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Complete Nucleotide Sequence and Transcription of *ermF*, a Macrolide-Lincosamide-Streptogramin B Resistance Determinant from *Bacteroides fragilis*

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DNA sequence analysis of a portion of an *Eco*RI fragment of the *Bacteroides fragilis* R plasmid pBF4 has allowed us to identify the macrolide-lincosamide-streptogramin B resistance (MLS^r) gene, *ermF*. *ermF* had a relative moles percent G+C of 32, was 798 base pairs in length, and encoded a protein of approximately 30,360 daltons. Comparison between the deduced amino acid sequence of *ermF* and six other *erm* genes from gram-positive bacteria revealed striking homologies among all of these determinants, suggesting a common origin. Based on these and other data, we believe that *ermF* codes for an rRNA methylase. Analysis of the nucleotide sequences upstream and downstream from the *ermF* gene revealed the presence of directly repeated sequences, now identified as two copies of the insertion element IS*4351*. One of these insertion elements was only 26 base pairs from the start codon of *ermF* and contained the transcriptional start signal for this gene as judged by S1 nuclease mapping experiments. Additional sequence analysis of the 26 base pairs separating *ermF* and IS*4351* disclosed strong similarities between this region and the upstream regulatory control sequences of *ermC* and *ermA* (determinants of staphylococcal origin). These results suggested that *ermF* was not of *Bacteroides* origin and are discussed in terms of the evolution of *ermF* and the expression of drug resistance in heterologous hosts.

Coincident resistance to macrolide, lincosamide, and streptogramin B-type antibiotics (MLS^r) has been identified in both gram-positive and gram-negative microorganisms (4, 8, 10, 12, 14, 41, 44, 67). In those organisms studied, the mechanism of naturally occurring MLS^r was invariably determined to be due to mono- or dimethylation of adenine residues in the 23S rRNA (8, 17, 50, 53, 63). This modification reduces affinity between MLS antibiotics and the 50S ribosomal subunit. MLS^r bacteria often exhibit inducibly expressed resistance to these antibiotics, although constitutive resistance has been noted (10, 30, 64, 67). Induction appears to occur posttranscriptionally by a mechanism of translational attenuation (27, 28, 39), requiring the presence of a 5′ leader sequence upstream in the mRNA of the MLS determinant. This mode of induction has been reviewed recently by Dubnau (14) and by Weisblum (67).

Resistance to MLS antibiotics among gram-negative, anaerobic *Bacteroides* species was first documented in 1979 (41, 60, 69) and has been recently reviewed (58). Three *Bacteroides* R plasmids that specify constitutive MLS^r have been identified (59, 61, 70): pBF4 (41 kilobases [kb]) and pBFTM10 (15 kb), which were isolated from strains of *Bacteroides fragilis*; and pBI136 (82 kb), which was isolated from *Bacteroides ovatus*. The MLS^r determinant from each plasmid has been localized to a single *Eco*RI restriction fragment. Although they vary in size and restriction endonuclease cleavage patterns, these fragments contain homologous sequences, presumably due to a similar MLS^r gene (23, 48, 54). Additional homology among these fragments was due to directly repeated DNA sequences (DR) which flank the MLS^r determinant of each of these plasmids (23, 57; J. L. Rasmussen, D. A. Odelson, and F. L. Macrina, manuscript in preparation). Likewise, the MLS^r determinants and the flanking DRs of both pBF4 (51, 52) and pBFTM10 (45) have also been shown to undergo transposition in both *E. coli* and *Bacteroides* spp. and have been designated as transposons Tn*4351* and Tn*4400*, respectively.

Our interest in MLS^r in *Bacteroides* spp. has centered on understanding the genetic basis of this antibiotic resistance. Accordingly, in this paper we report on the nucleotide sequence of the pBF4 MLS^r gene which we propose to designate *ermF*. The nucleotide as well as the deduced amino acid sequence of this constitutively expressed MLS^r element from an anaerobic gram-negative organism was compared with the sequences of six MLS^r genes from gram-positive organisms (21, 27, 29, 38, 44, 65). Striking similarities among all of the gene products were noted. We also examined the expression of the pBF4 MLS^r determinant and present evidence that transcriptional control of this gene is dependent on signals contained in a copy of an insertion element situated immediately upstream of the *ermF* structural gene.

MATERIALS AND METHODS

Bacterial strains and media. Bacterial strains and plasmids are shown in Table 1. *Escherichia coli* JM101 was used for the propagation of M13 recombinant phage constructed in this study. The chimeric plasmid pFD176 (55) was used to isolate fragments employed in S1 nuclease mapping and also for Northern blot hybridization experiments. Strain V831 was maintained on LB medium (11) containing 10 μg of tetracycline per ml. Strain JM101 was maintained on YT medium (36). Strains V479-1 and V600 were maintained on supplemented brain heart infusion medium (31) containing 5

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TABLE 1. Bacterial strains and plasmids

Species	Strain	Plasmid	Description[a]	Source or reference
Bacteroides fragilis	V479-1	pBF4	MLS^r Tc^r	(69)
	V600	pBF4Δ1[b]	MLS^s Tc^r	(69)
Escherichia coli	V831	pVA831 (pBR325::*Eco*RI-D)	*hsdR4* Gal^- Tc^r Ap^r	(33)
	JM101	F′	Δ(*lac proAB*)/F′ *traD36* $ProA^+B^+$ *lacI*^{q}ZΔM13	New England Biolabs

[a] Description abbreviations: MLS^r, operationally, resistant to 10 μg of clindamycin or erythromycin; Tc^r, tetracycline resistance (10 μg/ml); *hsd*R4, host-specific restriction deficient; Gal^-, galactose nonfermenting; Ap^r, ampicillin resistant (25 μg/ml); *tra*D, conjugation deficient; *proAB*, proline auxotroph.
[b] pBF4Δ1 is a spontaneous deletion derivation of pBF4 missing the *Eco*RI D fragment and, hence, the MLS^r gene.

μg of clindamycin per ml and 10 μg of tetracycline per ml, respectively.

Genetic transformation of *E. coli*. *E. coli* JM101 was transformed with M13 recombinant phage according to the protocol supplied by New England Biolabs. Competent cells were cryogenically preserved as described by Morrison (37).

