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Characterization of a New *erm*-Related Macrolide Resistance Gene Present in Probiotic Strains of *Bacillus clausii*

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The mechanism of resistance to macrolides, lincosamides, and streptogramins B was studied in four *Bacillus clausii* strains that are mixed in a probiotic administered to humans for prevention of gastrointestinal side effects due to oral antibiotic chemotherapy and in three reference strains of *B. clausii*, DSM8716, ATCC 21536, and ATCC 21537. An 846-bp gene called *erm*(34), which is related to the *erm* genes conferring resistance to these antibiotics by ribosomal methylation, was cloned from total DNA of *B. clausii* DSM8716 into *Escherichia coli*. The deduced amino acid sequence presented 61% identity with that of Erm(D) from *B. licheniformis*, *B. halodurans*, and *B. anthracis*. Pulsed-field gel electrophoresis of total DNA digested by I-*Ceu*I, followed by hybridization with an *erm*(34)-specific probe, indicated a chromosomal location of the gene in all *B. clausii* strains. Repeated attempts to transfer resistance to macrolides by conjugation from *B. clausii* strains to *Enterococcus faecalis* JH2-2, *E. faecium* HM1070, and *B. subtilis* UCN19 were unsuccessful.

Spores of *Bacillus* sp. are administered to humans for prevention of gastrointestinal side effects due to oral antibiotic therapy. The potential effects of spores are to restore an intestinal flora following destruction of commensals by antibiotics, immunostimulation, and increased secretion of immunoglobulins A (22, 23). It has been shown in a murine model that *Bacillus* spores can germinate in significant numbers in the jejunum and ileum (5). Enterogermina is a mixture of antibiotic-resistant *Bacillus* strains NR, OC, SIN, and T (7, 23). These strains have been recently identified as belonging to the species *Bacillus clausii* (30). Since administration of the probiotic is often combined with oral antibiotic treatment, the strains of *Bacillus* Enterogermina were antibiotic resistant (7, 22). Little is known about the origin of the Enterogermina strains, and each has a specific pattern of antibiotic resistance (7, 22). They are supposed to be mutants from a parental *Bacillus* following multiple-step selection. The low genetic diversity among these strains is consistent with the notion that they derive from closely related strains or from an unknown common ancestor (30). Erythromycin resistance is one of the reported characteristics of *B. clausii* strains (7). Oral administration of high numbers of multiply drug-resistant microorganisms might be a cause for concern if clinically important resistance determinants happened to be located on transferable genetic elements. A potential hazard is transfer of resistance to microorganisms pathogenic for humans. The risk that this event will occur and the consequences in terms of morbidity and mortality have not been evaluated. Parameters required for risk assessment include studies on the nature and mobility of the resistance genes of probiotics.

The aim of this work was to identify the mechanism of macrolide resistance in the *B. clausii* probiotic strains and to characterize the genetic support for the resistance determinant.

MATERIALS AND METHODS

Bacterial strains. The four *B. clausii* strains used for production of Enterogermina, OC, NR, SIN, and T, were obtained from Sanofi-Synthelabo OTC SpA (Milan, Italy) as separate spore suspensions. *B. clausii* DSM8716, ATCC 21536, and ATCC 21537 were used as reference strains.

Antibiotic susceptibility. The disk diffusion method was used to determine bacterial susceptibility to antibiotics as recommended by the Comité de l'Antibiogramme de la Société Française de Microbiologie (8). Disks impregnated with 40 μg of pristinamycin I were prepared in the laboratory. Interpretive criteria for susceptibility or resistance were those recommended by the Comité de l'Antibiogramme de la Société Française de Microbiologie (8). MICs were determined by agar dilution in accordance with the NCCLS (26, 27).

Plasmid analysis. Plasmid DNA was extracted from *Bacillus* strains as described by Ehrenfeld and Clewell (11). Briefly, bacterial cells were lysed with lysozyme and sodium dodecyl sulfate-NaOH. After treatment with potassium acetate, plasmid DNA was extracted with phenol-chloroform. *Enterococcus faecalis* JH2-2 containing plasmid pAD1 (59.6 kb) was used as a control (11). Plasmid size was estimated by comparison with a standard after digestion with *Eco*RI and electrophoretic migration.

