*. Six LS_AP-resistant *in vitro* mutants of *E. faecium* HM1070 as well as three different pairs of clinical isolates (pre- and postexposure to Q-D) were studied. The full genome sequence of an *in vitro* mutant (*E. faecium* UCN90B) was determined by using 454 sequencing technology and was compared with that of the parental strain. Single-nucleotide replacement was carried out to confirm the role of this mutation. By comparative genomic analysis, a point mutation was found within a 1,503-bp gene coding for an ABC homologue showing 66% amino acid identity with Lsa(A). This mutation (C1349T) led to an amino acid substitution (Thr450Ile). An identical mutation was identified in all *in vitro* and *in vivo* resistant strains but was not present in susceptible strains. The wild-type allele was named *eat*(A) (for Enterococcus ABC transporter), and its mutated allelic variant was named $eat(A)_v$. The introduction of $eat(A)_v$ from UCN90B into HM1070 conferred the LS_AP phenotype, whereas that of *eat*(A) from HM1070 into UCN90B restored susceptibility entirely. This is the first description of the molecular mechanism of acquired LS_AP resistance in *E. faecium*. Characterization of the biochemical mechanism of resistance and the physiological role of this ABC protein need further investigations.

Enterococci, particularly *Enterococcus faecalis* and *Enterococcus faecium*, are responsible for numerous infections worldwide, being the second or third most common pathogens in hospitals (1). For several years, these opportunistic pathogens have become more and more resistant to antibiotics with the emergence of vancomycin-resistant *E. faecium* (VREF) isolates belonging mostly to clonal complex 17 (CC17) (2). Since VREF isolates are resistant to β-lactam and glycopeptide antibiotics, only a few molecules are still active against these isolates, such as quinupristin-dalfopristin (Q-D), linezolid, and daptomycin (2, 3).

Q-D is an injectable streptogramin approved by the U.S. FDA for the treatment of severe VREF infections associated with bacteremia (3). Streptogramins form with macrolides (e.g., erythromycin) and lincosamides (e.g., lincomycin and clindamycin), a group of structurally distinct antibiotics (referred to as MLS) that present similar mechanism of action and cross-resistance patterns (4). Actually, streptogramins correspond to a mixture of two compounds that act synergically: streptogramins A (e.g., dalfopristin) and streptogramins B (e.g., quinupristin). In addition, pleuromutilins (e.g., tiamulin) are also a class of protein synthesis inhibitors that share ribosomal binding sites with lincosamide and streptogramin A antibiotics (5).

In enterococci, MLS resistance is due mainly to a ribosomal alteration mediated by a ribosomal methylase encoded by the *erm*(B) or *erm*(A) [formerly designated *erm*(TR)] gene, which is responsible for cross-resistance to all macrolides, lincosamides, and streptogramins B (MLS_B phenotype) that can be constitutive or inducible (4). Besides this common mechanism of resistance, there is a peculiar phenotype exhibiting cross-resistance to lincosamides, streptogramins A, and pleuromutilins, called the LS_AP phenotype (formerly the LS_A phenotype) due to ABC proteins. For instance, intrinsic LS_AP resistance in *E. faecalis* is due to the production of the ABC (ATP-binding cassette) homologue Lsa(A) (6–8). Other Lsa-like proteins have been involved in LS_AP resistance: Lsa(B) and Lsa(E), identified in *Staphylococcus* spp. (9–12), and Lsa(C), described in *Streptococcus agalactiae* (13, 14). As opposed to *E. faecalis*, *E. faecium* is intrinsically susceptible to all macrolides and related compounds, but this LS_AP phenotype may be selected *in vivo* after Q-D therapy exposure (15). Even if the support of resistance is very likely mediated through a chromosomal mutation(s), the biochemical and genetic basis of this resistance was not elucidated.

The aim of the study was then to (i) identify the molecular mechanism of LS_AP resistance in *E. faecium* mutants selected both *in vitro* and *in vivo*, (ii) confirm the role of a single point mutation in LS_AP resistance by allelic replacement, and (iii) evaluate the prevalence of this resistance mechanism among a collection of well-characterized VREF clinical isolates.

(A preliminary report of this work was presented at the 22nd European Congress of Clinical Microbiology and Infectious Diseases, London, United Kingdom, 31 March to 3 April 2012 [16].)

Received 13 May 2013 Returned for modification 10 June 2013
Accepted 29 June 2013
Published ahead of print 8 July 2013
Address correspondence to Vincent Cattoir, cattoir-v@chu-caen.fr.
† Deceased.
This paper is dedicated to the memory of Brigitte Malbruny.
Copyright © 2013, American Society for Microbiology. All Rights Reserved.
doi:10.1128/AAC.01030-13