Plasmid DNA and M13 replicative-form DNA isolation. Preparations of covalently closed circular DNA were obtained by the method of Guerry et al. (22) adjusted for 100 ml of cell culture. Bacterial cultures were grown at 37°C with aeration and harvested by standard procedures (13, 32). Plasmid pVA831 was amplified as described by Maniatis et al. (32). Phage M13 replicative-form DNA (34) was prepared by separate inoculation of 2.5 and 0.25 ml of YT broth with JM101 and with a loopful of phage cultivated in YT overlay, respectively. The bacterial and phage cultures were incubated at 37°C with aeration for 2.5 h, combined in 100 ml of fresh YT, and incubated at 37°C with aeration for an additional 5.5 h. The cell pellet from a 100-ml culture was used in the preparation of M13 phage replicative-form DNA. Plasmid or replicative-form DNA was further purified by dye-buoyant density centrifugation (43) of cleared lysates and analyzed by electrophoresis through agarose gels (35). *E. coli* V517 was used as a source of size reference covalently closed circular DNA molecules (31), whereas *Hin*dIII-digested lambda phage DNA or the 1-kb ladder (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was used for linear size reference molecules.

RNA isolation. RNA was extracted from logarithmic-phase cultures of *B. fragilis* essentially as described by Sarmientos et al. (47). A 10-ml sample of medium containing the appropriate antibiotic was inoculated with 1 ml of an overnight culture of bacteria, harvested after 2 to 3 h of growth at 37°C, and suspended in 2 ml of Tris hydrochloride-EDTA (32). After lysis of bacteria, samples were treated with DNase I (Worthington Diagnostics, Freehold, N.J.), extracted twice with phenol, and ethanol precipitated. The amount of RNA in samples was estimated by assuming that 40 μg of RNA per ml had an absorbance of 1.0 optical density unit at 260 nm (32).

Construction of M13 clones. Purified phage replicative-form DNA (mp8 or mp9) was cleaved with the desired restriction endonuclease and ligated with pVA831 DNA that had been cleaved with a compatible restriction endonuclease. T4 DNA ligase was used at a concentration of 2 and 400 U per reaction for cohesive and blunt end ligations, respectively. Purified recombinant phage replicative-form DNA was used in the construction of overlapping deletion derivatives (40). Recombinant plaques of interest were identified by a modification of the Benton and Davis plaque screening method (2). Plaques were lifted from agar plates with nitrocellulose (Schleicher & Schuell Co., Keene, N.H.) or Gene-Screen (New England Nuclear Corp., Boston, Mass.) filters. Phage-laden filters were treated as described by Maniatis et al. (32), except incubations were extended to 10 min. The filters then were rinsed for 1 min in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0) and baked for 2 h at 80°C. Prewashing, prehybridization, and hybridization were as described by Maniatis et al. (32). Nick translation kits were obtained from New England Nuclear and used as recommended by the supplier.

DNA sequencing. Single-stranded DNA from recombinant phage was isolated and employed as a template in the dideoxy sequencing method of Sanger et al. (46). Sequencing reactions were performed according to the protocol supplied by New England Biolabs, except before incubation template, primer, and sequencing buffer were mixed in a 1.5-ml polypropylene tube, incubated in a boiling water bath for 3 min, and then allowed to cool for 30 min. After incubation samples were fractionated by electrophoresis in 6% polyacrylamide gels containing 8 M urea.

S1 nuclease mapping. DNA-RNA hybridization and digestion with S1 nuclease were carried out as described by Christie and Calendar (7), except that hybrids were incubated at 40°C and were digested for 20 min at room temperature with 300 U of S1 nuclease (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). mRNA from *B. fragilis* V479-1 was used to protect a *Hin*dIII fragment from pFD176 (55) that contained the MLS^r gene of pBF4. In general 20 to 50 μg of RNA was used for each reaction. ^{32}P end-labeled 1-kb or 123-base-pair (bp) ladders (Bethesda Research Laboratories) were used as linear size reference molecules for estimation of S1-protected fragments.

Northern hybridization. Electrophoresis and Northern blot transfer of RNA were performed as described by Maniatis et al. (32) with 1.0% (wt/vol) agarose gels containing 2.2 M formaldehyde and Gene-Screen Plus (New England Nuclear), respectively.

Enzymes and nucleotides. Restriction endonucleases were purchased from Bethesda Research Laboratories, Boehringer Mannheim, International Biotechnologies, Inc. (New Haven, Conn.), or New England Biolabs (Beverly, Mass.) and used as recommended by the suppliers. Exonuclease *Bal* 31 was purchased from Boehringer Mannheim. *E. coli* DNA polymerase I large fragment, M13 oligonucleotide sequencing primer (17-mer), and T4 DNA ligase were purchased from New England Biolabs. Deoxy- and dideoxynucleotide triphosphates were obtained from Pharmacia, Inc. (Piscataway, N.J.). Radiolabeled nucleotides ([α-^{32}P]dATP, 800 Ci/mmol; [γ-^{32}P]ATP, 7,000 Ci/mmol; [α-^{32}P]dCTP, 800 Ci/mmol) were purchased from New England Nuclear.

Computer analysis. DNA sequence data were analyzed by the program of Conrad and Mount (9) or by the Microgenie program (42).

