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A Second Tylosin Resistance Determinant, Erm B, in *Arcanobacterium pyogenes*

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***Arcanobacterium pyogenes*, a common inhabitant of the mucosal surfaces of livestock, is also a pathogen associated with a variety of infections. In livestock, *A. pyogenes* is exposed to antimicrobial agents used for prophylaxis and therapy, notably tylosin, a macrolide used extensively for the prevention of liver abscessation in feedlot cattle in the United States. Many, but not all, tylosin-resistant *A. pyogenes* isolates carry *erm*(X), suggesting the presence of other determinants of tylosin resistance. Oligonucleotide primers designed for conserved regions of *erm*(B), *erm*(C), and *erm*(T) were used to amplify a 404-bp fragment from a tylosin-resistant *A. pyogenes* isolate, OX-7. DNA sequencing revealed that the PCR product was 100% identical to *erm*(B) genes, and the *erm*(B) gene region was cloned in *Escherichia coli*. The *A. pyogenes* Erm B determinant had the most DNA identity with an Erm B determinant carried by the *Clostridium perfringens* plasmid pIP402. However, the *A. pyogenes* determinant lacked direct repeat DR1 and contained a deletion in DR2. Flanking the *A. pyogenes erm*(B) gene were partial and entire genes similar to those found on the *Enterococcus faecalis* multiresistance plasmid pRE25. This novel architecture suggests that the *erm*(B) element may have arisen by recombination of two distinct genetic elements. Ten of 32 tylosin-resistant isolates carried *erm*(B), as determined by DNA hybridization, and all 10 isolates carried a similar element. Insertion of the element was site specific, as PCR and Southern blotting analysis revealed that the *erm*(B) element was inserted into *orfY*, a gene of unknown function. However, in three strains, this insertion resulted in a partial duplication of *orfY*.**

A common commensal organism on the mucous membranes of cattle and swine, *Arcanobacterium pyogenes* is also an opportunistic pathogen in these animals. *A. pyogenes* is responsible for a number of suppurative infections of the skin, joints, and visceral organs, including liver abscesses in feedlot cattle (13) and pneumonia (11) and arthritis (28) in pigs. Liver abscesses in feedlot cattle are a substantial problem for the beef cattle industry and are second only to respiratory diseases in terms of economic losses. The primary etiologic agent of liver abscessation is *Fusobacterium necrophorum* (22). However, *A. pyogenes* acts as a synergistic pathogen in this disease, being present in up to 90% of abscesses (17).

The use of antimicrobial agents as feed additives for disease prophylaxis and growth promotion is a common practice in the U.S. beef cattle industry. Tylosin is the most effective and commonly used feed additive for the prevention of bovine liver abscessation (16, 29). A study involving almost 7,000 feedlot cattle demonstrated that tylosin use reduced the incidence of liver abscessation by 73% and increased weight gain and feed conversion by 2.3 and 2.6%, respectively (30). Correspondingly, tylosin use is extensive, with 42.3% of U.S. feedlot cattle receiving tylosin as a feed additive (29). Interestingly, in cattle that were fed tylosin, the incidence of liver abscesses containing *A. pyogenes* increased from 10 to 53% (15).

While tylosin resistance in *A. pyogenes* has been documented (10, 27, 32), it is only recently that the mechanisms of tylosin resistance in *A. pyogenes* have been investigated. We identified an *erm*(X) determinant in the majority of tylosin-resistant *A. pyogenes* isolates (12). However, the identification of tylosin-resistant *A. pyogenes* isolates that did not carry *erm*(X) (12) highlighted the presence of other tylosin resistance mechanisms.

In this study, we report the identification of a novel Erm B determinant found in 31.2% of tylosin-resistant *A. pyogenes* isolates. The *A. pyogenes erm*(B)-associated sequences may have arisen by recombination and/or rearrangement of two distinct genetic elements. Furthermore, insertion of this element is site specific in *A. pyogenes*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The 110 *A. pyogenes* strains used in this study were field isolates obtained from veterinary diagnostic laboratories or personal collections. These strains were isolated from cattle ($n = 76$), swine ($n = 24$), birds ($n = 5$), dogs ($n = 2$), a deer ($n = 1$), a sheep ($n = 1$), and a cat ($n = 1$). *A. pyogenes* strains were grown on brain heart infusion (BHI; Difco) agar plates supplemented with 5% bovine blood at 37°C and 5% CO_2 or in BHI broth supplemented with 5% newborn calf serum (Omega Scientific Inc.) at 37°C with shaking. *Escherichia coli* DH5αMCR strains (Gibco-BRL) were grown at 37°C on Luria-Bertani (LB; Difco) agar or in LB broth with shaking. Antibiotics were added as appropriate at the following concentrations: for *A. pyogenes*, erythromycin (EM) or tylosin at 15 μg/ml and kanamycin (KM) at 30 μg/ml; for *E. coli*, chloramphenicol at 30 μg/ml, EM at 200 μg/ml, and KM at 50 μg/ml.

