Tylosin Resistance in *Arcanobacterium pyogenes* Is Encoded by an Erm X Determinant

B. Helen Jost, Adam C. Field, Hien T. Trinh, J. Glenn Songer and Stephen J. Billington

Tylosin Resistance in *Arcanobacterium pyogenes* Is Encoded by an Erm X Determinant

B. Helen Jost,* Adam C. Field, Hien T. Trinh, J. Glenn Songer, and Stephen J. Billington

Department of Veterinary Science and Microbiology, The University of Arizona, Tucson, Arizona 85721

Received 4 April 2003/Returned for modification 11 June 2003/Accepted 22 July 2003

*Arcanobacterium pyogenes*, a commensal on the mucous membranes of many economically important animal species, is also a pathogen, causing abscesses of the skin, joints, and visceral organs as well as mastitis and abortion. In food animals, *A. pyogenes* is exposed to antimicrobial agents used for growth promotion, prophylaxis, and therapy, notably tylosin, a macrolide antibiotic used extensively for the prevention of liver abscessation in feedlot cattle in the United States. Of 48 *A. pyogenes* isolates, 11 (22.9%) exhibited inducible or constitutive resistance to tylosin (MIC of $\geq 128 \mu g/ml$). These isolates also exhibited resistance to other macrolide and lincosamide antibiotics, suggesting a macrolide-lincosamide resistance phenotype. Of the 11 resistant isolates, genomic DNA from nine hybridized to an *erm*(X)-specific probe. Cloning and nucleotide sequencing of the *A. pyogenes* *erm*(X) gene indicated that it was $\geq 95\%$ similar to *erm*(X) genes from *Corynebacterium* and *Propionibacterium* spp. Eight of the *erm*(X)-containing *A. pyogenes* isolates exhibited inducible tylosin resistance, which was consistent with the presence of a putative leader peptide upstream of the *erm*(X) open reading frame. For at least one *A. pyogenes* isolate, 98-4277-2, *erm*(X) was present on a plasmid, pAP2, and was associated with the insertion sequence IS6100. pAP2 also carried genes encoding the repressor-regulated tetracycline efflux system determinant Tet 33. The *rep4* gene from pAP2 was nonfunctional in *Escherichia coli* and at least one *A. pyogenes* isolate, suggesting that there may be host-encoded factors required for replication of this plasmid.

The use of antimicrobial agents as feed additives for disease prophylaxis and growth promotion in the beef cattle industry is a common practice in the United States. One of the major targets is liver abscission, which is second only to respiratory disease in terms of economic losses to cattle feedlots. Control of liver abscesses has primarily depended on the use of control additives, such as the macrolide tylosin, chlortetracycline, and oxytetracycline (10, 26), with tylosin the most effective and commonly used feed additive (11). A study involving almost 7,000 feedlot cattle demonstrated that the use of tylosin as a feed additive reduced the incidence of the disease abscission by 73% and increased weight gain and feed conversion by 2.3 and 2.6%, respectively (G. J. Vogel and S. B. Laudert, abstract, J. Anim. Sci. 72[Supp 1]:293, 1994). Correspondingly, tylosin use is extensive, with 42.3% of feedlot cattle receiving tylosin as a feed additive, primarily for the prevention of liver abscesses (26).

*Fusobacterium necrophorum* is the primary etiological agent of bovine hepatic abscession (18), but *Arcanobacterium pyogenes* is a synergistic pathogen, being found in 10 (9) to 90% (12) of abscesses. Interestingly, in cattle fed tylosin, the incidence of hepatic abscesses containing *A. pyogenes* increased from 10 to 53% (9).

The resistance of *A. pyogenes* to tylosin has only recently been documented. In a survey of *A. pyogenes* strains conducted in our laboratory, we observed that resistance of *A. pyogenes* to tylosin was prevalent, with 22.9% of isolates tested (n = 48) having MICs of tylosin of $\geq 64 \mu g/ml$ (25). In addition, for the tylosin-resistant strains, MICs of a wide spectrum of macrolide and lincosamide antimicrobial agents were increased (25), suggesting a macrolide-lincosamide, and streptogramin B resistance phenotype. However, the mechanism(s) of resistance to tylosin in *A. pyogenes* was not investigated.