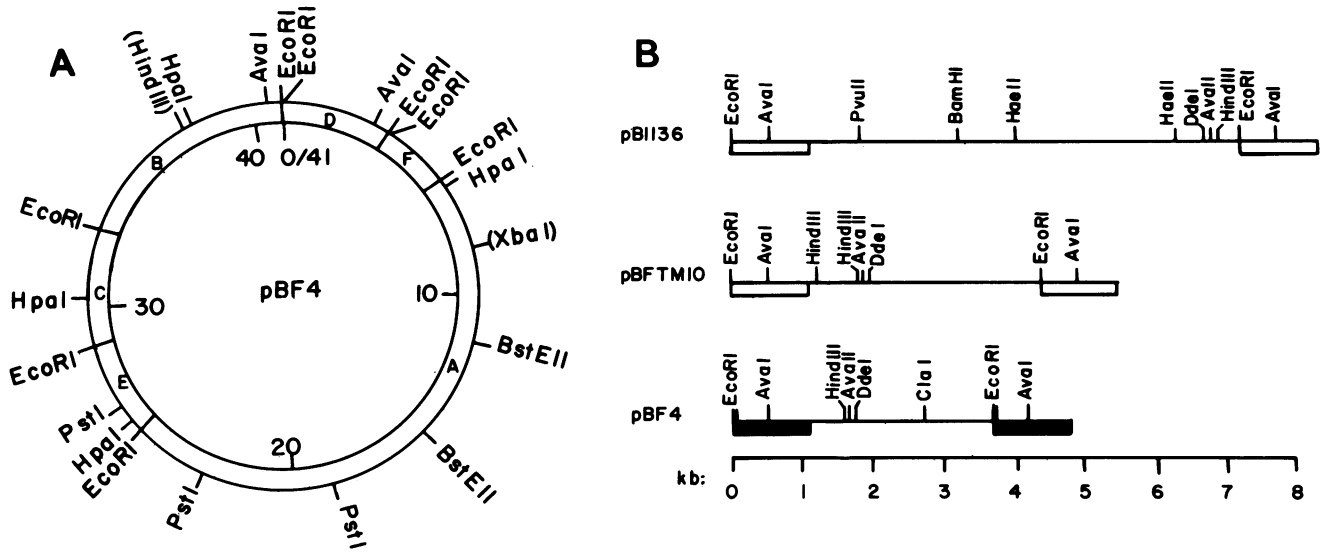

FIG. 1. (A) Restriction endonuclease map of the 41-kb R plasmid pBF4 from *B. fragilis*. Site designations in parentheses indicate the presence of additional unmapped cleavage sites. Capital letters in the inner circle refer to *Eco*RI-generated fragments. (B) Linear restriction endonuclease map of the MLS^r region and the flanking DNA of three *Bacteroides* R plasmids: pBI136 (54); pBFTM10 (61); and pBF4 (69). Each region shown contains additional unmapped *Ava*II and *Dde*I cleavage sites. The directly repeated sequences of pBF4 known to be copies of the insertion sequence element IS*4351* (see the text) are shown as the thick black lines. Comparable but unconfirmed sequences flanking the MLS^r genes on pBFTM10 and pBI136 are shown as open rectangles.

RESULTS

DNA sequence analysis of *ermF*. A restriction endonuclease map of pBF4 is illustrated in Fig. 1A. Early evidence (68) suggested that the MLS^r determinant encoded by this *Bacteroides* plasmid was contained on the *Eco*RI D fragment (0 to 3.8 kb on Fig. 1 circular map). Specifically, spontaneously occurring MLS-susceptible strains could be readily isolated that always carried pBF4 deletion derivatives missing the *Eco*RI D fragment. Shoemaker and co-workers (52) first showed that the *Eco*RI D fragment conferred MLS^r when tested with an *E. coli-Bacteroides* shuttle plasmid system. This work was extended by Smith (55), who demonstrated that a 1.9-kb *Ava*I-*Hae*II fragment from *Eco*RI-D conferred MLS^r in *Bacteroides* spp. This *Ava*I-*Hae*II fragment contained sequential *Hin*dIII, *Ava*II, and *Dde*I cleavage sites (57). Also noteworthy was the observation that this *Hin*dIII-*Ava*II-*Dde*I pattern was flanked by a directly repeated sequence of approximately 1.1 kb (57). This restriction pattern and its flanking DRs were found on two other different MLS^r *Bacteroides* plasmids (pBFTM10 and pBI136), suggesting the dissemination of a common MLS^r gene in *Bacteroides* spp. Restriction maps of these corresponding regions of the three different *Bacteroides* plasmids (pBF4, pBFTM10, and pBI136) are shown in Fig. 1B.

Based on these data, we concentrated our DNA sequencing efforts on the *Hin*dIII-*Ava*II-*Dde*I region. Two strategies were used to clone fragments of *Eco*RI-D for subsequent DNA sequence analysis. First, restriction endonuclease fragments of *Eco*RI-D were subcloned into the M13 vectors mp8 and mp9 from the chimeric plasmid pVA831 (pBR325::*Eco*RI-D [33]). Second, certain cloned restriction fragments were used as substrates for *Bal* 31 exonuclease to generate overlapping deletion derivatives. Figure 2 shows the sequencing strategies we employed to determine the nucleotide sequences in and around the area believed to contain the MLS^r gene based on the above data.

DNA sequences upstream from the terminus of the right

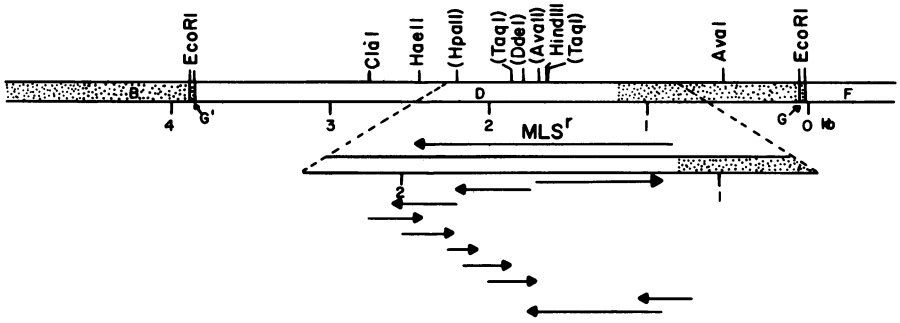

FIG. 2. Sequencing strategy and restriction endonuclease map of the *Eco*RI D fragment and the flanking DNA of pBF4. Restriction sites in parentheses refer to multiple sites within the D fragment. The arrow beneath MLS^r indicates the location of the MLS^r gene as well as its direction of transcription. Other arrows indicate the approximate base pair length of recombinant M13 clones as well as the direction of sequencing analyses. The stippled areas represent the directly repeated sequences (IS*4351*) flanking the MLS^r region.

member of the DR (Fig. 1 and 2) were analyzed by computer for an open reading frame which could correspond to the MLS^r gene. An open reading frame of 798 bp was identified starting 1,180 bp from the 3′ terminus of the right member of the DR. As expected, this region included the *Hin*dIII, *Ava*II, and *Dde*I sites believed to indicate the MLS^r genes of pBF4, pBFTM10, and pBI136 (see above). This open reading frame corresponded to a 266-amino-acid polypeptide with a calculated molecular weight of 30,360. The amino acid sequence deduced from this open reading frame revealed this protein to be strikingly similar to several MLS^r-associated gene rRNA methylases (see below). From this observation and genetic data cited above, we inferred that the open reading frame represented the pBF4 MLS^r determinant. Hereafter, we shall refer to this gene as *ermF*. The nucleotide sequence of *ermF*, with selected restriction sites and its deduced amino acid sequence as well as 240 bp of upstream sequence, is displayed in Fig. 3. A 6-bp sequence, 5′ AAGAAG 3′ (S-D in Fig. 3), just upstream from the start codon of *ermF* shows a match of 5 of 6 bp with the 3′ terminus of *Bacteroides* 16S rRNA (67), making this a likely candidate for the ribosome-binding site of the *ermF* mRNA. The frequency of codon usage of the *ermF* gene is shown in Fig. 4. Note the occurrence of *E. coli* minor tRNA species (underlined) used in this gene.

Comparison of *erm* determinants at the nucleotide and amino acid sequence level. Computer-generated restriction endonuclease cleavage site data of the published DNA sequences of four gram-positive *erm* determinants were compared with that of *ermF*. Figure 5 shows these representative restriction endonuclease cleavage sites maps aligned at their start codons. Previously published results revealed that nucleotide homology approached 60% when *ermA*, *ermS*, and *ermC* were compared (38). Despite this, these determinants and *ermF* failed to show any relatedness when examined by restriction enzyme cleavage patterns (Fig. 5). The percent G+C content of *ermF* was calculated to be 34% compared with 32% for *ermA*, 32% for *ermS*, 25% for *ermC*, and 39% for *ermD*.