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s) and antibiotic resistance(s)[a]	Reference or source
Strains		
E. faecium HM1070	Wild-type parental strain; Fus^r Rif^r	31
E. faecium UCN90A	LS_AP^r mutant derived from HM1070 (selected *in vitro* with Lin)	This study
E. faecium UCN90B	LS_AP^r mutant derived from HM1070 (selected *in vitro* with Lin)	This study
E. faecium UCN91A	LS_AP^r mutant derived from HM1070 (selected *in vitro* with Da)	This study
E. faecium UCN91B	LS_AP^r mutant derived from HM1070 (selected *in vitro* with Da)	This study
E. faecium UCN92A	LS_AP^r mutant derived from HM1070 (selected *in vitro* with Tia)	This study
E. faecium UCN92B	LS_AP^r mutant derived from HM1070 (selected *in vitro* with Tia)	This study
E. faecium UCN94	HM1070 derivative with allelic replacement of *eat*(A) by *eat*(A)$_v$ from UCN90B (LS_AP phenotype)	This study
E. faecium UCN95	UCN90B derivative with allelic replacement of *eat*(A)$_v$ by *eat*(A) from HM1070 (wild-type phenotype)	This study
E. faecium UCN80	Susceptible strain (isolated from patient B)	15
E. faecium UCN80-1	LS_AP^r mutant derived from UCN80 (selected *in vivo* with Q-D)	15
E. faecium UCN81	Susceptible strain (isolated from patient C)	15
E. faecium UCN81-1	LS_AP^r mutant derived from UCN81 (selected *in vivo* with Q-D)	15
E. faecium UCN82	Susceptible strain (isolated from patient F)	15
E. faecium UCN82-1	LS_AP^r mutant derived from UCN82 (selected *in vivo* with Q-D)	15
E. coli TOP10	Cloning strain	Invitrogen
Plasmids		
pCR2.1-TOPO	General AT cloning vector; Kan^r Amp^r	Invitrogen
pG1KT	Derivative of the thermosensitive shuttle plasmid pG(+)host5 (containing promoterless and terminatorless Kan^r cassette AphA-3); used for mutagenesis; Ery^r	19
pG1KTΩ*eat*(A)	pG1KT derivative carrying the 3′ end of *eat*(A); used for allelic replacement in UCN90B	This study
pG1KTΩ*eat*(A)v	pG1KT derivative carrying the 3′ end of *eat*(A)$_v$; used for allelic replacement in HM1070	This study

[a] Amp^r, ampicillin resistance; Da, dalfopristin; Ery^r, erythromycin resistance; Fus^r, fusidic acid resistance; Kan^r, kanamycin resistance; Lin, lincomycin; LS_AP, lincosamides-streptogramins A-pleuromutilins (LS_AP phenotype); Q-D, quinupristin-dalfopristin; Rif^r, rifampin resistance; Tia, tiamulin; *eat*(A), wild-type allele; *eat*(A)$_v$, allele of *eat*(A) with a single mutation (C1349T) leading to an amino acid substitution (Thr450Ile).

MATERIALS AND METHODS

Bacterial strains and antimicrobial susceptibility testing. Strains selected both *in vitro* and *in vivo* were studied (Table 1). Six LS_AP-resistant mutants of *E. faecium* HM1070 were selected *in vitro* with a frequency of ca. 10^{-9} by using lincomycin, dalfopristin, or tiamulin (at 1 μg/ml, 16 μg/ml, and 2 μg/ml, respectively). Approximately 10^9 CFU of exponentially growing bacteria was plated onto brain heart infusion (BHI) agar containing antibiotic concentrations (i.e., 4× MIC), and mutants were recovered after 48 h of incubation in ambient air at 37°C. Also, three pairs of clinical isolates collected from three different patients who were treated with Q-D were included (15). For each patient, one LS_AP-susceptible isolate (preexposure to Q-D) and one LS_AP-resistant isolate (postexposure to Q-D) were obtained. Noteworthy, strains from each pair were indistinguishable by pulsed-field gel electrophoresis (PFGE) (15).

A collection of 60 clonally unrelated (different PFGE profiles) VREF clinical isolates were also screened for the presence of the putative point mutation responsible for LS_AP resistance. These strains were received at the French Reference Centre for Enterococci between 2006 and 2008 and were extensively characterized by phenotypic and genotypic methods (17).

E. faecium HM1070 and *E. faecium* UCN90B were used for allelic replacement experiments. *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* TOP10 were used as a control for antimicrobial susceptibility testing and as a cloning strain, respectively.

MICs of erythromycin, lincomycin, clindamycin, dalfopristin, quinupristin, Q-D, and tiamulin were determined by the broth microdilution method (tested range, 0.06 to 64 μg/ml) according to CLSI guidelines (18).

Whole-genome sequencing. Genomic DNA was extracted from mid-log-phase cultures of *E. faecium* HM1070 (LS_AP-susceptible strain) and *E. faecium* UCN90B (LS_AP-resistant mutant derived from *E. faecium* HM1070) by using NucleoBond buffer set III and the NucleoBond AX-G 100 system (Macherey-Nagel, Hoerdt, France) according to the manufacturer's instructions. High-throughput sequencing was performed by using a 454 Life Sciences (Roche) GS-FLX system (DNAVision, Charleroi, Belgium). Shotgun sequencing for *E. faecium* HM1070 led to an assembly of 138 contigs with sizes ranging from 619 to 83,932 bp, with an aggregate genome size of 2,591,399 bp and an 11.3× average coverage of the genome, while data for *E. faecium* UCN90B were as follows: 268 contigs (sizes from 557 to 91,723 bp), an aggregate genome of 2,562,062 bp, and an 8.5× average genomic coverage. Comparative genomic analysis of the two strains was performed by using the Mosaik (http://code.google.com/p/mosaik-aligner/) and Samtools (http://samtools.sourceforge.net/) bioinformatics software tools (DNAVision, Charleroi, Belgium). The nucleotide and deduced protein sequences for each contig were analyzed with the BLASTN and BLASTX programs available on the Internet at the National Center for Biotechnology Information website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

PCR amplification and sequencing. Bacterial genomic DNA was extracted by using the QIAamp DNA minikit (Qiagen, Courtaboeuf, France). PCR experiments for the detection of the point mutation putatively involved in LS_AP resistance were carried out under standard conditions by using primers synthesized by Sigma-Aldrich France (Table 2). Briefly, 5 μl of total DNA was subjected to PCR in a 50-μl reaction mixture containing 1× PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl), 1.5 mM $MgCl_2$, 200 μM each deoxynucleotide triphosphate, 0.4 μM each primer, and 1 U of GoTaq Flexi DNA polymerase (Promega, Charbonnières-les-Bains, France). PCR amplifications were performed by using a Mastercycler gradient thermal cycler (Eppendorf, Le Pecq, France) as follows: (i) an initial denaturation step for 5 min at 95°C; (ii) 30 cycles of PCR, with 1 cycle consisting of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C; and (iii) a final extension step for 5 min at 72°C. Purified PCR products were then directly sequenced with the same sets of primers in both directions (GATC Biotech, Konstanz, Germany).