In this study, we report the identification and characterization of a plasmid-encoded Erm X determinant in a tylosin-resistant *A. pyogenes* isolate.

MATERIALS AND METHODS

**Bacteria and growth conditions.** The 48 *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 = 27), swine (n = 17), dogs (n = 2), a bird (n = 1), and a cat (n = 1). Specifically, strain BBR1 was isolated from a bovine abscess and strain 98-4277-2 was isolated from a case of bovine mastitis. *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% bovine calf serum (Omega Scientific Inc.) at 37°C with shaking. *Escherichia coli* DSMsMC strains (Gibco-BRL) were grown at 37°C on Luria-Bertani (Difco) agar or in Luria-Bertani broth with shaking. Antibiotics were added as appropriate for *A. pyogenes* (tylosin, 15 µg/ml; kanamycin [KM], 30 µg/ml; tetracycline [TC], 1 µg/ml) and for *E. coli* (chloramphenicol, 30 µg/ml; erythromycin, 200 µg/ml; KM, 50, µg/ml; and TC, 2 µg/ml).

**DNA techniques.** Genomic DNA from *A. pyogenes* was isolated by the method of Pospischil and Neumann (14). *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). Preparation of DNA probes using oligonucleotide primers designed to specific genes, DNA hybridization, and probe detection were performed using the digoxigenin (DIG) DNA labeling and detection kit (Roche), as recommended by the manufacturer. PCR DNA amplification was performed using TaqDNA polymerase (Promega) with the supplied reaction buffer for 35 cycles consisting of 1 min at 94°C, 1 min at 55°C, and 1 min/kb at 72°C, with a final extension step of 72°C for 5 min. The plasmids and oligonucleotide primers used in this study are shown in Tables 1 and 2, respectively.
Nucleotide sequence determination. The sequence of the \textit{erm}(X) gene region was determined from pGJS392, pGJS466, and pGJS551 and their subclones, using automated DNA sequencing. Sequencing was performed on both strands, crossing all restriction sites, using KS, SK, T3, or T7 sequencing primers or oligonucleotide primers designed to the sequence of the \textit{erm}(X) gene region. Sequencing reactions were performed by the Genomic Analysis and Technology Core at The University of Arizona, using a 377 DNA sequencer (Applied Biosystems Inc.).

Computer sequence analysis. Nucleotide sequence data were compiled using the Sequencher program (GeneCodes). Database searches were performed using the suite of programs available through the Genetics Computer Group (Accelrys). Multiple sequence alignments were performed using CLUSTAL W (24).

Determination of MICs. Determination of MICs for \textit{A. pyogenes} used National Committee for Clinical Laboratory Standards methodology (13), with the modifications described by Trinh et al. (25). The antimicrobial agents to be tested were diluted in a doubling dilution pattern over the range of 0.06 to 2,048 \textmu g/ml in the wells of sterile, 96-well, round-bottom microtiter plates in 50-\mu l volumes. The MIC was read visually as the lowest concentration of the antimicrobial agent to prevent growth (turbidity), compared with the control (no antimicrobial agent added). Each isolate was tested in duplicate, on two separate occasions, and the endpoints for each antimicrobial agent did not differ. In order to determine MICs following induction, the \textit{A. pyogenes} isolates were grown on BHI agar containing 5\% bovine blood and 1 \mu g of the appropriate antimicrobial agent/ml, prior to MIC determination, as described above.

Nucleotide sequence accession number. The sequence of pAP2 was submitted to the GenBank database under accession number AF255627.