The deduced amino acid sequences of the *erm* determinants also were aligned to evaluate homology (Fig. 6). Alignment was made to give the most homology between *ermF* and the other *erm* determinants. Included in these analyses were two recently sequenced genes, one from *Streptomyces erythreus* (65; *ermE*) and one from an *Arthrobacter* sp. (44; *ermA′*). The percent G+C content of *ermE* was 72%, whereas that of *ermA′* was 76%. Their restriction endonuclease cleavage patterns showed no resemblance to *ermA*, *ermS*, *ermC*, *ermD*, or *ermF* as expected from their elevated G+C content. Because the unique G+C content of *ermE* and *ermA′* suggested their distant relatedness to the other alleles, only *ermA*, *ermS*, *ermC*, *ermD*, and *ermF* were subjected to extensive comparison beyond simple amino acid alignment studies.

Previous results (Fig. 5) suggested that the *erm* genes have diverged significantly such that simple colinear alignment of their nucleotide sequences was not particularly informative. However, we aligned nucleic acid sequences of *ermA*, *ermS*, *ermC*, *ermD*, and *ermF* by codon in a manner corresponding to the previously determined amino acid sequence alignment (Fig. 6, Table 2). Codons specifying identical amino acids were analyzed to determine what percentage was identical and what percentage differed only in the third position. All aligned codons were compared to determine what percentage had at least one base in common and what percentage had at least two bases in common. In all cases the percentage match was found to exceed that for randomly aligned codons (Table 2).

Transcription of *ermF*. Most of the *erm* determinants found in pathogenic bacteria are inducibly expressed owing to a posttranscriptional, translational attenuation mechanism (14, 66). Because we had previously established that the pBF4-mediated MLS^r was constitutively expressed in *B. fragilis*, it was of interest to examine the nucleotide sequences upstream of *ermF* for transcriptional start signals (26). The first important feature found was that one member of the direct repeated sequence of pBF4 (Fig. 1) had its terminus 26 bp from the *ermF* translational initiation codon. We have now demonstrated that this directly repeated sequence is a novel insertion sequence element that appears unique to *B. fragilis*. A description of this element (IS*4351*) will be published elsewhere (Rasmussen et al., in preparation). Robillard et al. (45) have demonstrated that an analogous sequence on pBFTM10 is a functional insertion element. The proximity of IS*4351* to the *ermF* start codon led us to predict that the transcriptional start site for this gene was contained within this insertion sequence element.

The location of the transcriptional start site of *ermF* was determined by S1 nuclease mapping of the 5′ end of the transcripts (Fig. 7A). Protection from S1 digestion of a *Hin*dIII fragment from pFD176 (55) with RNA from V479-1 yielded a major fragment of 638 ± 11 (x ± standard error of the mean; n = 6) bases as well as a minor fragment of about 500 bases (Fig. 7A, lane 1). The intensity of the protected fragments was proportional to the amount of V479-1 RNA used in reactions (data not shown). Moreover, no S1 signals were observed when RNA from V600, a spontaneous MLS^s derivative of V479-1, was used in place of V479-1 RNA (Fig. 7A, lane 2). Using these data along with those of Fig. 3, we conclude that a major transcriptional start site for *ermF* exists at approximately nucleotide position 40. The minor signal would correspond to a start site around the IS*4351* terminus (nucleotide 215).

The presence of one major transcriptional start site was further buttressed by Northern blot analysis of total cellular RNA from V479-1 as the target and pFD176 as the probe. DNA used as probe hybridized to a single RNA transcript of about 1,500 nucleotides in length (Fig. 7B).

Analysis of sequences upstream of the *ermF* determinant. Because of the nucleotide and amino acid similarity of *ermF* to other *erm* determinants, it was of interest to examine upstream sequences associated with *ermF*. Comparable sequences for *ermA*, *ermS*, *ermC*, and *ermD* are recognized as providing the basis for the posttranscriptional regulation (i.e., inducibility) of MLS resistance (14, 20, 25, 67). Only MLS resistance which is contitutively expressed has been described in *Bacteroides* spp. thus far. In the *Bacteroides* R plasmid pBF4, 26 nucleotides separate the end of IS*4351* from the *ermF* start codon (Fig. 3). We aligned the start codons of *ermF* and *ermC* and examined their upstream sequences for homology (Fig. 8). The thick line below the sequence denotes the *ermC* nucleotides postulated to participate in the mRNA secondary structure thought to sequester the ribosomal binding site (14, 20, 28, 38, 67). The end of IS*4351* is illustrated in the *ermF* display as the stippled area. Homology of the *ermF* sequence to the *ermC* sequence (arbitrarily terminated at the end of the inverted repeat structure) was 54%. Identical bases are denoted by open or half-filled circles. Half-filled circles correspond to bases occurring in the postulated secondary structure of the *ermC* mRNA. This result further suggested a common origin for *ermF* and *ermC*. Similar comparisons with *ermA* sequences