DNA techniques. Genomic DNA from *A. pyogenes* was isolated by using the method of Pospiech and Neumann (20). *E. coli* plasmid DNA extraction, transformation, DNA restriction, ligation, agarose gel electrophoresis, and Southern transfer of DNA to nylon membranes were performed essentially as described previously (2). The preparation of DNA probes by PCR with oligonucleotide primers internal to specific genes, DNA hybridization, and probe detection were performed by using the digoxigenin DNA labeling and detection kit (Roche), as recommended by the manufacturer. The preparation of DNA-containing aga-

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TABLE 1. Sequences of oligonucleotide primers used in this study

Primer	Gene (orientation)	Nucleotide positions in the *erm*(B) gene region	Sequence (5′–3′)
ermBCTF	*erm*(B) (forward)	1742–1763	GAAATTGGAACAGGTAAAGG
ermBCTR	*erm*(B) (reverse)	2126–2145	TTTACTTTTGGTTTAGGATG
ermB6	*orf*ζ (reverse)	3568–3589	CTGACCCTGGTTGCCCACCAAG
tetW43	*orfY* (forward)	1059–1080, 3862–3883[a]	TATCGTATTAGCGGATTAGAGG
tetW47	*orfY* (reverse)	4228–4249	TCATCTCTTTAGGCGGTTCTGC

[a] Due to the partial duplication of *orfY* in OX-7, this primer binds at two sites in the *erm*(B) gene region.

rose plugs and pulsed-field gel electrophoresis (PFGE) were performed essentially as described previously (4). PCR DNA amplification was performed by using *Taq* DNA polymerase (Promega) with the supplied reaction buffer for 35 cycles, consisting of 1 min at 94°C, 1 min at 50 to 55°C, and 1 min/kb at 72°C with a final extension step of 72°C for 5 min. Oligonucleotide primer sequences are shown in Table 1, and their locations on the *erm*(B) gene map are indicated in Fig. 1A.

Nucleotide sequence determination. The sequence of the *erm*(B) gene region was determined from pJGS579 and its subclones by using automated DNA sequencing. Sequencing was performed with both strands, crossing all restriction sites, with KS or T7 sequencing primers or oligonucleotide primers designed for the sequence of the *erm*(B) gene region. The sequencing reactions were performed by the Genomic Analysis and Technology Core at the University of Arizona with a model 377 DNA sequencer (Applied Biosystems Inc.).

Computer sequence analysis. Nucleotide sequence data were compiled by using the Sequencher program (GeneCodes). Database searches were performed by using the BlastX and BlastP algorithms (1). Sequence analysis was performed by using the suite of programs available through the Genetics Computer Group (Accelrys). Multiple sequence alignments were performed by using CLUSTAL W (26).

Determination of MICs. The determination of MICs for *A. pyogenes* was conducted according to the National Committee for Clinical Laboratory Standards methodology (18) with the modifications described by Trinh et al. (27). The antimicrobial agents to be tested were diluted in a doubling-dilution pattern at concentrations ranging from 0.06 to 2,048 μg/ml in the wells of sterile, 96-well, round-bottom microtiter plates in 50-μl volumes. The MIC was read visually as the lowest concentration of the antimicrobial agent to prevent growth (turbidity) compared with the growth of the control (no antimicrobial agent added). Each