Mating experiments. *E. faecalis* JH2-2 (16), *E. faecium* HM1070 (resistant to rifampin and fusidic acid) (4), and *B. subtilis* UCN19 (resistant to ciprofloxacin) (3) were used as recipients in mating experiments. In every transfer experiment, *E. faecalis* BM4110 or *B. subtilis* BM450 containing the conjugative plasmid pAMβ1 (10, 21) was used as a control. Agar plates for selection of transconjugants contained rifampin (50 μg/ml) plus fusidic acid (20 μg/ml) or ciprofloxacin (8 μg/ml) combined with erythromycin (20 μg/ml). All mating experiments were repeated a minimum of three times.

PCR. Deoxyoligonucleotide primers specific for the *erm*(A), *erm*(B), *erm*(C), and *erm*(TR) genes were those designed previously (1, 31). PCR experiments were carried out with a Perkin-Elmer 4600 thermal cycler with a denaturation step (94°C, 5 min), followed by 35 cycles of amplification (30 s of denaturation at 94°C, 45 s of annealing at 47°C, and 45 s of elongation at 72°C) and a final elongation step (72°C for 10 min). Primers 5′-GAGCTTAAAAAAATGA AAAA and 5′-TTTCTTTAACATTCTCTC were used to amplify the entire *erm*(34) gene.

Cloning experiments and gene analysis. Extraction of total DNA from *B. clausii* and cloning were performed by standard techniques (29). DNA from *B. clausii* was digested with various restriction enzymes, including *Hin*dIII and *Eco*RI. The fragments were cloned into plasmid pUC18 and introduced by electrotransformation into *E. coli* DH10B, and transformants were selected on agar containing ampicillin (200 μg/ml) and erythromycin (50 μg/ml). Subcloning

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in *E. faecalis* JH2-2 was done by using the shuttle plasmid pAT28 as a vector (32). Nucleotide and amino acid sequences were analyzed by using the BLAST and FASTA softwares available over the Internet at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/). Multiple-sequence alignment and phylogenetic tree preparation were performed with the ClustalX and PHYLIP programs available at the Centre de Ressources Infobiogen website (http://www.infobiogen.fr/). Secondary structures of the attenuator mRNA were analyzed by using the Mu-fold software (34).

Southern hybridization. DNA from *B. clausii* was digested with *Sma*I or I-*Ceu*I, separated by pulsed-field gel electrophoresis by a technique similar to that used for enterococci (2), transferred onto a nylon membrane, and hybridized to a probe specific for *erm*(34) of *B. clausii*. The probe consisted in the entire gene amplified by PCR and labeled with digoxigenin (Boehringer Mannheim France, Meylan, France). Similar hybridization experiments were performed with plasmid DNAs from *B. clausii* OC and T digested with *Eco*RI.

Nucleotide sequence accession number. The nucleotide sequence of the *erm*(34) gene from *B. clausii* DSM8716 has been deposited in the GenBank nucleotide sequence database under accession number AY234334.

RESULTS

Macrolide resistance in *B. clausii*. All of the *B. clausii* strains studied, including the three reference strains, displayed similar phenotypes of resistance to macrolides. By the disk diffusion technique, no inhibition zone was visible around disks of erythromycin (14-membered ring macrolide), azithromycin (15-membered ring macrolide), spiramycin (16-membered ring macrolide), lincomycin, clindamycin (lincosamides), and pristinamycin I (streptogramin B). MICs of erythromycin, spiramycin, lincomycin, clindamycin, and pristinamycin I were greater than 128 μg/ml. All strains were susceptible to pristinamycin (a combination of oral streptogramins A and B). This pattern of resistance defines an MLS_B phenotype generally due to the presence of an *erm* gene encoding a ribosomal methylase (19).