RESULTS AND DISCUSSION

Identification and prevalence of \textit{erm}(X) in \textit{A. pyogenes}. Of 48 \textit{A. pyogenes} isolates previously tested, for 11 (22.9\%), MICs of tylosin were \textequal 64 \mu g/ml (25). rRNA methylases, encoded by \textit{erm} genes, are common determinants of macrolide resistance (16). More specifically, Erm X is a common determinant of macrolide resistance in the coryneform bacteria, to which \textit{A. pyogenes} is related. A DIG-labeled probe to the \textit{erm}(X) gene of the \textit{ Corynebacterium diphteriae} plasmid, pNG2 (5), was generated (Table 2) and used to probe genomic DNA from 48 \textit{A. pyogenes} isolates under high-stringency conditions. The DNA from the 11 tylosin-resistant, but none of the tylosin-susceptible, \textit{A. pyogenes} isolates hybridized to the \textit{erm}(X) probe (Fig. 1), suggesting that \textit{erm}(X) is a predominant determinant of tylosin resistance in \textit{A. pyogenes}.

\textbf{The \textit{erm}(X) gene confers inducible resistance to tylosin.} MICs of tylosin were determined for the 11 tylosin-resistant isolates, with and without induction. The \textit{erm}(X)-containing strains generally exhibited inducible resistance to tylosin, with the exception of one strain, 2977, for which the uninduced MIC was already at the limit of detection (Table 3). For seven of the nine isolates, high-level uninduced MICs of tylosin were \textequal 128 \mu g/ml, which increased to >2,048 \mu g/ml upon induction with tylosin. For two of the \textit{erm}(X)-containing strains, MICs were low at \textequal 8 \mu g/ml, which increased to 128 \mu g/ml following induction (Table 3). In comparison, for susceptible strains of \textit{A. pyogenes}, MICs of tylosin were \textless 0.06 \mu g/ml (25). The two tylosin-resistant, non-\textit{erm}(X) containing strains, OX-1 and OX-7, did not exhibit inducible resistance to tylosin (Table 3).

The resistance phenotypes observed among the \textit{erm}(X)-containing strains generally exhibited inducible resistance to tylosin, with the exception of one strain, 2977, for which the uninduced MIC was already at the limit of detection (Table 3). For seven of the nine isolates, high-level uninduced MICs of tylosin were \textequal 128 \mu g/ml, which increased to >2,048 \mu g/ml upon induction with tylosin. For two of the \textit{erm}(X)-containing strains, MICs were low at \textequal 8 \mu g/ml, which increased to 128 \mu g/ml following induction (Table 3). In comparison, for susceptible strains of \textit{A. pyogenes}, MICs of tylosin were \textless 0.06 \mu g/ml (25). The two tylosin-resistant, non-\textit{erm}(X) containing strains, OX-1 and OX-7, did not exhibit inducible resistance to tylosin (Table 3). The resistance phenotypes observed among the \textit{erm}(X)-containing strains (OX-1) and OX-7) were similar to those previously described for other \textit{A. pyogenes} isolates (16).

\begin{table}[h]
\centering
\caption{Plasmids used in this study}
\begin{tabular}{lll}
\hline
Plasmid & Relevant characteristics & Source or reference \\
\hline
pAP2 & 9.3-kb \textit{A. pyogenes} plasmid, \textit{erm}(X), \textit{tet}(33) & This work \\
pBC KS & 3.4-kb, ColEl origin, Km' & Stratagene \\
pEP2 & 3.1-kb derivative of pNG2, Km', replicates in \textit{A. pyogenes} & 15 \\
pJGS392 & pBC KS HindIII Ω 3.2-kb HindIII fragment of pAP2 carrying \textit{erm}(X), Km' \textit{Em}' & This work \\
pJGS406 & pEP2 Sall-BamHI Ω 2.8-kb Sall-BamHI fragment of pJGS392 carrying \textit{erm}(X), \textit{Em}' Km' & This work \\
pJGS446 & pBC KS SacI Ω 8.4-kb SacI fragment of pAP2, Km' \textit{Te} & This work \\
pJGS551 & pBC KS HindIII Ω 6.2-kb HindIII fragment of pAP2, Km' \textit{Te} & This work \\
PNG2 & 15.1-kb \textit{C. diphtheriae} plasmid, \textit{erm}(X), replicates in \textit{A. pyogenes} & 19 \\
\hline
\end{tabular}
\end{table}