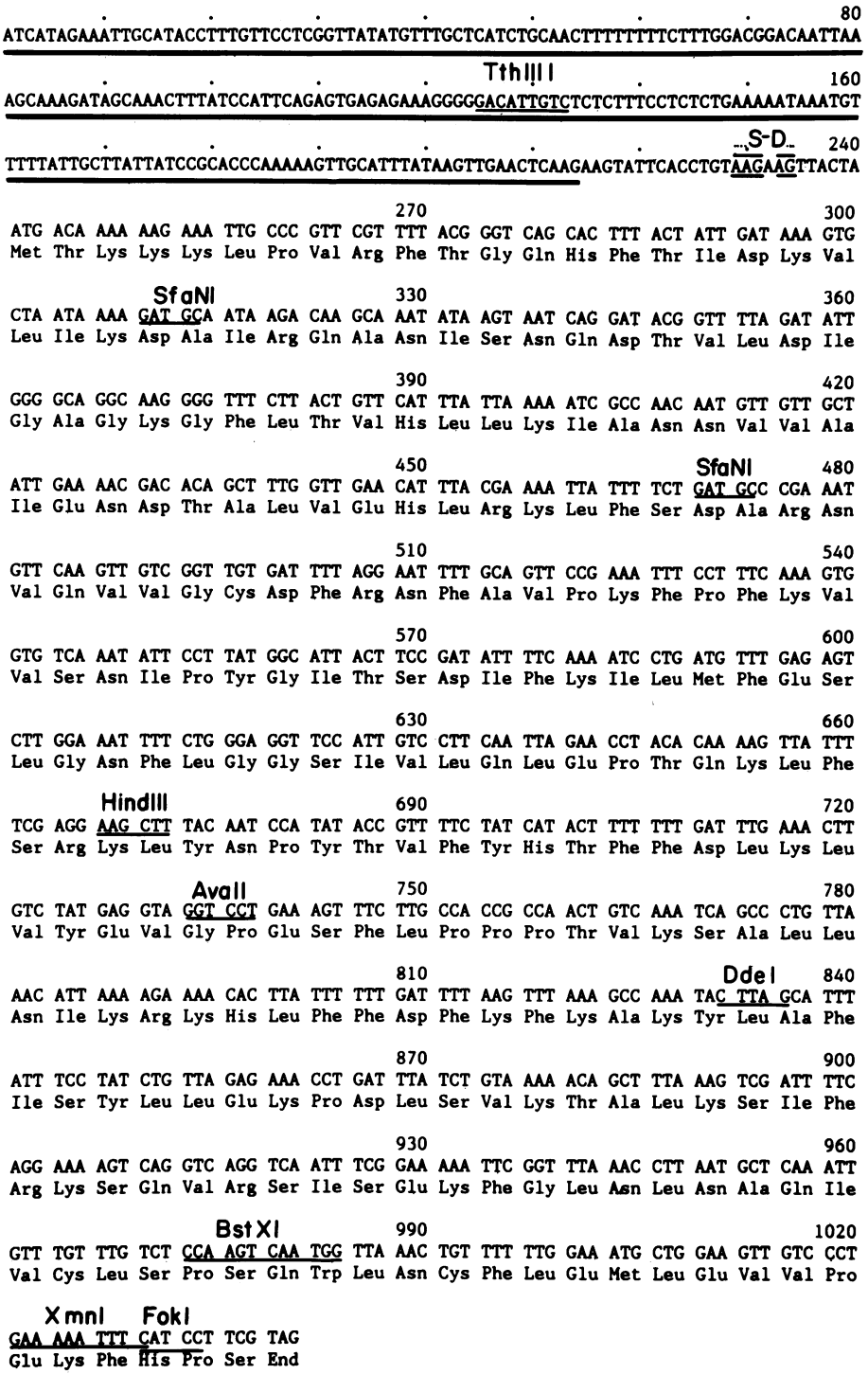

FIG. 3. Nucleotide and deduced amino acid sequence of *ermF* and upstream DNA. Sequence is shown 5′ → 3′. Endonuclease sites are underlined. The putative Shine-Dalgarno sites (S-D) is under- and overlined. The partial IS*4351* sequence is underlined with the thick line.

revealed 45% homology to *ermF* over the same corresponding area (data not shown).

We also examined sequences at the other end of the IS*4351* element and compared them with sequences that occurred at the 3′ terminus of *ermC* (from the plasmid pE194 [29]). Only 164 downstream nucleotides beyond the termination codon of *ermF* were available for our use. This sequence displayed a 34% homology with pE194 sequences at the 3′ terminus of *ermC* (data not shown).

DISCUSSION

DNA sequence analysis of a portion of the *Eco*RI D fragment of the *B. fragilis* R plasmid pBF4 has enabled us to

TTT Phe	19 (7.1)	TCT Ser	3 (1.1)	TAT Tyr	5 (1.9)	TGT Cys	3 (1.1)
TTC Phe	6 (2.2)	TCC Ser	3 (1.1)	TAC Tyr	2 (0.7)	TGC Cys	0 (0.0)
TTA Leu	15 (5.6)	TCA Ser	3 (1.1)	TAA End	0 (0.0)	TGA End	0 (0.0)
TTG Leu	6 (2.2)	TCG Ser	4 (1.5)	TAG End	1 (0.4)	TGG Trp	1 (0.4)
CTT Leu	6 (2.2)	CCT Pro	7 (2.6)	CAT His	4 (1.5)	CGT Arg	1 (0.4)
CTC Leu	0 (0.0)	CCC Pro	1 (0.4)	CAC His	2 (0.7)	CGC Arg	0 (0.0)
CTA Leu	1 (0.4)	CCA Pro	4 (1.5)	CAA Gln	6 (2.2)	CGA Arg	2 (0.7)
CTG Leu	5 (1.9)	CCG Pro	2 (0.7)	CAG Gln	3 (1.1)	CGG Arg	0 (0.0)
ATT Ile	12 (4.5)	ACT Thr	5 (1.9)	ATT Asn	9 (3.4)	AGT Ser	5 (1.9)
ATC Ile	2 (0.7)	ACC Thr	1 (0.4)	AAC Asn	5 (1.9)	AGC Ser	0 (0.0)
ATA Ile	3 (1.1)	ACA Thr	4 (1.5)	AAA Lys	20 (7.5)	AGA Arg	2 (0.7)
ATG Met	3 (1.1)	ACG Thr	2 (0.7)	AAG Lys	6 (2.2)	AGG Arg	4 (1.5)
GTT Val	12 (4.5)	GCT Ala	4 (1.5)	GAT Asp	10 (3.7)	GGT Gly	5 (1.9)
GTC Val	6 (2.2)	GCC Ala	4 (1.5)	GAC Asp	1 (0.4)	GGC Gly	2 (0.7)
GTA Val	2 (0.7)	GCA Ala	5 (1.9)	GAA Glu	8 (3.0)	GGA Gly	2 (0.7)
GTG Val	3 (1.1)	GCG Ala	0 (0.0)	GAG Glu	3 (1.1)	GGG Gly	2 (0.7)

FIG. 4. Frequency of codon usage in *ermF*. The numbers of amino acids encoded by codons are followed by percentages of total amino acids in parentheses. Codons corresponding to minor tRNA species in *E. coli* (18, 19) are underlined.

identify and characterize the MLS^r gene, *ermF*. These molecular data are reinforced by genetic studies (55) with a 1.9-kb portion of the *Eco*RI D fragment which confers MLS^r in *Bacteroides* spp. and contains the entire MLS^r sequence (*ermF*) described in this paper. Based on the phenotypic characteristics of this type of resistance as well as the similarity of the deduced amino acid sequence to those of other *erm* genes, we believe that this gene codes for an rRNA methylase of approximately 30,360 daltons (or *N*-methyl transferase [14, 67]). This conclusion is supported by the data of Tally et al. (62), who demonstrated that MLS^r in *Bacteroides* species is not due to impaired uptake or inactivation of the antibiotic.