Allelic replacement by site-directed mutagenesis. Fragments containing the 3′ ends of the *eat*(A) gene and a variant of the *eat*(A) gene

TABLE 2 Deoxynucleotide primers used in this study

Primer[a]	Nucleotide sequence (5′–3′)	Positions[b]	Purpose
lsaF-Efm-F	TTTGAACAACCTCCGAAAGC	660490–660509	Detection of the point mutation in *eat*(A)
lsaF-Efm-R	TTTCTGTGCCTGCATCTGTC	660927–660946	
lsaF-Efm-mut-F	GCAATCGTGAATCGGATGG	660098–660116	Allelic replacement of *eat*(A) and checking of pG1KT derivatives
lsaF-Efm-mut-R	GGAATCGTACAGCGAACGC	661148–661166	
M13-Fm	GTTGTAAAACGACGGCCAG	NA	
M13-Rm	GGATAACAATTTCACACAGG	NA	
kana-R	GCTTATATACCTTAGCAGGAG	NA	
lsaF-Efm-3′-R	TCCCCTTAACCATACCTTGTTG	660791–660812	
lsaF-Efm-cont-F	TCGAGCAGATGAGTATGGG	660543–660561	
lsaF-Efm-cont-R	TTCTAATTGTTCTTGATTGAAG	660649–660670	
lsaF-Efm-GSP-R1	TTGCATCAATTGGAGCT	659565–659581	Determination of start transcription site of *eat*(A) by 5′ RACE
lsaF-Efm-GSP-R2	AGGCAAATTCTTGCTGATGG	659455–659474	
lsaF-Efm-GSP-R3	TTGTCTTTCCTCGACCGTTT	659386–659405	

[a] cont, control; Efm, *Enterococcus faecium*; F, forward primer; GSP, gene-specific primer; mut, mutagenesis; R, reverse primer.
[b] Primer positions were determined according to the genome sequence of *E. faecium* Aus0004 (GenBank accession no. NC_017022). NA, not applicable.

[$eat(A)_v$] were amplified by using primers lsa-Efm-mut-F and lsa-Efm-mut-R (Table 2) from *E. faecium* HM1070 and *E. faecium* UCN90B, respectively. PCR products were cloned into the pCR2.1-TOPO plasmid (Invitrogen) and transformed into *E. coli* TOP10 cells. Recombinant plasmids were extracted by using a QIAprep Spin Miniprep kit (Qiagen, Courtaboeuf, France), according to the manufacturer's instructions, and then digested with the EcoRI enzyme (New England BioLabs, Evry, France). Restriction products were then cloned into the thermosensitive plasmid pG1KT, which is a derivative of the shuttle plasmid pG(+)host5 containing the promoterless and terminatorless kanamycin resistance cassette AphA-3 (19), as previously described (20). Briefly, *E. coli* transformants were selected on medium containing erythromycin (150 μg/ml), and construction was checked by specific PCR amplifications (Table 2). Recombinant plasmids pG1KTΩ*eat*(A) and pG1KTΩ*eat*(A)v were then introduced by electrotransformation into *E. faecium* UCN90B and *E. faecium* HM1070, respectively (Table 1). *E. faecium* transformants were selected on medium containing kanamycin (500 μg/ml) and erythromycin (15 μg/ml) after an incubation step at 42°C, allowing plasmid integration into the chromosome. The spontaneous loss of pG1KT was obtained by daily subculture in tryptone-soy broth (AES Laboratories, Combourg, France) at 30°C with no antibiotic. Candidate colonies were tested for their susceptibility to MLS and checked by PCR sequencing.

5′ RACE. Total RNAs were extracted from cultures of *E. faecium* HM1070 by using the ZR Fungal/Bacterial RNA Miniprep kit (Zymo Research, Irvine, CA). The transcription start site (TSS) and promoter sequences were then determined by using the 5′ rapid amplification of cDNA ends (RACE) system kit (Invitrogen), according to the manufacturer's instructions, with different specific primers (Table 2).

Multiple alignment and phylogenetic analysis. Sequence comparison and phylogenetic analysis were performed by using the neighbor-joining algorithm with ClustalX software (version 1.83).

Nucleotide sequence accession numbers. The nucleotide sequences of *eat*(A) and $eat(A)_v$ were deposited in the GenBank database under accession no. KF010778 and KF010779, respectively.

