ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 1997, p. 2719–2723 Vol. 41, No. 12
0066-4804/97/$04.00+0
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Cloning and Characterization of a Novel Macrolide Efflux Gene, *mreA*, from *Streptococcus agalactiae*

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Received 7 March 1997/Returned for modification 23 April 1997/Accepted 3 October 1997

A strain of *Streptococcus agalactiae* displayed resistance to 14-, 15-, and 16-membered macrolides. In PCR assays, total genomic DNA from this strain contained neither *erm* nor *mef* genes. *Eco*RI-digested genomic DNA from this strain was cloned into λ Zap II to construct a library of *S. agalactiae* genomic DNA. A clone, pAES63, expressing resistance to erythromycin, azithromycin, and spiramycin in *Escherichia coli* was recovered. Deletion derivatives of pAES63 which defined a functional region on this clone that encoded resistance to 14- and 15-membered, but not 16-membered, macrolides were produced. Studies that determined the levels of incorporation of radiolabelled erythromycin into *E. coli* were consistent with the presence of a macrolide efflux determinant. This putative efflux determinant was distinct from the recently described Mef pump in *Streptococcus pyogenes* and *Streptococcus pneumoniae* and from the multicomponent MsrA pump in *Staphylococcus aureus* and coagulase-negative staphylococci. Its gene has been designated *mreA* (for macrolide resistance efflux).

The first macrolide antibiotic was isolated in 1950 as a fermentation product of a *Streptomyces* strain (17). Natural and semisynthetic macrolides, all characterized by the possession of a macrocyclic lactone ring, are used extensively in human medicine. Resistance to these antibiotics emerged within a few years of their introduction as therapeutic agents. The following three functional classes of macrolide resistance mechanisms exist in pathogenic bacteria: those that modify the ribosome, which is the target of the antibiotic; those that modify the antibiotic itself; and those that affect the rate of transport of the antibiotic across the cell membrane. So far, only target modification and transport modification have been well documented to convey resistance to 14- and 15-membered macrolides in streptococci. Target modification is conveyed by the action of a family of methyltransferase enzymes encoded by the *erm* (for erythromycin ribosome methylation) genes (23). A second gene has recently been shown to encode resistance by increasing the transport of 14- and 15-membered macrolides from cells of *Streptococcus pyogenes* (9). A very similar gene has recently been cloned from macrolide-resistant strains of *Streptococcus pneumoniae* (8). This gene family is designated *mef* (for macrolide efflux) and is believed to encode a hydrophobic membrane protein which uses the energy of the proton motive force to pump macrolides from the interior of the cell (9). Other mechanisms of macrolide resistance in other species of bacteria have previously been described (11, 13, 18, 23). Here we report the molecular cloning and characterization of a previously uncharacterized macrolide efflux gene from a strain of *Streptococcus agalactiae.*

MATERIALS AND METHODS

Chemicals. Erythromycin, azithromycin, clarithromycin, roxithromycin, oleandomycin, and josamycin were from the Charles Pfizer sample bank. Spiramycin, tylosin, clindamycin, lincomycin, ampicillin, kanamycin, carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP), 2,4-dinitrophenol (DNP), acriflavine hydrochloride, rhodamine 6G, ethidium bromide, acridine orange, and arsenic acid (sodium salt and heptahydrate) were from Sigma (St. Louis, Mo.). Norfloxacin was from Merck, Sharp & Dohme (Munich, Germany). Tetraphenylphosphonium chloride was from Aldrich (Milwaukee, Wis.). Durapore HV filters were from Millipore (Bedford, Mass.). Polymyxin B nonapeptide (PMBN) was from Boehringer Mannheim (Indianapolis, Ind.). [*N-methyl*-^{14}C]erythromycin was from Dupont, NEN. Brain heart infusion (BHI) broth and Mueller-Hinton medium with cations (20 mg of Ca^{2+} per liter and 12.5 mg of Mg^{2+} per liter; MHC) were from Remel, Lenexa, Kans. Restriction endonucleases were from New England Biolabs (Beverly, Mass.). Exonuclease III, S1 nuclease, DNA ligase, pGEM-T, and the Wizard DNA cleanup system were from Promega (Madison, Wis.). *Taq* polymerase was from Perkin-Elmer (Foster City, Calif.). The λ Zap II library construction kit, pBluescript (SK−), and pBluescript (KS+) were from Stratagene (La Jolla, Calif.). The Fast Link DNA ligation kit was from Epicentre Technologies (Madison, Wis.).