\begin{table}[h]
\centering
\caption{Sequences of oligonucleotide primers used in this study}
\begin{tabular}{lll}
\hline
Primer & Sequence (5'–3') & Gene Position (bp) Size (bp) \\
\hline
ermA5 & CACCCGCCTCTAGAAATAATAG & \textit{erm}(X) –243–788 1,031 \\
ermA3 & CGATGTTGCTAGAGAAGGAA & \textit{erm}(X) \\
ermX28 & GGTCTGTAACGCTGGCGCCG & repA 68–291 224 \\
ermX29 & TGTCGCCCGCCCAAGGTCCTCACG & repA \\
ermX12 & GATGCGATTTCITCGCAGTCG & tetA(33) 84–1224 1,089 \\
ermX19 & CCACCGTCATGAGAATCAGGCC & tetA(33) \\
ermX8 & TTTGACGAGCGAAGATGACG & tnpA 15–736 722 \\
ermX2A & GCCCAATGCGCAGAGTCGCTCTC & tnpA \\
ermX1 & GTCGCAGCTCAGGCTAAAGGC & \textit{erm}(X) –205–453 657 \\
ermX5 & CATGCGCGACTGAGGCGGACTC & \textit{erm}(X) \\
\hline
\end{tabular}
\end{table}

\begin{table}[h]
\centering
\caption{MICs of tylosin for \textit{A. pyogenes} strains with and without induction}
\begin{tabular}{lcc}
\hline
Strain & \textit{erm}(X)* & MIC of tylosin (\mu g/ml) following: \\
& & No induction \hspace{1cm} Induction with tylosin (1 \mu g/ml) \\
\hline
3 & + & 256 \hspace{1cm} \textless 0.05 \mu g/ml \\
4 & + & 256 \hspace{1cm} \textless 0.05 \mu g/ml \\
2977 & + & >2,048 \hspace{1cm} >2,048 \\
4759 & + & 128 \hspace{1cm} >2,048 \\
14373-00-1 & + & 1,024 \hspace{1cm} >2,048 \\
52789-99 & + & 8 \hspace{1cm} 128 \\
98-4272-2 & + & 2 \hspace{1cm} 128 \\
B107 & + & 128 \hspace{1cm} >2,048 \\
E11E & + & >2,048 \hspace{1cm} >2,048 \\
OX-1 & + & >2,048 \hspace{1cm} >2,048 \\
OX-7 & + & >2,048 \hspace{1cm} >2,048 \\
BBR(1pEP2) & + & >2,048 \hspace{1cm} >2,048 \\
BBR(1pGJS406) & + & 64 \hspace{1cm} 128 \\
\hline
*a Presence of absence of the \textit{erm}(X) gene is denoted by + and –, respectively. \\
*b Not determined, since BBR(1pEP2) will not grow on 1 \mu g of tylosin/ml.
\end{tabular}
\end{table}
taining strains varied considerably. This could be due to the presence of other resistance mechanisms, such as additional \textit{erm} genes, genes encoding efflux pumps, or ribosomal mutations. In addition, any \textit{erm}(X)-carrying plasmids could be present at variable copy number, giving rise to different MICs through a gene dosage effect.

The nine \textit{erm}(X)-containing isolates were also tested to determine MICs of other macrolide and lincosamide antimicrobial agents. All the isolates showed increased MICs of erythromycin, oleandomycin, spiramycin, clindamycin, and lincomycin (data not shown), indicating that \textit{erm}(X) confers a macrolide-lincosamide resistance phenotype. It is probable that the \textit{A. pyogenes} \textit{erm}(X) also confers resistance to the streptogramin B class of antimicrobial agents, as seen with other \textit{erm}(X) genes (17). However, susceptibility to these agents was not tested.

Cloning and nucleotide sequence determination of \textit{erm}(X).