RESULTS

Identification of a single mutation in an *lsa*-like gene. By comparing the entire genome of *E. faecium* HM1070 (LS_AP-susceptible strain) and that of *E. faecium* UCN90B (LS_AP-resistant mutant derived from *E. faecium* HM1070), we found 50 different mutations, including one within a 1,503-bp gene coding for a 500-amino-acid (ca. 58-kDa) ABC protein homologue of Lsa-like proteins. This mutation was responsible for a transition (C1349T) leading to the amino acid substitution Thr450Ile (Fig. 1). By using specific PCR primers, a strictly identical mutation (C1349T) was identified among all *in vitro* and *in vivo* resistant strains but was not present in susceptible strains. According to the recommendations for tetracycline and MLS nomenclature (http://faculty.washington.edu/marilynr/), the wild-type gene was named *eat*(A) (for *Enterococcus* ABC transporter), whereas the allele with the single point mutation C1349T was designated $eat(A)_v$. A BLAST analysis showed that *eat*(A) was present in all sequenced *E. faecium* genomes but not in other enterococcal species (data not shown), suggesting that *eat*(A) is species specific for *E. faecium* and is an intrinsic gene of this species. The Eat(A) protein displayed 66%, 44%, 43%, and 42% amino acid identities with other proteins conferring LS_AP-type resistance in various Gram-positive organisms, Lsa(A), Lsa(E), Lsa(B), and Lsa(C), respectively. Like other Lsa-like proteins, the structure of Eat(A) showed duplications of Walker A and B motifs, the ABC signature, and the H-loop switch (Fig. 1).

Drug susceptibility patterns conferred by the single mutation C1349T. By single-nucleotide allelic replacement, we demonstrated that the unique substitution Thr450Ile was responsible for the LS_AP phenotype (Table 3). Indeed, the introduction of the mutated allele $eat(A)_v$ from *E. faecium* UCN90B into susceptible *E. faecium* strain HM1070 (also known as strain UCN94) conferred the LS_AP phenotype with an increase of MICs of lincomycin (from 0.25 to 8 μg/ml), clindamycin (from 0.12 to 2 μg/ml), dalfopristin (from 4 to >64 μg/ml), Q-D (from 0.5 to 2 μg/ml), and tiamulin (from 0.5 to 64 μg/ml), whereas MICs of erythromycin and quinupristin did not change (Table 3). Conversely, the introduction of the wild-type allele *eat*(A) from *E. faecium* HM1070 into LS_AP-resistant *E. faecium* strain UCN90B (also known as strain UCN95) restored entire susceptibility to lincomycin (from 8 to 0.25 μg/ml), clindamycin (from 2 to 0.06 μg/ml), dalfopristin (from >64 to 4 μg/ml), Q-D (from 2 to 0.5 μg/ml), and tiamulin (from 32 to 0.5 μg/ml), with no alteration of MICs of erythromycin and quinupristin (Table 3).