Identification of the *erm*(34) gene from *B. clausii* DSM8716. No DNA could be amplified with primers specific for the *erm*(A), *erm*(B), *erm*(C), and *erm*(TR) genes responsible for acquired MLS_B resistance in gram-positive organisms pathogenic for humans and animals and total DNA of *B. clausii* strains as a template. Total DNAs from *B. clausii* DSM8716 and the probiotic strains were used to clone the determinant responsible for macrolide resistance. Three DNA fragments that conferred erythromycin resistance on *E. coli* DH10B were cloned, a 10-kb *Hin*dIII fragment from *B. clausii* DSM8716, a 4-kb *Hin*dIII fragment from *B. clausii* T, and a 6-kb *Eco*RI fragment from *B. clausii* SIN. A 1.4-kb *Eco*RI-*Hin*dIII fragment from strain DSM8716 was then subcloned and sequenced on both strands. Analysis of the sequence revealed an open reading frame of 846 bp preceded at 10 bp by an AGGGG sequence similar to the ribosome-binding site consensus sequence. This open reading frame could possibly code for a 281-amino-acid protein (Fig. 1). Comparison of the deduced sequence with proteins showed homology with various Erm proteins. These proteins are ribosomal methylases that monomethylate or dimethylate adenine at position 2058 (*E. coli* numbering) in 23S rRNA, which binds macrolides. The methylation confers cross-resistance to macrolides, lincosamides, and streptogramins B, the so-called MLS_B resistance phenotype, because these molecules all have A2058 in their ribosomal binding site. The closest homology for the Erm sequence of *B. clausii* was with Erm(D) from *B. licheniformis*, *B. halodurans*, and *B. anthracis* (61% identity and 71% homology) and with Erm(W) from *Micromonospora griseorubida* (13, 14, 15, 17, 18) (Fig. 2). Although to a lesser extent, homology was also found with the other Erm proteins. *erm* genes with deduced amino acid sequences with less than 79% identity are given different letter or number designations (28). The *erm*-related gene of *B. clausii* DSM 8716 was thus designated *erm*(34). The 1.4-kb *Eco*RI-*Hin*dIII fragment containing the *erm*(34) gene was subcloned into shuttle plasmid pAT28 and introduced into *E. faecalis* JH2-2, where it conferred an MLS_B phenotype characterized by cross-resistance between erythromycin and lincomycin (MIC, >128 μg/ml), showing that this gene could also be expressed in a heterologous gram-positive background.

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1
AAAATACGTTGACTTTTTCGTGATATTCATGTATGGTAAACGAGTAATGAAGTCAAAACAACTGGA
                  81
GGATTGTTAAAAAT ATG CAT TTC ATA AGA TTG CGT TTT CTC GTT TTG AAC AAG
                M   H   F   I   R   L   R   F   L   V   L   N   K
122                        Leader Peptide
TAA AATTCTTCATGGAACAAATATGCAATAGTGAAATGTGAAGAAAGGGGACTCGTTCTGCCTTT
END
CTTTTTTGTGTGCCTAAACGTTCGTTGTAGGCGTATTTTGGCAAACGTTTGTTTGCGAAATGCGCT