A Southern blot of \textit{Hin}dIII-digested 98-4277-2 genomic DNA was hybridized with the \textit{erm}(X) probe under high-stringency conditions, and a 3.2-kb hybridizing band was identified (data not shown). A \textit{Hind}III library of \textit{A. pyogenes} 98-4277-2 genomic DNA was prepared in pBC KS (Stratagene) and introduced into \textit{E. coli} DH5\alphaMCR by electroporation. Clones carrying the \textit{erm}(X) gene were identified by colony hybridization with the \textit{erm}(X)-specific probe. One such clone, pJGS392, was selected for further analysis. pJGS392 contained a 3.2-kb \textit{Hind}III insert, and sequence analysis revealed that \textit{erm}(X) was indeed present in this clone. The \textit{A. pyogenes} \textit{erm}(X) gene had significant DNA identity with the \textit{erm}(X) genes from the \textit{C. diphtheriae} plasmid, pNG2 (97.7%; GenBank accession no. AF492560), the \textit{Corynebacterium striatum} plasmid, pTP10 (97.5%; GenBank accession no. AF024666), and the \textit{Corynebacterium jeikeium} and \textit{Propionibacterium acnes} transposon, Tn5432 (94.7% and 97.5%; GenBank accession no. AF338705 and AF411029, respectively). The \textit{A. pyogenes} Erm(X) protein shared 97.5% identity and 98.2% similarity with Erm(X) from pNG2. In order to confirm that \textit{erm}(X) was responsible for the tylosin resistance phenotype, a 2.8-kb SalI-BamHI fragment of pJGS392, containing \textit{erm}(X), was cloned into the KM resistance vector, pEP2 (15), which replicates in \textit{A. pyogenes} (6). This plasmid, pJGS406, was used to transform \textit{A. pyogenes} BBR1 to KM resistance, and the MIC for tylosin was determined. For BBR1(pEP2), the MIC of tylosin was 0.06 g/ml, compared to that for BBR1(pJGS406) of 64 g/ml, which increased to 128 g/ml following induction with tylosin (Table 3). The higher uninduced MIC for BBR1(pJGS406) compared with 98-4277-2 may reflect that for BBR1(pJGS406), \textit{erm}(X) is carried on a multicopy plasmid.

As with its homologues, upstream of the \textit{A. pyogenes} \textit{erm}(X) gene is an open reading frame (ORF) encoding a 15-amino-acid leader peptide (Fig. 2A), which has been postulated to be involved in translational attenuation similar to that seen with...
**erm(C),** leading to an inducible resistance phenotype (27). However, in strain 98-4277-2, there was an 86-bp duplication, including the ribosome binding site and the first 24 codons of *erm(X)*, creating a small ORF, *orf28*, which overlaps stop codon to start codon with *erm(X)* (Fig. 2A). To determine whether this duplication was found in all *erm(X)*-containing isolates, PCR was used to amplify the region around the duplication. Primers *ermX1* and *ermX5* (Table 2) amplified a 657-bp product in strain 98-4277-2 (Fig. 2B). The other eight strains apparently did not possess this duplication, since the PCR products amplified were approximately 86 bp smaller (Fig. 2B). Furthermore, the PCR products from two of these strains were sequenced, confirming the lack of duplication (data not shown). The duplication in 98-4277-2 may explain the lower MIC of tylosin observed for this isolate (Table 3), since (data not shown). The duplication in 98-4277-2 may explain the lower MIC of tylosin observed for this isolate (Table 3), since the duplication may alter any secondary structures in the leader peptide region of the duplication. The *erm(X)* gene region was circularly permuted, indicating that *erm(X)* was carried on a plasmid. This plasmid was designated pAP2. The nucleotide sequence of the entire 9,304-bp pAP2 plasmid was determined, and the salient features of pAP2 are indicated on the restriction map in Fig. 3. In addition to *erm(X)* and its associated small ORFs, pAP2 contained six other ORFs, designated *grcY*, *repA*, *orf95*, *tetR* (33), and *tnpA*, which was associated with the insertion sequence IS6100 (Fig. 3). IS6100 is found associated with integrons and antibiotic resistance genes in a variety of gram-negative and gram-positive bacteria, including *Pseudomonas aeruginosa*, *Salmonella enterica*, and *Mycobacterium fortuitum*, from where it was originally isolated (8).