In addition, all *in vitro* mutants harboring the C1349T mutation presented the same LS_AP phenotype as strain UCN90B, with MICs of lincomycin, clindamycin, dalfopristin, Q-D, and tiamu-

```
Lsa(B)    MSMIHVQNLTFSYPSSFDNIFEDVSFQIDTDWKLGFIGRNGRGKTTLFNLLLDKFEYRGKIISSVDFNYFPYPVEDKSKYTH 82
Lsa(E)    MSLINVSNLTFSYEGSYDNIFENVSFQIDTDWKLGFIGRNGRGKTTFLNLLLGKYAYSGNISSTVKFEYFPYDVEDKSLYTI 82
Lsa(C)    MSTIKIENLTFSYYGYVKPVFENVSFSFDTNWKTGLIGRNGIGKSTLFKLLLNQEVYKGKISKSVDFIKFPPNLSDTSKLGI 82
Eat(A)    MSKIEIKNLTFGYDSQGTLLFEQANLNFDTQWKLGLIGRNGRGKTTLLNILQNKLPYQGQVIHQQEFAYFPQQTKDKERLTY 82
Eat(A)v   MSKIEIKNLTFGYDSQGTLLFEQANLNFDTQWKLGLIGRNGRGKTTLLNILQNKLPYQGQVIHQQEFAYFPQQTKDKERLTY 82
Lsa(A)    MSKIELKQLSFAYDNQEALLFDQANITMDTNWKLGLIGRNGRGKTTLLRLLQKQLDYQGEILHQVDFVYFPQTVAEEQQLTY 82
          ** *.:.:*:*.* .    :*::..: :**:** *:***** **:*::.:*  :  * *::    .*  **    : .
                                            Walker A1

Lsa(B)    EILEEICPQAEDWEFLREIAYLNVDAEAMYRPFETLSNGEQTKVLLVALFLNEGQFLLIDEPTNHLDTEARKTVSNYLRKKK 164
Lsa(E)    EVMKSICTECMDWEIFREISLLDVQEDALYRPFNTLSNGEQTKVLLAALFLTASCFLLIDEPTNHLDIDARNVVQNYLKRKK 164
Lsa(C)    ELYRELISDEEEWKLFRELHLLKVDESLIYRKFETLSKGEQTKILLAILFTREDGFLLIDEPTNHLDMDGRKIVSEYLKNKK 164
Eat(A)    YVLNDIT-DFEIWEIERELQLMQTDPEILWREFSTLSGGEKTKVLLALLFVDDTHFPLIDEPTNHLDISGRKQVAAYLKKKK 163
Eat(A)v   YVLNDIT-DFEIWEIERELQLMQTDPEILWREFSTLSGGEKTKVLLALLFVDDTHFPLIDEPTNHLDISGRKQVAAYLKKKK 163
Lsa(A)    YVLQEVT-SFEQWKLERELTLLNVDPEVLWRPFSSLSGGEKTKVLLGLLFIEENAFPLIDEPTNHLDLAGRQQVAEYLKKKK 163
           : ..:  .   *:: **:  :..: . ::* *.:** **:**:**  **     * **********  .*: *  **:.**
                                        ABC signature 1        Walker B1

Lsa(B)    -GNILISHDRNFLDGSVDHILSINRADIEVQSGNYSSWKLNFDRQQGHEQATNERLQKDIGRLEQSTKRSAGWSNRVEASKN 245
Lsa(E)    -GFILVSHDRSLLDQCVDHILSINKTNIEIQKGNFTSWWENKTLQDNFELAENKKLLKEIGRLSYAAKRSSNWSNKVEKSKY 245
Lsa(C)    -GFLLISHDRDFLDGCINHIISINRNSIDVQSGNFTSWYENKLMKDQFEISQNEKLRKDIKRLKEAARQSQIWSDKVENTKN 245
Eat(A)    QGFIVVSHDRGFIDEVVDHVLAIEKSQLELYQGNFSIYEEQKKLRDEFEMAQNEKLKKEVSRLKKTAAEKAEWSRSREGDKT 245
Eat(A)v   QGFIVVSHDRGFIDEVVDHVLAIEKSQLELYQGNFSIYEEQKKLRDEFEMAQNEKLKKEVSRLKKTAAEKAEWSRSREGDKT 245
Lsa(A)    HGFILVSHDRAFVDEVVDHILAIEKSQLTLYQGDFSIYEEQKKLRDAFELAENEKIKKEVNRLKETARKKAEWSMNREGDKY 245
           * :::****  ::*  ::*:::*:: .: : .*::: :  :   :: .* : *::: *:: **. :: ..  **   *  *
             Switch 1

Lsa(B)    GTTN-----SGSKLDKGFVGHKAAKMMKRSKNLEARQQKSIEEKSKLLKNIEKTESLQFEPVEYKSKELIQLTDVSVIYDGQ 322
Lsa(E)    GTTN-----SGSKLDKGYVGHKAAKAMKRAKNIESRHQEAVLQKSELLHNIEQYDDLKISPLEFHKECLIEANDLSLSYGDK 322
Lsa(C)    GVKV-----SGVKPDKGHIGHQSAKMMKKSKNLENRQNKAIEEKQNLLKDIETKESLLLHPLHHHKNPLISVCDLSSYYGKK 322
Eat(A)    KKQVGFIDTESRRVNKGAVGADAARTMKRSKAIVNRMETQISEKEKLLKDIEYIDSLTMNSQASHHKRLLSVEDLQLGYEN- 326
Eat(A)v   KKQVGFIDTESRRVNKGAVGADAARTMKRSKAIVNRMETQISEKEKLLKDIEYIDSLTMNSQASHHKRLLSVEDLQLGYEN- 326
Lsa(A)    GNAK--EKGSGAIFDTGAIGARAARVMKRSKHIQQRAETQLAEKEKLLKDLEYIDPLSMDYQPTHHKTLLTVEELRLGYEKN 325
                   ..   :.* :*  :*: **::* :  * :  : :*.:**:::*  : * :     : : *:   ::   *

Lsa(B)    VVNKPISFNVEQGDRIVLDGKNGSGKSSILKLILGD-PIQYTGTLNTGSNLITSYVQQDTSHLKGMLADFIEENEIDESLFK 403
Lsa(E)    EVCSNLNFRVNIGDRVAIIGKNGSGKSSILKLINGD-DIKFTGNFMLASGLKISYISQDTSYLKGNLSEFAYNNKIDETLFK 403
Lsa(C)    QILSNISFDIKQGDIVAIYGGNGSGKSTLIKILLGL-NHEYSGDVKLASNLKISYVPQDTSNLTGSLNEYIHKQGVDETLCK 403
Eat(A)    LLFEPIHFTIEPHQRVAISGPNGAGKSSIIHYLLGAFNGKVIGEKYQPKHLSISYASQNYEDNRGTLAEFAEKNQVDYQAFL 408
Eat(A)v   LLFEPIHFTIEPHQRVAISGPNGAGKSSIIHYLLGAFNGKVIGEKYQPKHLSISYASQNYEDNRGTLAEFAEKNQVDYQAFL 408
Lsa(A)    WLFTPISFSINAGEIVGITGKNGSGKSSLIQYLLGDFSGDSEGEATLAHQLTISYVRQDYEDNQGTLSEFAEKNQLDYTQFL 407
           :   : * ::  : : : * **:***:::  : *    .  *       *  **  *: .   * * ::  :: :*
                  Walker A2

Lsa(B)    AILRKLDFDRVQFEKDISHYSGGQKKKLLIAKSLCEKAHLYIWDEPLNFIDIYSRMQIEELIQTFNPTMVFVEHDQTFQETI 485
Lsa(E)    TILRKLDFNREQFDKNMVDFSAGQKKKVLIAKSLCESAHLYIWDEPLNYIDIFSRIQIEKMILEYCPTLLFVEHDDAFCNNI 485
Lsa(C)    TILRKLDFARELFEIDMKNYSDGQKKKVLIAVSLSKSAHIFIWDEPLNYLDVISRIQIEEIIKEANPTLIFVEHDKSFVEDI 485
Eat(A)    NNLRKLGMERDVFHNKIEQMSMGQRKKVELAKSLSQPAELYTWDEPLNYLDVFNQEQLEQLILNVKPAMLLVEHDQTFLDKV 490
Eat(A)v   NNLRKLGMERDVFHNKIEQMSMGQRKKVELAKSLSQPAELYIWDEPLNYLDVFNQEQLEQLILNVKPAMLLVEHDQTFLDKV 490
Lsa(A)    NNLRKLGMERAVFTNRIEQMSMGQRKKVEVAKSLSQSAELYIWDEPLNYLDVFNHQQLEALILSVRPAMLVIEHDAHFMKKI 489
            ****.: *  *   : . * **:**: :* **.: *.:: ******::*: .: *:* :*    *:::.:***  * . :
                 ABC signature 2        Walker B2                      Switch 2

Lsa(B)    STKIIKI--- 492
Lsa(E)    CTKNINLGL- 494
Lsa(C)    ANKIIRL--- 492
Eat(A)    STEIISLERI 500
Eat(A)v   STEIISLERI 500
Lsa(A)    TDKKIALKS- 498
            : * :
```