Our comparison of the amino acid sequence of *ermF* to the other *erm* genes led to conclusions similar to those of Uchiyama and Weisblum (65), Roberts et al. (44), Murphy (38), and Dubnau (14). These procaryotic *erm* genes clearly are related. In addition to the seven regions of conserved amino acids pointed out by Uchiyama and Weisblum (65), our analysis (Fig. 6) has shown that the homology of all of the *erm* genes extends to 23 identical amino acids. Moreover, analysis of the codon usage of *ermF* and the other *erm* genes (Table 2) suggests that the homology at the amino acid level is not random. Common codon usage ranged from 34 to 46%.

At face value it appears that *ermD* (from *Bacillus licheniformis* [21]) is the most closely related to *ermF*; alignment of the deduced amino acid sequences of these

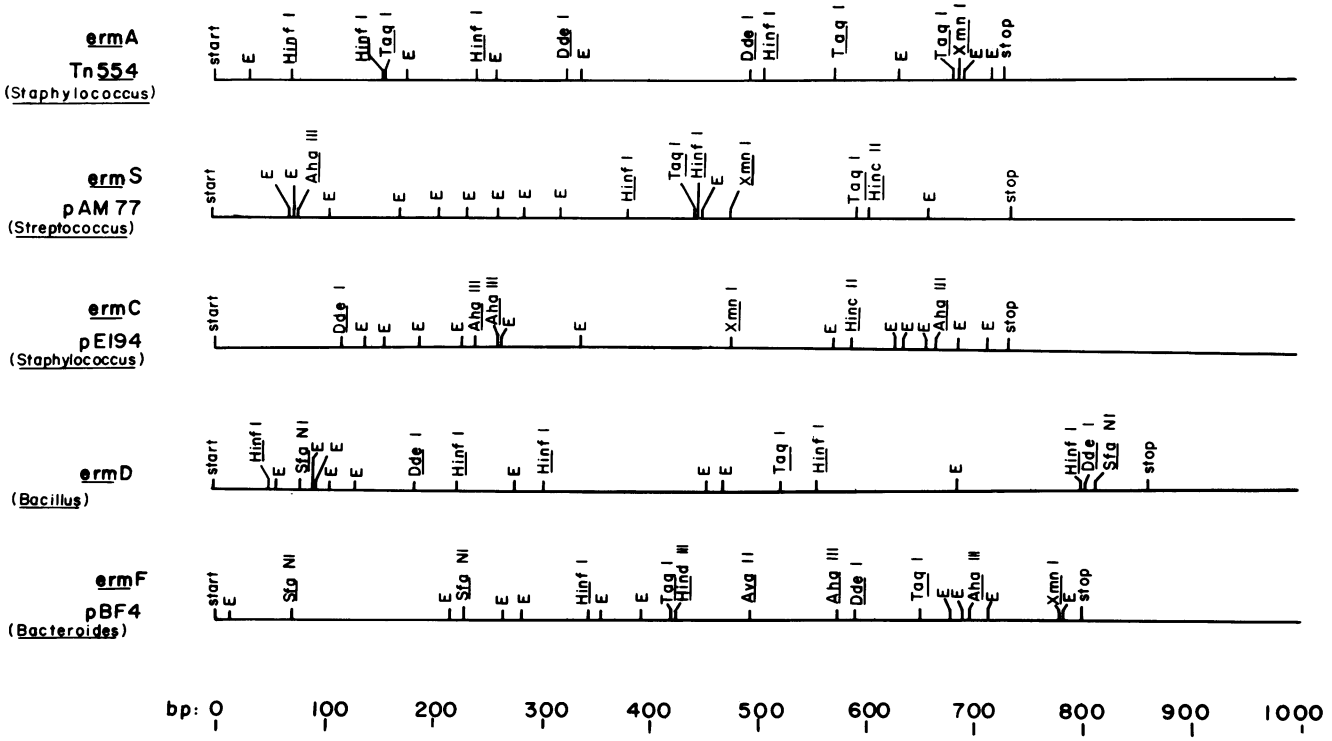

FIG. 5. Comparison of restriction endonuclease maps of five *erm* genes: *S. aureus ermA* from transposon Tn*554* (38); *Streptococcus sanguis ermS* from plasmid pAM77 (27); *S. aureus ermC* from plasmid pE194 (29); *Bacillus licheniformis ermD* from chromosomal DNA (21); and *B. fragilis ermF* from plasmid pBF4. E, *Eco*RI*.