A region of pAP2 from map coordinates 8585 to 3123 had 100% DNA identity with bases 21859 to 25701 of the *Corynebacterium glutamicum* plasmid pTET3 (GenBank accession no. NC_003227). This region encompassed the last 126 bp of the left IS6100, *orf95*, *tetR* (33), *tetA* (33), and the entire right IS6100 of the pTET3 IS6100-bound *tet* gene element, possibly suggesting defective insertion of this element into a pAP2 progenitor plasmid. *orf95* was not identified in the sequence of pTET3, but the stop codon of *tetR* (33) and the start codon of the putative *orf95* overlap, suggesting translational coupling.

Downstream of *orf95* is *repA*, which encodes a replication protein with similarity to RepA from pNG2 (46.8% identity, sert (Fig. 3). Nucleotide sequencing of pJGS466 and pJGS551 revealed that the *erm(X)* gene region was circularly permuted, indicating that *erm(X)* was carried on a plasmid. This plasmid was designated pAP2. The nucleotide sequence of the entire 9,304-bp pAP2 plasmid was determined, and the salient features of pAP2 are indicated on the restriction map in Fig. 3. In addition to *erm(X)* and its associated small ORFs, pAP2 contained six other ORFs, designated *grcY*, *repA*, *orf95*, *tetR* (33), and *tnpA*, which was associated with the insertion sequence IS6100 (Fig. 3). IS6100 is found associated with integrons and antibiotic resistance genes in a variety of gram-negative and gram-positive bacteria, including *Pseudomonas aeruginosa*, *Salmonella enterica*, and *Mycobacterium fortuitum*, from where it was originally isolated (8).

A region of pAP2 from map coordinates 8585 to 3123 had 100% DNA identity with bases 21859 to 25701 of the *Corynebacterium glutamicum* plasmid pTET3 (GenBank accession no. NC_003227). This region encompassed the last 126 bp of the left IS6100, *orf95*, *tetR* (33), *tetA* (33), and the entire right IS6100 of the pTET3 IS6100-bound *tet* gene element, possibly suggesting defective insertion of this element into a pAP2 progenitor plasmid. *orf95* was not identified in the sequence of pTET3, but the stop codon of *tetR* (33) and the start codon of the putative *orf95* overlap, suggesting translational coupling.

Downstream of *orf95* is *repA*, which encodes a replication protein with similarity to RepA from pNG2 (46.8% identity,
erm(X) ENCODES TYLOSIN RESISTANCE IN A. PYOGENES

54.7% similarity) and pTET3 (39.5% identity, 54.4% similarity). The pAP2 repA gene is truncated at the 5’ end compared with that from pNG2, which may have resulted from the fusion of the truncated IS6100 sequence during insertion of the tet gene element. One hundred eighty-two base pairs downstream of repA is a copy of the 22-bp box (5’T-CGGTACGAAATATAC GTTCCGC-3’) thought to be required for replication of corynebacterial rolling circle plasmids (20).

pAP2 also carries gerY, so named because of the similarity of its translated product to GcrY, a protein of unknown function, encoded by pTP10 (71.8% identity, 87.7% similarity). The 6-bp GTATAC direct repeats downstream of gerY in pTP10 (23) were not present in pAP2.

Given the similarity of the pAP2 repA to that of pNG2, which is broad host range (19), we tried to transform E. coli to erythromycin resistance with 98-4277-2 genomic DNA, containing pAP2, but were unsuccessful after numerous attempts (data not shown). Similar experiments were performed with A. pyogenes strain BBR1 using either 98-4277-2 genomic DNA or pJGS551, which carries an intact repA gene and its associated 22-bp box. No transformants were obtained in several attempts, indicating that pAP2 does not replicate in A. pyogenes strain BBR1 (data not shown). As a control, strain 98-4277-2 was transformed to CM resistance with pJGS551, demonstrating that pJGS551 is replication competent in the appropriate host. We conclude that pAP2 likely requires additional host-encoded factors, not present in strain BBR1, for efficient replication. One reason for this may be the apparent 5’ deletion of repA, which may have occurred during plasmid evolution.