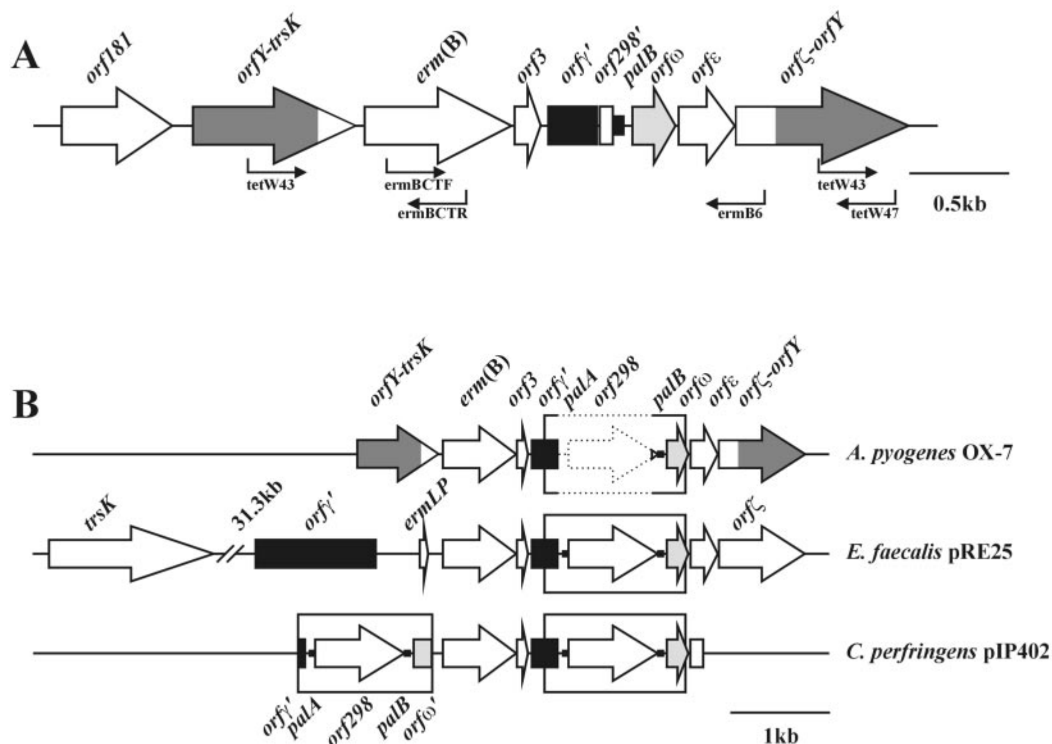

FIG. 1. Genetic organization of the *erm*(B) element from *A. pyogenes* and comparison with related *erm*(B) elements. (A) Map of the *A. pyogenes* OX-7 *erm*(B) gene region. ORFs and their orientations are represented by arrows. Partial ORFs are represented by rectangles. In all cases, the partial ORFs are oriented in the same direction as the other ORFs. The shaded portions of the *orfY* gene fusions delineate the *orfY* sequences and the insertion points of the *erm*(B) element. *orf*γ and *orf*ω are represented by black and gray, respectively. The *palB* sequence is indicated by a small black rectangle. The positions and directions of the oligonucleotide primers used in this study are represented by bent arrows below the gene map. (B) Comparative genetic organization of *erm*(B) elements from *A. pyogenes* strain OX-7, *C. perfringens* plasmid pIP402 (GenBank accession no. U18931), and *E. faecalis* plasmid pRE25 (GenBank accession no. X92945). The open rectangles denote the DRs originally identified in the pIP402 sequence. The dashed lines in the *A. pyogenes erm*(B) element delineate the deletion in DR2. The two *orf*γ′ sequences in pRE25 are different, but partially overlapping, sequences encoding the C-terminal portion of γ.

isolate was tested in duplicate on two separate occasions, and the end points for each antimicrobial agent did not differ. To determine the MICs following induction, the *A. pyogenes* isolates were grown on BHI agar containing 5% bovine blood and 1 μg of the appropriate antimicrobial agent/ml prior to MIC determination, as described above.

Nucleotide sequence accession number. The nucleotide sequence of the *erm*(B) gene and associated sequences were submitted to the GenBank database under accession number AY334073.

RESULTS AND DISCUSSION

Identification and prevalence of *erm*(B) in *A. pyogenes*. Of 110 *A. pyogenes* isolates tested, there were 32 (29.1%) for which the tylosin MIC was ≥64 μg/ml (27; data not shown). Of these 32 resistant isolates, 23 (71.9%) carried the *erm*(X) gene, as determined by DNA hybridization with an *erm*(X)-specific probe (12; data not shown), indicating that the remaining isolates carried another mechanism of tylosin resistance. As *erm*(B) and *erm*(C) are predominant Erm determinants found in a wide variety of bacterial species (21), PCR primers were designed for conserved regions of *erm*(B) (GenBank accession no. M36722) and *erm*(C) (GenBank accession no. J01755). In addition, these primers also amplify *erm*(T) (GenBank accession no. AF310974). PCRs were performed on the 32 tylosin-resistant *A. pyogenes* isolates by using primers ermBCTF and ermBCTR (Table 1; Fig. 1A), and a product of 404 bp was obtained from 10 isolates. Sequencing of the PCR product from *A. pyogenes* strain OX-7 revealed that it had 100% nucleotide sequence identity with several *erm*(B) genes (e.g., GenBank accession no. M36722). To confirm the presence of *erm*(B) in the other isolates, DNA hybridization with an *erm*(B)-specific probe was performed. The 404-bp probe spanned bases 103 to 506 of the *erm*(B) open reading frame (ORF) and was hybridized under high-stringency conditions to genomic DNA from the 10 PCR-positive isolates. All of these isolates, but not BBR1 or 98-4277-2, which carries *erm*(X) (12), hybridized to the *erm*(B) probe, indicating the presence of the *erm*(B) gene (data not shown). Therefore, the prevalence of *erm*(B) in *A. pyogenes* isolates is 9.1% (n = 110), and this gene is present in 31.2% of tylosin-resistant isolates. One isolate, JGS496, carries both the *erm*(B) and *erm*(X) genes.