TCGGGCGTATTCTTGTTCTCGTTTACTTTAAGCCGCAGACGCTTTCGTCTGCGGCTTTTTTTGTGC
                                                    355
AGGTAGAGCTTAAAAAAATGAAAAAGGGGCGAGTAGCC  ATG ACG AAA AAA ATG AAC
                                          M   T   K   K   M   N
AAG TAT AAT GGG AAA AAA CTT AGC CGT GGA GAA CCT CCC AAT TTT AGC
 K   Y   N   G   K   K   L   S   R   G   E   P   P   N   F   S
GGT CAG CAT TTT ATG CAC AAT AAA CGG CTA CTG AAG GAA ATT GTT GAT
 G   Q   H   F   M   H   N   K   R   L   L   K   E   I   V   D
AAA GCT GAC GTC TCT GTT CGT GAT ACG GTT TTA GAG CTG GGA GCA GGA
 K   A   D   V   S   V   R   D   T   V   L   E   L   G   A   G
AAA GGC GCG TTG ACG ACG ATT TTA AGC GAA CGC GCG GAC CGG GTT CTA
 K   G   A   L   T   T   I   L   S   E   R   A   D   R   V   L
GCC GTC GAG TAT GAC CAA AAA TGT ATT GAA GCG CTG CAA TGG AAA CTA
 A   V   E   Y   D   Q   K   C   I   E   A   L   Q   W   K   L
GTT GGG TCA AAA AAC GTG TCC ATT CTC CAT CAA GAT ATT ATG AAG GTG
 V   G   S   K   N   V   S   I   L   H   Q   D   I   M   K   V
GCA TTG CCA ACG GAA CCG TTT GTT GTT GTT TCC AAC ATC CCT TAT TCG
 A   L   P   T   E   P   F   V   V   V   S   N   I   P   Y   S
ATC ACA ACG GCA ATC ATG AAA ATG CTG TTA AAC AAT CCA AAA AAC AAA
 I   T   T   A   I   M   K   M   L   L   N   N   P   K   N   K
CTA CAA CGA GGG GCA ATT GTA ATG GAG AAA GGA GCA GCA AAG CGG TTT
 L   Q   R   G   A   I   V   M   E   K   G   A   A   K   R   F
ACA AGC GTT TCG CCG AAA GAC GCT TAT GTG ATG GCT TGG CAT ATG TGG
 T   S   V   S   P   K   D   A   Y   V   M   A   W   H   M   W
TTT GAC ATC CAC TAT GAA AGG GGA ATT TCC AGA AGT TCA TTT TCG CCG
 F   D   I   H   Y   E   R   G   I   S   R   S   S   F   S   P
CCG CCG AAA GTC GAT TCT GCC CTT GTC CGC ATT GTC CGC AAA CAG CAT
 P   P   K   V   D   S   A   L   V   R   I   V   R   K   Q   H
CCC CTT TTT CCA TAT AAA GAG GCG AAA GCG ATG CAT GAC TTT TTA TCG
 P   L   F   P   Y   K   E   A   K   A   M   H   D   F   L   S
TAC GCA CTA AAC AAC CCT AGA GCA CCC CTT GAT CAG GTA TTA CGA GGA
 Y   A   L   N   N   P   R   A   P   L   D   Q   V   L   R   G
ATT TTT ACC GCC CCT CAA GCA AAA AAA GTG CGG CAG GCA ATC GGC GTC
 I   F   T   A   P   Q   A   K   K   V   R   Q   A   I   G   V
AAA CCT GAG ACA CCA GTG GCC ATG CTT CAT GCC AGG CAG TGG GCG ATG
 K   P   E   T   P   V   A   M   L   H   A   R   Q   W   A   M
GTT TGT GAC GCG ATG GTT CGG CAT GTT CCA AAA GTG TAT TGG CCA AGG
 V   C   D   A   M   V   R   H   V   P   K   V   Y   W   P   R
                1200
CGA AAG AGA TAA
 R   K   R  END
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FIG. 1. *erm*(34) DNA sequence and deduced amino acid sequence. The nucleotide sequence of *erm*(34) is shown together with the deduced amino acid sequence of Erm34 methylase and its leader peptide. Putative ribosome-binding sites are underlined.

The structural gene for the putative methylase was preceded by a 68-nucleotide leader sequence, together with a ribosome-binding site, which could encode a 13-amino-acid peptide (MHFIRLRFLVLNK). In addition, series of inverted repeats that extended from the sequence of the leader peptide to the initiation sequences for the methylase (ribosome-binding site and initiation codon) were identified that could form stem-

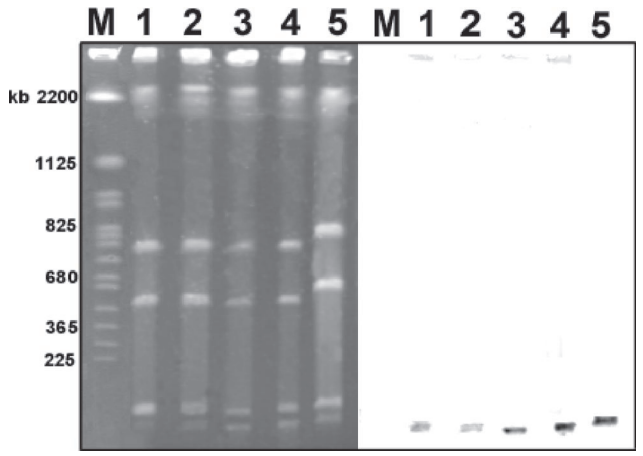

FIG. 2. Localization of the *erm*(34) gene in *B. clausii* NR, OC, SIN, T, and DSM8716. (Left) Total DNAs from *B. clausii* strains NR (lane 1), OC (lane 2), SIN (lane 3), T (lane 4), and DSM8716 (lane 5) were digested with the I-*Ceu*I restriction enzyme and submitted to pulsed-field gel electrophoresis. M, molecular size standard (*Saccharomyces cerevisiae* chromosomal DNA). (Right) DNA was transferred to a nylon membrane and hybridized with an *erm*(34) probe labeled with digoxigenin.