pAP2 is not carried by all erm(X)-containing A. pyogenes isolates. The finding that erm(X) in 98-4277-2 was plasmid borne led us to investigate whether this gene was carried on a similar plasmid in all erm(X)-containing strains. DIG-labeled probes were prepared for tnpA (IS6100), repA, and tetA (33) (Table 2) and were hybridized under high-stringency conditions against the 11 tylosin-resistant isolates and 37 tylosin susceptible isolates. IS6100 was preferentially associated with erm(X)-containing isolates, since 89.9% of erm(X) strains carried IS6100, compared with 10.3% of non erm(X) strains (Fig. 4A). However, PCR experiments indicated that IS6100 and erm(X) were adjacent only in strain 98-4277-2 (data not shown). repA was present in only two erm(X) strains, indicating that erm(X) was not pAP2 associated in all erm(X) strains. Furthermore, the finding that only two non erm(X) strains carried repA (Fig. 4B) indicates that pAP2-like plasmids are not widespread, correlating with the suggestion that replication of repA-containing plasmids may be strain specific. tetA (33) was present in 55.6% of erm(X)-containing strains but only in 5.1% of non erm(X) strains (Fig. 4C). Only one other erm(X)-containing isolate in addition to 98-4277-2 carried all three ORFs. The two non erm(X) strains carrying repA, also carried tetA (33), but only one also carried IS6100. The permuted combinations of resistance genes and insertion sequences with repA may suggest the presence of other pAP2-like plasmids.

erm(X) in many corynebacteria is associated with IS249 on the transposon Tn5432 (17, 22, 23), although this is not the case with erm(X) from pNG2 (20) or pAP2. The erm(X)-containing A. pyogenes isolates were subjected to PCR with IS249-specific primers. None of the A. pyogenes strains carried IS249 (data not shown), suggesting that the erm(X) genes in A. pyogenes are not derived from Tn5432.

tetA(33) confers low-level TC resistance in A. pyogenes. Sequence analysis of pAP2 indicated that like pTET3, this plasmid contained the repressor-regulated TC resistance determinant, Tet 33, which is similar to group I tetracycline efflux systems from gram-negative bacteria (21). This system was functional in E. coli, since we were able to clone the 6.2-kb HindIII fragment of pAP2 containing Tet 33 in pGGS551 by selection on TC. For C. glutamicum, tetA(33) results in an MIC of TC of 16 μg/ml (21). However, for A. pyogenes strains carrying tetA(33), this gene was only able to result in an MIC of TC of 1 μg/ml, compared with TC-susceptible strains, for which MICs of TC were ≤0.06 μg/ml (3). This is in contrast to strains carrying tet(W), the most prevalent TC resistance gene in A. pyogenes, which confers an MIC of TC of 8 μg/ml (3). For A. pyogenes strains harboring both genes, the MIC of TC is 16 μg/ml. Tauch et al. found that tetA(33) was inducible for C. glutamicum (21), but no induction was observed for A. pyogenes (data not shown).

Conclusions. In the United States, tylosin is widely used as a feed additive to control liver abscessation in feedlot cattle (11). Resistance of the liver abscess pathogen, A. pyogenes, to macrolide antibiotics has been reported (4, 25, 28), but the mechanisms of resistance were not determined. This work is the first report of the identification and characterization of a determinant encoding tylosin resistance in A. pyogenes. erm X is a prevalent determinant of tylosin resistance, with more than 80% of the tylosin-resistant A. pyogenes strains studied carrying this determinant. For strain 98-4277-2, erm(X) is encoded on a plasmid, pAP2, but this plasmid is not widespread among erm(X)-containing strains. pAP2 also carries a Tet 33 determinant, which is only the second Tet determinant identified for A. pyogenes. The finding that two tylosin-resistant A. pyogenes strains did not carry erm(X) suggests that there is at least one other determinant of tylosin resistance present in this organism.

ACKNOWLEDGMENT

Partial support for this work was provided by USDA/NRICGP award 99-35204-7818.

REFERENCES