Cloning and nucleotide sequence determination of *erm*(B). A *Bam*HI library of *A. pyogenes* OX-7 genomic DNA was prepared in pBC KS (Stratagene) and introduced into *E. coli* DH5αMCR by electroporation. As *erm*(B) genes also confer resistance to EM, for convenience, experiments with *E. coli* were performed using EM. EM-resistant colonies were selected by growth on LB agar containing chloramphenicol and EM. Plasmid DNA from one of these recombinants, pJGS579, contained an approximately 7-kb insert, encompassing the entire *erm*(B) gene region, and the nucleotide sequence of this region was deduced from pJGS579 and its overlapping subclones.

Comparative analysis of the *erm*(B) element. The *erm*(B) gene carried by OX-7 has substantial DNA identity with other *erm*(B) genes, such as that from the *Clostridium perfringens* plasmid pIP402 (GenBank accession no. U18931; 100% identity), the *Enterococcus faecalis* plasmid pRE25 (GenBank accession no. X92945; 99.6% identity), and the *Clostridium difficile* transposon Tn*5398* (GenBank accession no. AF109075; 99.1% identity). Like the *erm*(B) gene product from pIP402 (3), the *A. pyogenes erm*(B) gene product lacked a leader peptide, which is in contrast to the pRE25 (23) and Tn*5398* (9) *erm*(B) gene products.

The *A. pyogenes erm*(B) element had a novel genetic organization, containing genes and gene fragments from two known *erm*(B) elements, pIP402 and pRE25 (Fig. 1B). Overall, the *A. pyogenes erm*(B) element displayed the highest DNA identity with that carried on pIP402 (3). Identity with this element begins 51 bp upstream of the start of the *A. pyogenes erm*(B) gene, equivalent to base 1460 of the pIP402 sequence (GenBank accession no. U18931). The two elements displayed 100% DNA identity over a 1,205-bp span, ending at base 2664 of pIP402. This region encompasses the −10 box of the putative *erm*(B) promoter, *erm*(B), *orf3*, a partial *orf*γ, and part of direct repeat DR2. At this point, the *A. pyogenes erm*(B) element contains a 947-bp deletion, and DNA identity resumes at base 3612 of the pIP402 sequence. The *A. pyogenes erm*(B) element has 98.3% DNA identity with the remaining 518 bp of the available pIP402 sequence. This region downstream of the deletion carries the 3′ ends of *orf298*, *palB*, and *orf*ω and the 5′ end of *orf*ε. *orf*ω and *orf*ε are not annotated in the pIP402 sequence, and these ORFs were identified by their similarity to genes carried by pRE25, *orf16*, and *orf17*, respectively (23). It is not known if the DNA identity between the *erm*(B) elements from *A. pyogenes* and *C. perfringens* extends past the available pIP402 sequence, but this information may provide clues regarding the evolution of these elements.

The DR elements identified in the *C. perfringens* (3) and *C. difficile* (8, 9, 24) *erm*(B) elements consist of fragments of ORFs, probably from a pRE25-like plasmid. The 5′ end of the DR is homologous to the 3′ end of *orf*γ, a type I topoisomerase gene of pRE25; the central portion contains *orf298*, encoding an ATPase involved in plasmid partitioning (23); and the 3′ end contains the start of *orf*ω, a gene encoding a transcriptional repressor responsible for plasmid maintenance (7).