loops by base pairing. Computer analysis of the secondary structure of the mRNA proposed several alternative structural conformations. A final set of inverted repeats would sequester both the methylase ribosome-binding site and the codons for the first four amino acids of the methylase (data not shown). This structure resembles that involved in the expression of inducible *erm* genes, including *erm*(C), *erm*(A), and *erm*(D), which have been reported to function as translational or transcriptional attenuators (33).

Distribution and localization of the *erm*(34) gene. An 856-bp fragment could be amplified by PCR from the DNAs of all *B. clausii* strains. The sequences of all of the amplified DNA fragments were nearly identical. The total DNAs of the reference *B. clausii* strains and the four probiotic strains were digested with I-*Ceu*I or *Sma*I, submitted to pulsed-field gel electrophoresis, transferred to a nylon membrane, and hybridized successively with *erm*(34) and 16S rRNA probes. The I-*Ceu*I enzyme cuts in a 26-bp DNA sequence that is specific for rRNA operons (20). After digestion with this enzyme, the DNA from the *B. clausii* strains yielded seven fragments that hybridized with the rRNA probe, indicating that this species contained a minimum of seven rRNA operons (data not shown). The *erm*(34) probe hybridized to a single low-molecular-weight fragment in all of the strains studied. The *erm*(34) probe also hybridized to an approximately 20-kb *Sma*I fragment in all of the strains tested (data not shown).

The *B. clausii* probiotic strains were analyzed for their plasmid content. A large plasmid could be visualized only in *B. clausii* T and OC, confirming a previous report (22). After digestion with *Eco*RI and electrophoretic migration, the two plasmids yielded similar restriction patterns composed of four fragments. The size of the plasmid was estimated to be approximately 30 kb. The DNA fragments were transferred to a nylon membrane and hybridized with the *erm*(34) probe. No signal was detected. We therefore concluded that the *erm*(34) gene was chromosomally located.

In vitro transfer of resistance to macrolides. Repeated attempts to transfer resistance to macrolides by conjugation from *B. clausii* probiotic strains to *E. faecalis* JH2-2, *E. faecium* HM1070, and *B. subtilis* UCN19 were unsuccessful (frequencies inferior to the limit of detection, 10^{-9} per donor colony for *B. subtilis* and 5×10^{-10} for enterococci). By contrast, the 35-kb erythromycin resistance plasmid pAMβ1 could be transferred from *E. faecalis* BM4110/pAMβ1 or *B. subtilis* BM450/pAMβ1 to all recipient strains at frequencies approximately equal to 10^{-3} per donor colony for *E. faecalis* JH2-2 and *E. faecium* HM1070 and 10^{-4} per donor colony for *B. subtilis* UCN19.

DISCUSSION

The *B. clausii* probiotic strains are resistant to clinically important antibiotics, including macrolides and aminoglycosides (3, 22). We have recently shown that resistance to aminoglycosides was due to the synthesis of an aminoglycoside-inactivating enzyme encoded by an *aadD2* chromosomal gene (3). In this study, we have shown that resistance to macrolides was associated with the presence of an *erm*(34) gene that has not been characterized or found in other bacteria so far. A minimum of 21 *erm* gene classes have been reported, which are distinguished on the basis of sequence comparison (28). Some of the *erm* genes are found in the chromosome of microorganisms that produce antibiotics or in soil bacteria; others are found on plasmids and transposons in microorganisms pathogenic for humans and animals. The *erm*(34) gene differed from the other *erm* genes in *Bacillus* spp. As already mentioned, *erm*(D) genes, previously called *ermD*, *ermK*, and *ermJ*, were characterized in *B. licheniformis*, *B. halodurans*, and *B. anthracis*, respectively (13, 14, 17). The *ermD* and *ermK* genes are localized on the chromosome of the *Bacillus* strains, but the intrinsic or acquired nature of these determinants has not been established. By contrast, *ermJ* is probably acquired since *B. anthracis* strains are usually susceptible to macrolides. Since the sequences of ErmD, ErmK, and ErmJ are nearly identical, they were reclassified recently in a unique Erm(D) class (28). Another gene, *erm*(G), presumed to be chromosomal, has been characterized in *B. sphaericus* (25). A closely related gene (99.7% identity) borne by a conjugative transposon was found in *Bacteroides* sp. (9). Finally, a staphylococcal gene, *erm*(C), was detected in *B. subtilis*, where it is plasmid borne (24). Alignment of Erm methylases was used to construct a phylogenetic tree (12). The methylases from the antibiotic producers and those from pathogenic bacteria form two distinct groups, and Erm(34), although closely related to Erm(D) and Erm(W), was placed on a separate branch (Fig. 3).