FIG 1 Amino acid sequence comparison of the Eat(A) and Eat(A)$_v$ ABC proteins with Lsa-like proteins involved in MLS resistance, Lsa(A), Lsa(B), Lsa(C), and Lsa(E). The two copies of Walker A and B motifs and ABC signatures are boxed. Similarities in amino acid sequences are marked by asterisks (same amino acid), colons (strong similarity), and dots (family similarity). Multiple-sequence alignment was done with ClustalX 1.83 software. The location of the Thr450Ile substitution is indicated by an arrow.

lin at 8, 2, >64, 2, and 32 μg/ml, respectively (Table 3). Concerning clinical isolates, all were highly resistant to erythromycin, lincomycin, clindamycin, and quinupristin due to a MLS_B phenotype mediated by the *erm*(B) gene (data not shown), but only *in vivo* mutants with the C1349T mutation presented the LS_AP phenotype (Table 3).

Genetic environment of *eat*(A) in *E. faecium* Aus0004. In the genome of *E. faecium* Aus0004, the *eat*(A) gene corresponded to the gene *EFAU004_00630* (positions 659270 to 660772). This gene was surrounded by the two genes *EFAU004_00629* (positions 657891 to 659012) and *EFAU004_00631* (positions 659270 to 660772), coding for a glycerate kinase (373 amino acids) and a

TABLE 3 MICs of macrolides and related compounds for *E. faecium* strains

Strain	MIC (μg/ml)[a]						
	ERY	LIN	CLI	DAL	QUI	Q-D	TIA
HM1070	0.12	0.25	0.12	4	2	0.5	0.5
In vitro mutants							
UCN90A	0.12	8	2	>64	2	2	32
UCN90B	0.12	8	2	>64	2	2	32
UCN91A	0.12	8	2	>64	2	2	32
UCN91B	0.12	8	2	>64	2	2	32
UCN92A	0.12	8	2	>64	2	2	32
UCN92B	0.12	8	2	>64	2	2	32
UCN94	0.12	8	2	>64	2	2	64
UCN95	0.12	0.25	0.06	4	2	0.5	0.5
Clinical isolates (including *in vivo* mutants)							
UCN80	>64	>64	>64	2	>64	1	0.5
UCN80-1	>64	>64	>64	16	>64	4	32
UCN81	>64	>64	>64	2	>64	1	0.5
UCN81-1	>64	>64	>64	16	>64	4	32
UCN82	>64	>64	>64	2	>64	0.5	1
UCN82-1	>64	>64	>64	32	>64	4	64

[a] CLI, clindamycin; DAL, dalfopristin; ERY, erythromycin; LIN, lincomycin; Q-D, quinupristin-dalfopristin; QUI, quinupristin; TIA, tiamulin.

phosphoglycerate mutase (175 amino acids), respectively (Fig. 2). Bioinformatically, no operon structure was predicted, and we unambiguously determined the TSS to be 221 bp upstream of the start codon of *eat*(A), revealing a long 5′ untranslated region (UTR) (Fig. 2).

Prevalence of the mutated allele *eat*(A)$_v$ among VREF clinical isolates. Out of the 60 VREF isolates, the point mutation C1349T was detected in 14 (23.3%) strains by PCR sequencing. All the VREF isolates were highly resistant to macrolides, lincosamides, and quinupristin (MLS_B phenotype). On the other hand, all *eat*(A)$_v$-positive strains were also resistant to quinupristin-dalfopristin and tiamulin, whereas all susceptible strains (except two) remained entirely susceptible to these antibiotics (data not shown). This suggests that these molecules may be used as phenotypic markers of resistance in the case of combined resistance mechanisms.

DISCUSSION

ABC systems constitute one of the largest families of proteins, with most of them being involved in import and export, often called ABC transporters (21). These transporters share an organization with two hydrophobic transmembrane domains (TMDs) and two intracytoplasmic nucleotide-binding domains (NBDs) implicated in ATP hydrolysis and comprising specific motifs (Walker A and B motifs and the ABC signature) that are conserved in all ABC proteins (22). Besides importers and exporters, there is a third group

FIG 2 Schematic map of genetic environment of *eat*(A) (i.e., *EFAU004_00630*) in the genome of *E. faecium* Aus0004 (GenBank accession no. NC_017022). Open reading frames are shown as arrows indicating the orientation of their coding sequence. The genes *EFAU004_00628*, *EFAU004_00629*, and *EFAU004_00631* code for a 2-dehydropentoate 2-reductase, a glycerate kinase, and a phosphoglycerate mutase, respectively. The nucleotide sequence corresponding to the upstream region of the *eat*(A) gene is represented in detail. The −35 and −10 promoter boxes are underlined, and the transcription start site is represented by an arrow. The start codon of *eat*(A) and its putative ribosome-binding site (RBS) as well as the stop codon of *EFAU004_00629* are also indicated.

of ABC proteins (named class 2) that lack TMDs, consisting of two NBDs fused into a single protein (21–23). Several of these class 2 ABC systems have been involved in MLS resistance, such as Msr-, Vga-, or Lsa-like proteins (21).