The 66 bp of *orf298* encoded by the *A. pyogenes erm*(B) element is 100% identical to that from pIP402 (GenBank accession no. U18931) and 97.0% identical to *orf16* from pRE25 (GenBank accession no. X92945). Immediately downstream of the *orf298* stop codon is the 49-bp *palB* sequence, which, with *palA*, is a palindrome found flanking *orf298* in *erm*(B) elements from pIP402 (3), pRE25 (23), and *C. difficile* (9). *orf*ω carried by the *A. pyogenes erm*(B) element is 97.8% identical to that from pIP402 (GenBank accession no. U18931) and 96.7% identical to *orf17* from pRE25 (GenBank accession no. X92945). The *A. pyogenes erm*(B) element contains a complete copy of *orf*ε, which has 83.9% DNA identity with *orf18* of pRE25. A 194-bp fragment of *orf*ζ starts 1 bp downstream of *orf*ε. This partial gene has 77.8% DNA identity with the appropriate region of *orf19* from pRE25. *orf*ε and *orf*ζ encode an addiction system involved in plasmid maintenance, with ζ acting as a toxin and ε acting as its corresponding antidote (6).

The *A. pyogenes* element has DNA identity with that of pIP402 51 bp upstream of the *erm*(B) gene, which includes the −10 box of the putative *erm*(B) promoter. At this point, there is a 162-bp internal fragment of *trsK* (*orf33*), a gene encoding a conjugal transfer protein, which is also found in pRE25 (23) but which has not been identified in clostridial *erm*(B) elements. The region of *trsK* present in the *A. pyogenes erm*(B) element has 99.4% DNA identity with bases 486 to 647 of the

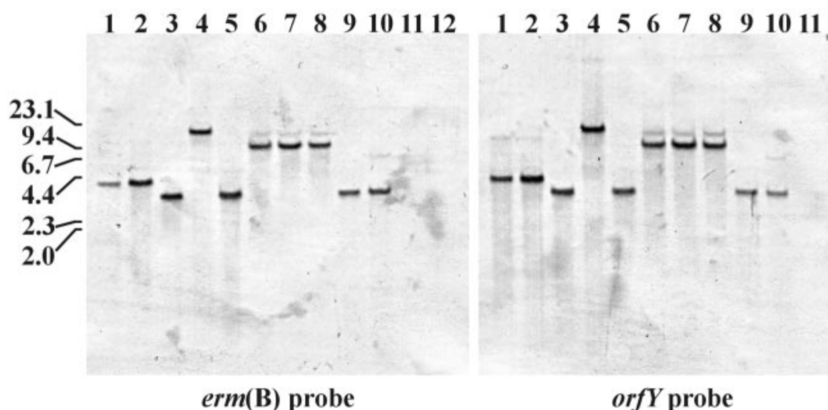

FIG. 2. Southern blot of *Bam*HI-*Xba*I-digested *A. pyogenes* genomic DNA with digoxigenin-labeled probes specific for *orfY* or *erm*(B). Lanes: 1, OX-1; 2, OX-7; 3, JGS496; 4, JGS573; 5, JGS574; 6, JGS597; 7, JGS598; 8, JGS599; 9, 01-4195; 10, D9509363; 11, BBR1 [*erm*(B) negative and *erm*(X) negative]; 12, 98-4277-2 [*erm*(B) negative and *erm*(X) positive]. The specific probe used is indicated under each Southern blot. DNA size standards in kilobases are shown to the left of the blots.

1,656-bp pRE25 gene (23). The fusion of the *trsK* fragment with the *erm*(B) upstream sequence resulted in the formation of a putative hybrid promoter with the −35 box (TTGTTA) contributed by the *trsK* sequence, presumably allowing expression of the *erm*(B) gene.

The entire *A. pyogenes erm*(B) element, delineated by the *trsK* gene fragment at the 5′ end and the truncated *orf*ζ at the 3′ end, is 2,224 bp in length with a G+C content of 33.6%. The average G+C content of known *A. pyogenes* housekeeping genes is 62.5% (our unpublished data), and the reduced G+C content of the *erm*(B) element more closely resembles those of genes from *Clostridium* or *Enterococcus* spp., from which this element is hypothesized to have originated. Indeed, while *erm*(B) is a widespread gene, it is not commonly found in bacterial species with high G+C contents (21).

The evolution of the *A. pyogenes erm*(B) element is unclear, but the DNA identity with pIP402 suggests that this, or a similar plasmid, was the original source. The acquisition of the *trsK* fragment probably occurred before the element inserted into *A. pyogenes*, as the target gene *orfY* (see below) is intact in non-*erm*(B)-containing strains and lacks *trsK* sequences (data not shown). The sequences that make up the pIP402 *erm*(B) element appear to have arisen from the duplication, deletion, and/or recombination of a pRE25-like plasmid. While the sequence of the *erm*(B) element from pIP402 lacks *trsK*, it is as yet unknown whether this plasmid carries a *trsK* homologue.