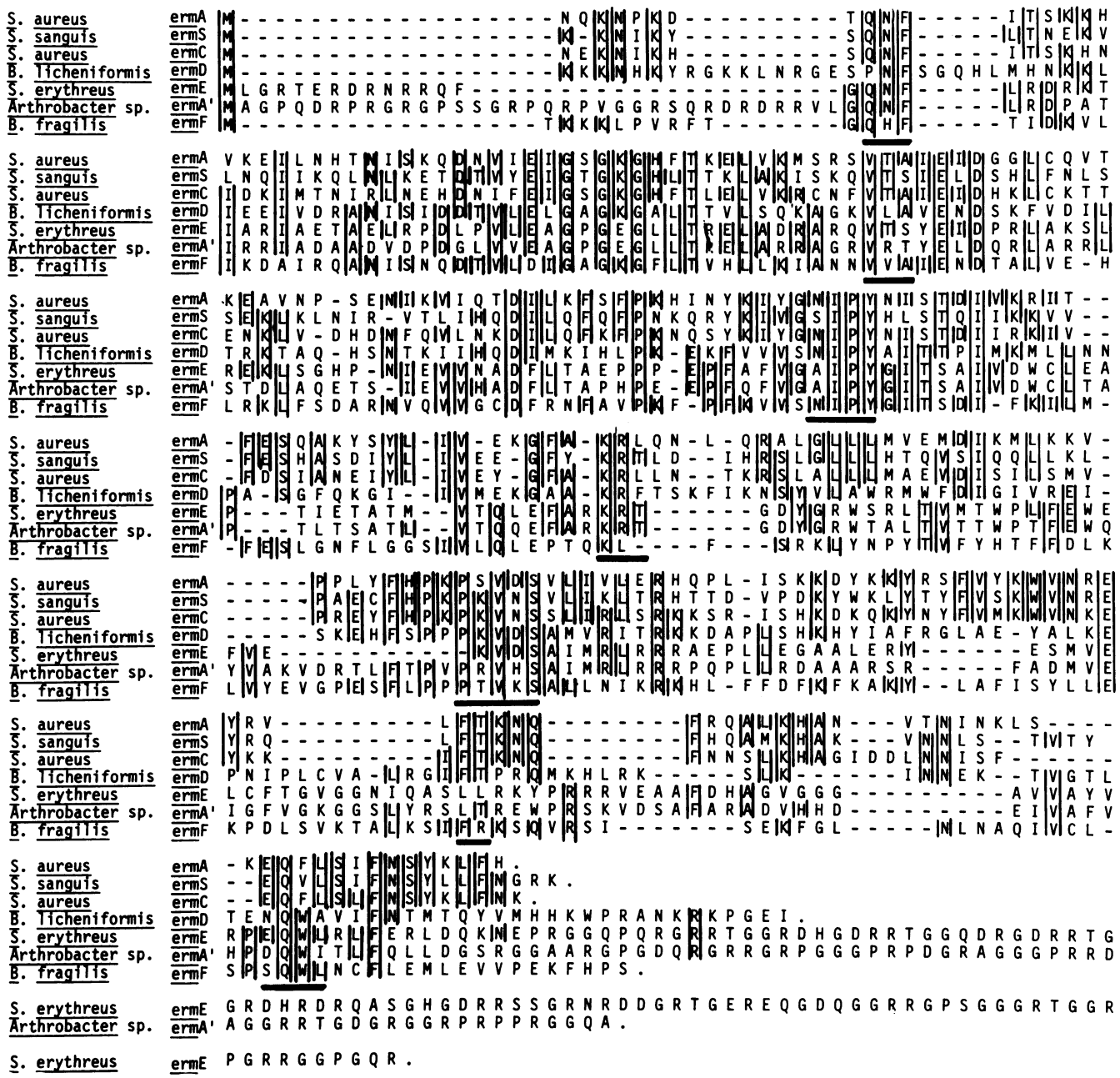

FIG. 6. Comparison of deduced amino acid sequence of seven *erm* determinants: *S. aureus ermA* (38); *S. sanguis ermS* (27); *S. aureus ermC* (29); *B. licheniformis ermD* (21); *Streptomyces erythreus ermE* (65); *Arthrobacter* sp. *ermA'* (44); and *B. fragilis ermF*. Conserved amino acids are bracketed. The seven regions of conserved amino acids noted by Uchiyama and Weisblum (65) are underlined.

genes (Fig. 6, Table 2) revealed 85 identical amino acids. However, based on the moles percent G+C of *ermF* as well as the unique nucleotide sequence homology between the leader sequence of *ermC* or *ermA* and the upstream DNA of *ermF* (Fig. 9), it seems reasonable that *ermF* may also have originated from a *Staphylococcus aureus* gene. Nevertheless, these comparisons further substantiate the clear relatedness of these genes. These results also support the hypothesis of Benveniste and Davies (3) and Walker and Walker (66), according to which some resistance mechanism which originated in an antibiotic-producing organism(s) has been transferred to pathogenic bacteria. MLS^r has also been described in *Clostridium perfringens* (4), another common soil inhabitant. Analysis of this MLS^r gene could provide additional clues to the dissemination of this resistant trait.

MLS resistance within *Bacteroides* species is also clearly related. Based on DNA blot hybridization studies (48, 54, 59) as well as conserved restriction endonuclease patterns (*Hin*dIII-*Ava*II-*Dde*I; Fig. 1), it seems likely that the three R plasmids, pBF4, pBFTM10, and pBI136, contain the *ermF* allele. Some *Bacteroides* species with putatively chromosome associated MLS^r may also contain the *ermF* gene (33). However, to date no attempt has been made to isolate this resistance gene. Although Southern hybridization experi-

TABLE 2. Codon usage analysis of four MLS genes[a]

Gene compared with *ermF*	No. of aligned codons	No. of aligned identical amino acids	Identical amino acids encoded by identical codons (%)	Identical amino acids with 2-out-of-3-base match (%)	Aligned codons with at least a 1-base match (%)	Aligned codons with at least a 2-base match (%)
ermA	225	63	46	49	76	45
ermS	234	71	44	44	72	42
ermC	233	72	49	47	73	43
ermD	238	85	34	49	70	43

[a] The DNA sequence of each determinant was aligned so as to correspond with the amino acid sequence alignment shown in Fig. 6. *ermF* was then compared with each sequence to determine the total number of aligned codons and the number of aligned codons representing identical amino acids in each case. The condons were then compared to determine the number of identical amino acids which were encoded by the same triplet and the number of identical amino acids encoded by triplets which differed only in the third position. Finally, aligned codons were compared to determine which of these matched in at least one position and which of these matched in at least two positions of the triplet. Values for randomly matched codons were 58% for a 1-out-of-3-base match and 16% for a 2-out-of-3-base match.

ments have shown that several plasmidless MLS^r *Bacteroides* species do contain homology to the *Eco*RI D fragment of pBF4 (5, 24, 33), the contributions underlying this homology (*ermF* gene versus flanking DNA) are not clear at present. In this respect it is worth noting that some MLS^r *Bacteroides* species contain no homology with the *Eco*RI D fragment (5, 24).

A most interesting finding is that transcription of *ermF* appears to originate within the upstream insertion element IS*4351*. This insertion element appears to be of *Bacteroides* origin, inasmuch as it has been found in chromosomal DNA (unpublished data), and its moles percent G+C is similar to that of *B. fragilis* (42% [6; Rasmussen et al., in preparation]). Nevertheless, we have recently shown that IS*4351* can efficiently activate promoterless antibiotic resistance genes in *E. coli* (Rasmussen et al., in preparation), further supporting the transcriptional function contributed by this insertion element in the *Bacteroides* plasmid pBF4. Based on restriction endonuclease cleavage maps, we presume that the DRs flanking the MLS^r genes of pBI136 and pBFTM10 represent iso-IS*4351* sequences (IS*4400* has been proposed to designate the sequences flanking the MLS^r region on pBFTM10 [45]). Unfortunately, it is not known whether the sequences in pBI136 and pBFTM10 also provide transcriptional control

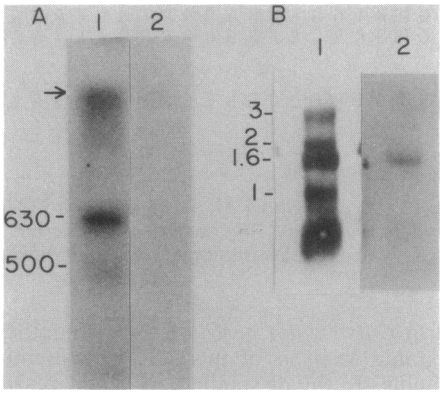

FIG. 7. Autoradiographic analysis of transcripts in *B. fragilis*. (A) S1 analysis. Total cellular RNA (50 μg) was hybridized with a 5′-^{32}P-end-labeled *ermF* probe (see the text) and incubated for 15 min with 300 U of S1 nuclease. Lanes 1, V479-1 RNA; 2, V600 RNA (negative control). Numbers indicate approximate sizes in nucleotides. Arrow indicates undigested probe. (B) Northern blot analysis. RNA (25 μg) from V479-1 (lane 2) was fractionated by electrophoresis in a 1% agarose-formaldehyde gel as described in the text and probed with nick-translated pFD176. Numbers indicate the approximate sizes in kilobases of markers seen in Lane 1.