The observed profile of cross-resistance to lincosamides, streptogramins A, and pleuromutilins conferred by Eat(A)$_v$ was similar to those conferred by other Lsa-like proteins. In *Enterococcus faecalis*, Lsa(A) is responsible for intrinsic LS_AP resistance (6, 14). Lsa(B), encoded by a plasmid-borne gene from *Staphylococcus sciuri*, confers an increase in MICs of lincosamides, whereas streptogramins A and pleuromutilins had not been tested; however, a LS_AP phenotype is very likely (9). A chromosomal gene, *lsa*(C), was demonstrated to be responsible for acquired LS_AP resistance in *Streptococcus agalactiae* clinical isolates (14). Very recently, a novel gene, named *lsa*(E), which likely originated from *E. faecalis*, was found in both methicillin-resistant and -susceptible *Staphylococcus aureus* isolates of animal and human origins (11, 12). Interestingly, all Lsa-like proteins conferring a LS_AP phenotype [i.e., Lsa(A), Lsa(B), Lsa(C), Lsa(E), and Eat(A)$_v$] possess an isoleucine (a hydrophobic amino acid) instead of a threonine (a polar neutral amino acid) (Fig. 1). This suggests an important role of position 450 in LS_AP resistance, since it is located within the Walker B motif of the second NBD, a domain known to be involved in ATP binding (24).

Another important question that remains poorly elucidated is the biochemical mechanism of resistance. Indeed, even if class 2 ABC proteins are presumed to act as efflux pump systems, only one study of Msr(A) suggests that these proteins might be able to hijack the TMDs of ABC transporters to mediate efflux (25). However, no membrane partners for Msr(A) have clearly been identified so far (26). A ribosomal protection mechanism of resistance might also be hypothesized. Since *eat*(A) is an innate gene in *E. faecium*, it is obvious that it has a physiological role in the bacterial cell, and Eat(A) might be involved in protein translation since Lsa-like proteins are homologous to the eukaryotic elongation factor eEF-3 from the fungus *Saccharomyces cerevisiae* (27).

Concerning the expression of the *eat*(A) gene, a long 5′ UTR has been identified, suggesting either a transcriptional mechanism or a posttranscriptional (translational) regulation mechanism. Transcriptional attenuation may occur since a Rho-independent transcription terminator has been bioinformatically predicted (data not shown). This regulatory strategy is largely used by bacteria for sensing intracellular signals (28) and has already been described for the regulation of *erm*(K), another MLS resistance determinant (29). Also, a 44-amino-acid putative peptide preceding the *eat*(A) start codon was identified, which may be part of a mechanism of translational attenuation; however, no obvious inverted repeat sequences have been found (data not shown). The presence of a leader peptide in the 5′ UTR has been reported or postulated to be involved in the posttranscriptional regulation of several MLS resistance genes, such as *erm*(A), *erm*(B), *erm*(C), *msr*(A), *lsa*(A), and *lsa*(B) (6, 9, 30). Further investigations are currently in progress to determine the exact mechanism of *eat*(A) expression regulation.

In conclusion, this is the first characterization of the molecular mechanism of the acquired LS_AP resistance phenotype in *E. faecium*. Even though the LS_AP phenotype could be phenotypically detected, PCR detection using specific primers may be needed, especially for *E. faecium* strains with combined resistance mechanisms (e.g., the MLS_B phenotype). As for other ABC proteins of this class, the biochemical mechanism of resistance and the physiological role will need to be further investigated.

ACKNOWLEDGMENTS

This work was funded by a grant from the Ministère de l'Enseignement Supérieur et de la Recherche (EA4655), Université Caen Basse-Normandie, France.

We gratefully acknowledge the technical assistance of Michel Auzou. We kindly thank Marilyn C. Roberts for providing us the gene name according to the nomenclature center for MLS genes (http://faculty.washington.edu/marilynr/).