It is not known whether this element is mobile in *A. pyogenes*. Furthermore, any mechanisms of mobility and/or insertion for this element are unknown. No entire genes involved in transposition or conjugal transfer were found associated with the *erm*(B) determinant, and there are no apparent direct or indirect repeats at either end of this element.

In *A. pyogenes* OX-7, the *erm*(B) element has inserted into *orfY*. Insertion of the *erm*(B) element in *A. pyogenes* strain OX-7 resulted in fusion of the *trsK* sequences with the first 638 bp of *orfY*, a gene of unknown function. The G+C content of *orfY* (37.3%) is substantially different than that of other *A. pyogenes* genes, and the translated product of this gene has 48.8% identity and 73.4% similarity to OrfY from the Tn*5405*-like transposon of *Staphylococcus intermedius* (GenBank accession no. AF299292). However, there is no significant DNA identity with the *orfY* harbored on this transposon, suggesting that in *A. pyogenes*, *orfY* was not acquired from this source. Interestingly, in this *S. intermedius* strain, the transposon is inserted immediately downstream of a region of DNA containing *erm*(B) and *orf3* (5).

orf181, which encodes a protein with 42.0% identity and 68.0% similarity to a hypothetical protein of unknown function from *Sinorhizobium meliloti* (GenBank accession no. NC_003047), is 96 bp upstream of the *orfY-trsK* fusion. Like *orfY*, *orf181* has a reduced G+C content of 40.3%, and this region of the *A. pyogenes* OX-7 genome may be part of a larger region acquired by horizontal transfer, probably prior to the acquisition of the *erm*(B) element. *orfY* exists uninterrupted and is adjacent to *orf181* in the genome of the tylosin-susceptible *A. pyogenes* strain OX-9 (S. J. Billington, unpublished data), suggesting a subsequent insertion of the *erm*(B) element. The insertion of the *erm*(B) element in strain OX-7 resulted in a partial duplication of *orfY*, as the partial *orf*ζ is fused to the last 673 bp of *orfY*. The 5′ *orfY* is truncated by 94 bp from the end of the 732-bp gene, while the 3′ *orfY* is truncated 59 bp from the start. Thus, the *erm*(B) element is flanked by 579-bp DRs composed of sequences internal to *orfY*.

The *erm*(B) element inserts in a site-specific manner in the *A. pyogenes* genome. An initial experiment was performed to determine whether the insertion point for the *erm*(B) element was random. *A. pyogenes* genomic DNA from the 10 *erm*(B) strains was digested with *Bam*HI-*Xba*I, and Southern blotting was performed. The blots were hybridized under high-stringency conditions with either the *erm*(B)-specific probe or an *orfY*-specific probe (spanning bases 271 to 658 of *orfY*). The *orfY* probe was amplified from *A. pyogenes* strain OX-9 by using primers tetW43 and tetW47 (Table 1; Fig. 1A). In all *erm*(B)-containing isolates, the *erm*(B) and *orfY* probes hybridized to DNA fragments of the same size (Fig. 2), indicating that *erm*(B) and *orfY* are linked and that integration of the *erm*(B) element is most likely site specific. The sizes of the hybridizing bands differed between isolates (Fig. 2), probably as a result of restriction fragment length polymorphism. To