of their respective MLS^r genes. Based on the proximity of these sequences to the genes (Fig. 1), we would predict a similar role, however. Indeed, genetic studies by Smith (55) have suggested that expression of MLS^r in pBI136 is dependent on the adjacent DR; however, in pBI136 the DR is in the opposite orientation with respect to the MLS^r locus (Fig. 1).

ermF is not expressed in *E. coli*; this observation has been noted by other laboratories (52, 57) as well as our own (unpublished data). This inhibition may be due to a variety of factors, including problems related to codon usage. Although the putative Shine-Dalgarno sequence associated with *ermF* (Fig. 3) does show homology with the 3′ end of the 16S rRNA of *E. coli* (49; data not shown), this alone may not suffice for efficient translation initiation (16). Moreover, *ermF* codons recognized by the minor tRNAs of *E. coli* (18, 19) are found throughout this gene (Fig. 4) and may result in inefficient translation or a truncated protein or both. Unfortunately, little is known about the expression of *Bacteroides* genes in *E. coli*. To date, only two other genes from *Bacteroides* species have been cloned into *E. coli*: a chondroitin lyase gene from *Bacteroides thetaiotaomicron* (25) and a pilin gene from *Bacteroides nodosus* (1, 15). Both of these genes are expressed in this host, although the former gene product shows several differences from the natural *Bacteroides* chondroitin lyase (25). However, inasmuch as *ermF* does not appear to be of *Bacteroides* origin, it is worth noting that expression in *E. coli* of the *ermC* gene, the possible progenitor of *ermF*, is greatly diminished compared with expression in *S. aureus* (63).

A summary of the molecular nature of *ermF* and its flanking DNA is illustrated in Fig. 9. Based on our sequence data, restriction endonuclease sites are indicated above the map, and the relative moles percent G+C is indicated below the map. The relative position of *ermF* as well as a putative

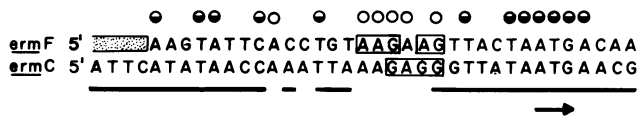

FIG. 8. Comparison of the 5′ nucleotide sequence of the *B. fragilis* MLS^r gene, *ermF*, to the leader sequence of the *S. aureus* MLS^r gene, *ermC* (29). Sequences were aligned at their respective start codons (arrow). Underlines denote regions of the *ermC* leader sequence involved in secondary structure of translational attenuation. The stippled box indicates the end of IS*4351*. Regions of homology with respective Shine-Dalgarno sequences are boxed. Symbols: ⊖, nucleotide homology between *ermF* and the *ermC* leader sequence involved in secondary structure; ○, general nucleotide homology. See the text for details.

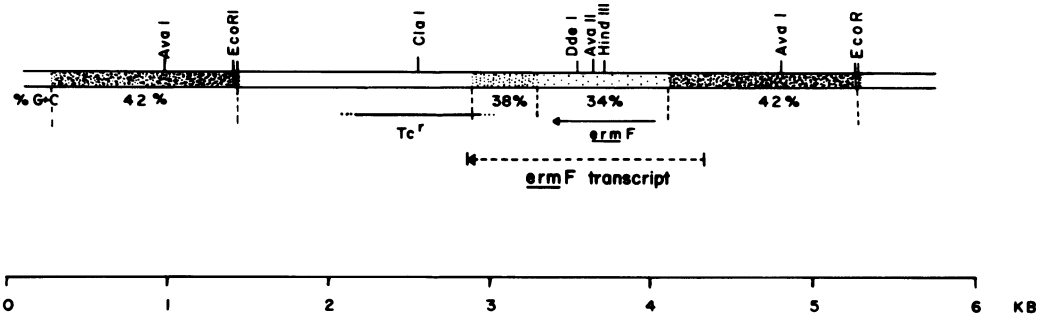

FIG. 9. Linear map of *ermF* and its flanking DNA. Dark shaded areas indicate the direct repeats corresponding to the IS*4351* sequence (Rasmussen et al., in preparation) flanking the *ermF* locus. Characteristic restriction endonuclease sites are indicated above the linear map. Numbers below the map indicate moles percent G+C of the denoted regions. Increased G+C content is represented by increased density in the stippling. The relative positions of *ermF*, a putative tetracycline resistance gene (57), and the corresponding *ermF* transcript are indicated below the map.

Tc^r gene, which is expressed in aerobically grown *E. coli* and not *Bacteroides* spp. (57), is indicated below the linear map. The putative transcript of the *ermF* gene is denoted by the dashed line. Although our sequence data failed to detect an obvious transcriptional terminator region, available sequence data only extended to 164 bp downstream from the *ermF* gene. Based on S1 nuclease mapping and Northern blot hybridization experiments, we would predict that the terminator region is about 400 bp downstream from this gene.

The sequences depicted in Fig. 9 in fact define the transposon Tn*4351*, described by Shoemaker et al. (51, 52). The molecular nature of this element represents that of a composite transposon, containing a drug resistance gene(s) flanked by insertion sequence elements (Rasmussen et al., in preparation). The disparity of the moles percent G+C content of this transposon suggests that *ermF* was transferred to *Bacteroides* sp. from a gram-positive organism on a plasmid or perhaps another transposon. Our data (Fig. 8) suggest that the invading *erm* sequence was transferred to the *Bacteroides* sp. together with its upstream regulatory sequences. This event was followed by movement of a copy of IS*4351* such that insertion rendered this element close (26 bp) to the start codon of the *erm* gene. This resulted in a takeover of transcriptional control of the gene by an IS*4351* promoter with concomitant "short circuiting" of the upstream regulatory sequences of the gene. Perhaps this was necessary to increase the efficiency of expression of the gene in the *Bacteroides* host. These events then were followed at some point by a duplication of IS*4351* such that copies of this element now flanked the gene, resulting in the generation of a composite transposon. Using strains of *B. fragilis* containing chromosomal copies of IS*4351* (Rasmussen et al., in preparation), we should be able to test this model in part by transformation of a *Bacteroides* sp. host (56) with a plasmid copy of *ermF* linked to a regulatory leader sequence (for example, from *ermC*). This would allow us to evaluate whether this gene is capable of inducible (or any) expression under the control of such regulatory sequences. Continued selective pressure imposed by clindamycin would enable us to screen for variants where the chromosomal IS*4351* had transposed next to the *ermF* allele so as to control its expression. In this fashion we may be able to simulate the events that led to the genesis of Tn*4351*, thus shedding light on the evolution of the expression of drug resistance in heterologous hosts.

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