An attenuator structure with a leader peptide and a set of inverted repeats similar to those regulating inducible expression of MLS_B resistance in several *erm* genes was identified upstream of *erm*(34). The induction mechanism has been intensively studied in the case of *erm*(C) from *Staphylococcus aureus*. It has been shown that *erm*(C) mRNA exists in a stable conformation in which the initiation sequences for the methylase are sequestered by base pairing and thus rendered inaccessible for ribosome binding (33). Binding of erythromycin to

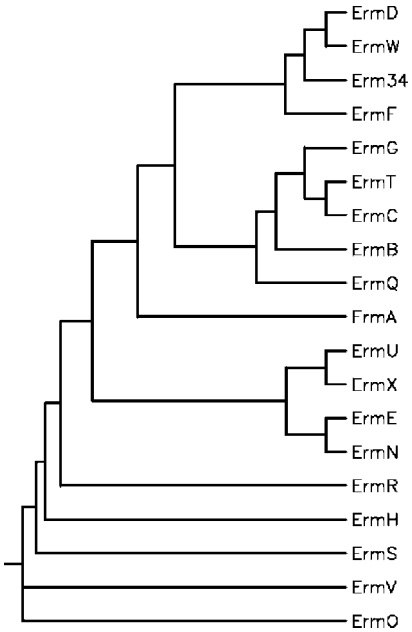

FIG. 3. Phylogenetic relationships among Erm methylases. The tree was constructed by using the neighbor-joining method. Sequences are from *S. aureus* [Erm(A) (accession no. X03216) and Erm(C) (accession no. J01755)], *Streptococcus pneumoniae* [Erm(B) (accession no. X52632)], *B. licheniformis* [Erm(D) (accession no. M29832)], *Saccharopolyspora erythraea* [Erm(E) (accession no. X51891)], *Bacteroides fragilis* [Erm(F) (accession no. M14730)], *B. sphaericus* [Erm(G) (accession no. M15332)], *Streptomyces thermotolerans* [Erm(H) (accession no. P13079)], *Streptomyces fradiae* [Erm(N) (accession no. X97721) and Erm(S)], *Streptomyces lividans* [Erm(O) (accession no. M74717)], *Clostridium perfringens* [Erm(R) (accession no. L22689)], *Arthrobacter* sp. [Erm(R) (accession no. M11276)], *Lactobacillus reuteri* [Erm(T) (accession no. M64090)], *Streptomyces lincolnensis* [Erm(U) (accession no. X62867)], *Corynebacterium diphtheriae* [Erm(X) (accession no. M36726)], *M. griseorubida* [Erm(W) (accession no. D14532)], and *B. clausii* [Erm34].

a ribosome during translation of the leader peptide yields ribosomal stalling. This stalling event results in opening of the structure, exposing the initiation sequences and allowing translation to occur. Translational regulation has also been proposed for the regulation of resistance to MLS_B antibiotics encoded by the *erm*(A) and *erm*(B) genes. In the case of *ermK* from *B. licheniformis*, both translational attenuation and transcriptional attenuation seem to contribute to the regulation of the gene (6, 18). Close similarities between the attenuators of *ermK* and *erm*(34) suggest that the same mechanisms might modulate the expression of macrolide resistance in *B. clausii*.

Although we could study only a few *B. clausii* strains, the *erm*(34) gene, which is chromosomal, is probably species specific and the MLS_B resistance is inherent to *B. clausii*. The stability of the macrolide resistance and the high level of resistance conferred by the *erm*(34) gene constitute an advantage, allowing the probiotic to be maintained in the gut when it is coadministered with oral macrolides.

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