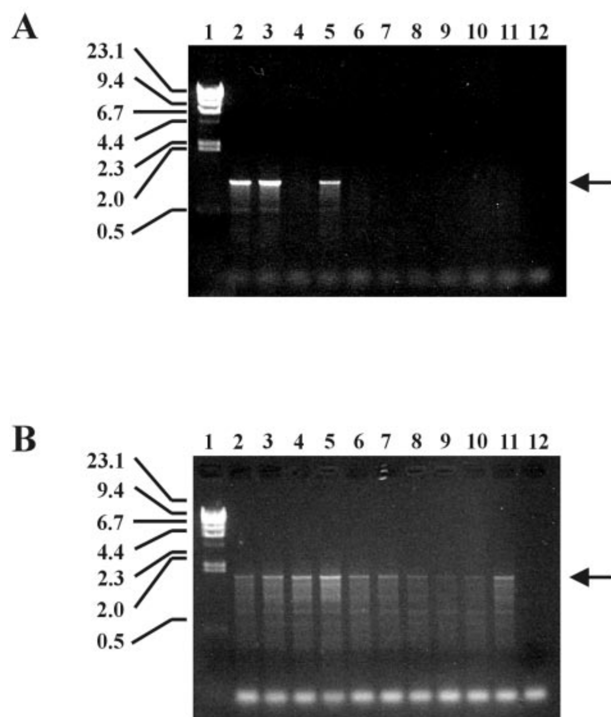

FIG. 3. The *A. pyogenes erm*(B) element inserts in a site-specific manner. The *erm*(B)-*orfY* gene region was amplified by PCR, and the amplicons were visualized following electrophoresis in a 1% agarose gel. Lanes: 1, λ *Hin*dIII standards; 2, OX-1; 3, OX-7; 4, JGS496; 5, JGS573; 6, JGS574; 7, JGS597; 8, JGS598; 9, JGS599; 10, 01-4195; 11, D9509363; 12, no-template control. The sizes of DNA standards in kilobases are shown to the left of the gels. (A) Primers tetW43 and ermBCTR (Table 1; Fig. 1A) were used. The 1,087-bp product is indicated by an arrow. (B) Primers ermBCTF and ermB6 (Table 1; Fig. 1A) were used. The 1,847-bp product is indicated by an arrow.

further delineate the insertion point, PCR was used to amplify sequences between *erm*(B) and *orfY*. The initial PCR experiment to amplify sequences 5′ from *erm*(B) used primers tetW43 and ermBCTR (Table 1; Fig. 1A), but a product was amplified from only three strains, including strain OX-7 (Fig. 3A), somewhat contradicting the Southern blot data. However, a PCR using primers ermBCTF and ermB6 (Table 1; Fig. 1A) amplified a product of 1,847 bp, including *orfY* sequences 3′ from *erm*(B), from all 10 isolates (Fig. 3B). It appears that *erm*(B) insertion is indeed site specific in *A. pyogenes*, but in three isolates, including OX-7, this insertion resulted in a partial duplication of *orfY* such that internal portions of *orfY* flanked the *erm*(B) element insertion point. There is no correlation between the strains carrying a duplicated *orfY* and the size of the *erm*(B)- and *orfY*-hybridizing band detected by Southern blotting (Fig. 2).

Interestingly, DNA hybridization experiments with the *orfY*-specific probe indicated that *orfY* was present in 100% of the strains containing *erm*(B) (Fig. 2). In contrast, this gene was present in only 8.7% of *erm*(B)-negative *A. pyogenes* strains ($n = 46$; data not shown). This finding provides further evidence for site-specific integration. *orfY* is probably not associated with the *erm*(B) element, as it is present in *A. pyogenes* strains that lack *erm*(B). However, its reduced G+C content suggests that *orfY* may have also been acquired by horizontal transfer and may be part of an additional element.

In order to determine if *erm*(B) was plasmid encoded, PFGE was performed on genomic DNA from the 10 *erm*(B)-containing isolates. PFGE conditions were chosen such that episomal DNA would be resolved in the gel, leaving undigested genomic DNA in the gel wells. Southern blotting was performed under high-stringency conditions with the *erm*(B) probe, and the presence of hybridization in only the well region suggested that the *erm*(B) elements appear to be chromosomally associated in all isolates (data not shown).

Determination of MICs and inducibility. The MICs of tylosin were determined for the 10 *erm*(B)-containing isolates, with and without induction. The MICs of tylosin for the isolates of porcine origin, which included OX-7, were 128 μg/ml, which did not increase following induction (Table 2), indicating constitutive expression of *erm*(B). This finding is not unexpected given that the product of the *A. pyogenes erm*(B) gene does not possess a leader peptide, which is responsible for the inducible

TABLE 2. MICs of tylosin for *A. pyogenes* strains, determined with and without induction

Strain	Animal species of isolation	MIC (μg/ml)			
		Tylosin	Tylosin after induction[a]	Clindamycin	EM
BBR1[b]	Bovine	≤0.06	ND[c]	≤0.06	≤0.06
01-4195	Bovine	>2,048	>2,048	512	1,024
D9509363	Bovine	>2,048	>2,048	512	1,024
JGS496	Bovine	>2,048	>2,048	512	1,024
JGS573	Bovine	>2,048	>2,048	512	1,024
JGS574	Bovine	>2,048	>2,048	512	1,024
JGS597	Porcine	128	128	256	256
JGS598	Porcine	128	128	256	256
JGS599	Porcine	128	128	256	256
OX-1	Porcine	128	128	256	256
OX-7	Porcine	128	128	256	256
JGS6000		≤0.06	>2,048		
JGS6001		≤0.06	>2,048		

[a] Induction involved 1 μg of tylosin per ml.
[b] BBR1 is a tylosin-susceptible strain whose results are shown for comparison.
[c] ND, not determined (as *A. pyogenes* strain BBR1 will not grow with 1 μg of tylosin/ml).

expression of some *erm* genes (31). Somewhat surprisingly, the MICs of tylosin for all the bovine isolates were >2,048 μg/ml (Table 2). Although bovine isolates were also tested following induction, the uninduced MICs were so high that any increase would not have been detected. These results suggested that there was some difference in the determinants of tylosin resistance in *erm*(B)-containing *A. pyogenes* strains isolated from different animal hosts. Such differences could be due to either differential expression of *erm*(B) or the presence of an additional tylosin resistance determinant. The MICs of clindamycin and EM were also determined for these 10 strains. As expected, *A. pyogenes* strains carrying *erm*(B) also displayed resistance to these antimicrobial agents (Table 2).