REFERENCES

1. **Arias CA, Murray BE.** 2012. The rise of the *Enterococcus*: beyond vancomycin resistance. Nat. Rev. Microbiol. **10:**266–278.
2. **Cattoir V, Leclercq R.** 2013. Twenty-five years of shared life with vancomycin-resistant enterococci: is it time to divorce? J. Antimicrob. Chemother. **68:**731–742.
3. **Eliopoulos GM.** 2003. Quinupristin-dalfopristin and linezolid: evidence and opinion. Clin. Infect. Dis. **36:**473–481.
4. **Leclercq R.** 2002. Mechanisms of resistance to macrolides and lincosamides: nature of the resistance elements and their clinical implications. Clin. Infect. Dis. **34:**482–492.
5. **Novak R, Shlaes DM.** 2010. The pleuromutilin antibiotics: a new class for human use. Curr. Opin. Investig. Drugs **11:**182–191.
6. **Singh KV, Weinstock GM, Murray BE.** 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.
7. **Dina J, Malbruny B, Leclercq R.** 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. **Singh KV, Murray BE.** 2005. Differences in the *Enterococcus faecalis lsa* locus that influence susceptibility to quinupristin-dalfopristin and clindamycin. Antimicrob. Agents Chemother. **49:**32–39.
9. **Kehrenberg C, Ojo KK, Schwarz S.** 2004. Nucleotide sequence and organization of the multiresistance plasmid pSCFS1 from *Staphylococcus sciuri*. J. Antimicrob. Chemother. **54:**936–939.
10. **Kehrenberg C, Aarestrup FM, Schwarz S.** 2007. IS21-558 insertion sequences are involved in the mobility of the multiresistance gene *cfr*. Antimicrob. Agents Chemother. **51:**483–487.
11. **Wendlandt S, Lozano C, Kadlec K, Gómez-Sanz E, Zarazaga M, Torres C, Schwarz S.** 2013. The enterococcal ABC transporter gene *lsa*(E) confers combined resistance to lincosamides, pleuromutilins and streptogramin A antibiotics in methicillin-susceptible and methicillin-resistant *Staphylococcus aureus*. J. Antimicrob. Chemother. **68:**473–475.
12. **Li B, Wendlandt S, Yao J, Liu Y, Zhang Q, Shi Z, Wei J, Shao D, Schwarz S, Wang S, Ma Z.** 2013. Detection and new genetic environment of the pleuromutilin-lincosamide-streptogramin A resistance gene *lsa*(E) in methicillin-resistant *Staphylococcus aureus* of swine origin. J. Antimicrob. Chemother. **68:**1251–1255.
13. **Malbruny B, Werno AM, Anderson TP, Murdoch DR, Leclercq R.** 2004. A new phenotype of resistance to lincosamide and streptogramin A-type antibiotics in *Streptococcus agalactiae* in New Zealand. J. Antimicrob. Chemother. **54:**1040–1044.
14. **Malbruny B, Werno AM, Murdoch DR, Leclercq R, Cattoir V.** 2011. Cross-resistance to lincosamides, streptogramins A, and pleuromutilins due to the *lsa*(C) gene in *Streptococcus agalactiae* UCN70. Antimicrob. Agents Chemother. **55:**1470–1474.
15. **Dowzicky M, Talbot GH, Feger C, Prokocimer P, Etienne J, Leclercq R.** 2000. Characterization of isolates associated with emerging resistance to quinupristin/dalfopristin (Synercid) during a worldwide clinical program. Diagn. Microbiol. Infect. Dis. **37:**57–62.
16. **Malbruny B, Leclercq R, Cattoir V.** 2012. Abstr 22nd Eur. Congr. Clin. Microbiol. Infect. Dis., London, United Kingdom, 31 March to 3 April 2012, abstr P1284.
17. **Bérenger R, Bourdon N, Auzou M, Leclercq R, Cattoir V.** 2011. In vitro activity of new antimicrobial agents against glycopeptide-resistant *Enterococcus faecium* clinical isolates from France between 2006 and 2008. Med. Mal. Infect. **41:**405–409.

18. **Clinical and Laboratory Standards Institute.** 2013. Performance standards for antimicrobial susceptibility testing; approved standard, 23rd ed., M100-S23. Clinical and Laboratory Standards Institute, Wayne, PA.
19. **Lalioui L, Pellegrini E, Dramsi S, Baptista M, Bourgeois N, Doucet-Populaire F, Rusniok C, Zouine M, Glaser P, Kunst F, Poyart C, Trieu-Cuot P.** 2005. The SrtA sortase of *Streptococcus agalactiae* is required for cell wall anchoring of proteins containing the LPXTG motif, for adhesion to epithelial cells, and for colonization of the mouse intestine. Infect. Immun. **73:**3342–3350.
20. **Arsène S, Leclercq R.** 2007. Role of a *qnr*-like gene in the intrinsic resistance of *Enterococcus faecalis* to fluoroquinolones. Antimicrob. Agents Chemother. **51:**3254–3258.
21. **Davidson AL, Dassa E, Orelle C, Chen J.** 2008. Structure, function, and evolution of bacterial ATP-binding cassette systems. Microbiol. Mol. Biol. Rev. **72:**317–364.
22. **Biemans-Oldehinkel E, Doeven MK, Poolman B.** 2006. ABC transporter architecture and regulatory roles of accessory domains. FEBS Lett. **580:** 1023–1035.
23. **Kos V, Ford RC.** 2009. The ATP-binding cassette family: a structural perspective. Cell. Mol. Life Sci. **66:**3111–3126.
24. **Falcón-Pérez JM, Martínez-Burgos M, Molano J, Mazón MJ, Eraso P.** 2001. Domain interactions in the yeast ATP binding cassette transporter Ycf1p: intragenic suppressor analysis of mutations in the nucleotide binding domains. J. Bacteriol. **183:**4761–4770.
25. **Ross JI, Eady EA, Cove JH, Cunliffe WJ, Baumberg S, Wootton JC.** 1990. Inducible erythromycin resistance in staphylococci is encoded by a member of the ATP-binding transport super-gene family. Mol. Microbiol. **4:**1207–1214.
26. **Reynolds E, Ross JI, Cove JH.** 2003. Msr(A) and related macrolide/streptogramin resistance determinants: incomplete transporters? Int. J. Antimicrob. Agents **22:**228–236.
27. **Andersen CB, Becker T, Blau M, Anand M, Halic M, Balar B, Mielke T, Boesen T, Pedersen JS, Spahn CM, Kinzy TG, Andersen GR, Beckmann R.** 2006. Structure of eEF3 and the mechanism of transfer RNA release from the E-site. Nature **443:**663–668.
28. **Merino E, Yanofsky C.** 2005. Transcription attenuation: a highly conserved regulatory strategy used by bacteria. Trends Genet. **21:**260–265.
29. **Kwak JH, Choi EC, Weisblum B.** 1991. Transcriptional attenuation control of *ermK*, a macrolide-lincosamide-streptogramin B resistance determinant from *Bacillus licheniformis*. J. Bacteriol. **173:**4725–4735.
30. **Depardieu F, Podglajen I, Leclercq R, Collatz E, Courvalin P.** 2007. Modes and modulations of antibiotic resistance gene expression. Clin. Microbiol. Rev. **20:**79–114.
31. **Bozdogan B, Leclercq R.** 1999. Effects of genes encoding resistance to streptogramins A and B on the activity of quinupristin-dalfopristin against *Enterococcus faecium*. Antimicrob. Agents Chemother. **43:**2720–2725.