The bovine isolate JGS574 carries an additional tylosin resistance determinant. To determine whether nucleotide changes within *erm*(B) or its promoter region were responsible for the increased MICs for bovine isolates, the *orfY-erm*(B) region of strain JGS573 was amplified with primers tetW43 and ermBCTR (Table 1; Fig. 1A). JGS573 was chosen as this was the only bovine isolate in which the *orfY* duplication had occurred, enabling the amplification of the start of the *erm*(B) element with existing primers. There were no differences between the OX-7 and JGS573 sequences (data not shown), suggesting that there may be an additional determinant of tylosin resistance carried by bovine *A. pyogenes* isolates. To confirm this hypothesis, inactivation of the *erm*(B) gene was undertaken. A 404-bp fragment of *erm*(B) was amplified by PCR with primers ermBCTF and ermBCTR. The ends were blunted with T4 DNA polymerase, and the fragment was cloned into *Sma*I-digested pHSS19 (19). This plasmid, which was unable to replicate in *A. pyogenes*, was used to transform JGS574, another bovine isolate, to KM resistance by the insertion of the plasmid via a single crossover event (data not shown). JGS574 was used in the knockout experiment instead of JGS573, as it was subsequently determined that JGS573 was resistant to KM. The MICs of tylosin for two independently derived *erm*(B) mutants, JGS6000 and JGS6001, were determined with and without induction. In the absence of induction, the MICs of tylosin for both *erm*(B) knockout mutants were, in contrast to that for the parental strain JGS574, ≤0.06 μg/ml (Table 2). However, when induction with tylosin was performed, the MICs for the two mutants were >2,048 μg/ml (Table 2). These data suggest the presence of an inducible tylosin resistance determinant in JGS574, and it is possible that this, or another, determinant is present in the other bovine isolates. While this second determinant is inducible and *erm*(B) is not, the MIC of tylosin for strain JGS574 is >2,048 μg/ml in the absence of induction. This is most likely due to the presence of *erm*(B) allowing the nonlethal uptake of tylosin, which subsequently induces expression of the other tylosin resistance determinant. The result is a high level of apparently noninducible tylosin resistance.

Hybridization with a *Staphylococcus aureus erm*(C)-derived probe (GenBank accession no. V01278) and PCR experiments with primers for conserved regions of the macrolide efflux genes *mefA* and *mefE* (14) suggest that none of these genes are responsible for the additional tylosin resistance observed in bovine *erm*(B) isolates (data not shown). While it would be interesting to identify this additional tylosin resistance determinant, it was beyond the scope of the present investigation.

Conclusions. The *A. pyogenes erm*(B) element is most likely derived from an element similar to that carried on the *C. perfringens* plasmid pIP402. However, at least one component of the *A. pyogenes* element is similar to a region of pRE25, suggesting the occurrence of some recombination event during its evolution. While *erm*(B) is widespread (21), its presence in bacterial species with high G+C contents is uncommon. The *A. pyogenes erm*(B) element appears to be similar for all strains, in contrast to those identified for *C. difficile*, which differ considerably (9, 24). Insertion of the *erm*(B) element in *A. pyogenes* appears to be restricted to isolates carrying another horizontally acquired gene, *orfY*. This may explain why the prevalence of *erm*(B) is lower than that of *erm*(X), which is carried by 71.9% of tylosin-resistant *A. pyogenes* isolates (12; data not shown).

While tylosin is not used for humans, it is still able to select for bacterial cross-resistance to drugs used in human therapy, such as EM and clindamycin, through determinants like Erm B and Erm X. Surveillance studies have reported an increase in the incidence of antibiotic resistance among pathogenic and commensal bacteria as a result of the veterinary use of these drugs (25), contributing to selective pressure for microbial resistance and resulting in the generation of resistance reservoirs. The presence of multiple macrolide-lincosamide-streptogramin B resistance determinants in the commensal organism *A. pyogenes*, possibly as a result of exposure to tylosin, is consistent with this hypothesis.

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