Bacterial strains, plasmids, and growth conditions. Strain COH31 γ/δ was isolated from a foot ulcer of a diabetic patient (20). *S. agalactiae* strains were grown in MHC broth overnight in a 5% CO_2 incubator at 37°C without shaking. *Escherichia coli* strains were grown in MHC or BHI broth at 37°C with shaking and with appropriate selection for resident plasmids (ampicillin at 100 μg/ml).

Strain characterization. PCR analysis of *S. agalactiae* COH31 γ/δ for macrolide-lincosamide-streptogramin B resistance and *mef* resistance was performed as previously described (3, 9).

MICs of antibiotics. Broth dilution MICs were determined for *S. agalactiae* strains in MHC broth–2.5% lysed horse blood. Agar dilution MICs in MHC agar and in the presence of 100 μg of PMBN per ml (to permeabilize DH5α clones to macrolide antibiotics [22]) and 100 μg of ampicillin per ml were determined for *E. coli* DH5α strains containing streptococcal DNA inserts in pGEM-T. Determinations of broth and agar dilution MICs were performed under National Committee for Clinical Laboratory Standards conditions (15).

Plasmid preparation, restriction endonuclease analysis, transformations, preparation of genomic DNA, and sequencing. Plasmids were prepared with Qiagen columns (Chatsworth, Calif.) or by alkaline lysis techniques (4, 21). Restriction digestions were performed according to the suppliers' directions. Genomic DNA from *S. agalactiae* was prepared by the method of Ausubel et al. (4), after an initial incubation with 3 mg of lysozyme per ml in 7% sucrose–50 mM Tris–1 mM EDTA (pH 8.0). Automated fluorescent sequencing was performed on both DNA strands with an Applied Biosystems apparatus (model 373A). DNA and protein homologies were determined by using the FASTA, GAP, and PROSITE algorithms. Additional gene and protein analyses were performed with Geneworks (Intelligenetics, Campbell, Calif.) and Lasergene software (DNASTAR, Inc., Madison, Wis.).

Cloning into λ Zap II. Genomic DNA from *S. agalactiae* COH31 γ/δ was digested with *Eco*RI, the DNA was ligated overnight to 1 μg of dephosphorylated λ Zap II arms digested with *Eco*RI, and the ligation reaction was packaged into phage heads with Gigapack II packaging extract (Stratagene). Phage were resuspended and lysed, and the mixture was spun briefly in a microcentrifuge. The library phagemid stock was titered with the XL-1 Blue MRF′ strain. pBluescript (SK−) phagemids were excised from the λ Zap II vector by combining 100 μl of phage stock with 200 μl of XL-1 Blue MRF′ cells and 1 μl of ExAssist helper phage (Stratagene). This rescued phagemid stock suspension was used to infect 200 μl of SOLR cells. One hundred microliters of the mixture was plated on Luria-Bertani–ampicillin–X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)–IPTG (isopropyl-β-D-thiogalactopyranoside) plates and incubated overnight at 37°C.

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TABLE 1. *E. coli* plasmids

Strain	Plasmid	Description	Reference or source
DH5α	None		12
	pBluescript	2.9-kb vector	Stratagene
	pAES63	3.8-kb *Eco*RI fragment in pBluescript (SK−)[a]	This study
	pGEM-5Zf(+)	3.0-kb vector	Promega
	pF1R3	1.8-kb clone spanning 1.9 to 3.7 kb of pAES63 in pGEM[a]	This study
	pF5R5	1.1-kb clone spanning 2.2 to 3.3 kb of pAES63 in pGEM[a]	This study
	pF6R7	1.1-kb clone spanning 1.3 to 2.4 kb of pAES63 in pGEM[a]	This study
	pAES63.I	Inverted 3.8-kb *Eco*RI fragment in pBluescript (KS+)[a]	This study
	pMR3	1.7-kb PCR product containing *mefE* gene from *S. pneumoniae* 02J1052 in pGEM in an orientation opposite to that of the *lac* promoter on the vector	This study
XL-1 Blue MRF′	F′	Supports growth of λ Zap II vector	λ Zap II instruction manual (Stratagene)
SOLR	F′	Supports excision of pBluescript phagemid from λ Zap II vector	λ Zap II instruction manual (Stratagene)
BM694	pAT63	*ereA*	2
	pAT72	*ereB*	2

[a] See Fig. 1 and Materials and Methods.

Subcloning of insert in pAES63 into pBluescript (KS+). To isolate the insert in the opposite orientation in the vector, plasmid pAES63 was digested with *Bam*HI and *Xho*I and electrophoresed on a 0.7% agarose gel in Tris-acetate buffer at 5 V/cm and the 3.8-kb fragment containing the macrolide-resistant determinant was gel purified by the freeze-thaw phenol method. pBluescript (KS+) vector was digested with *Bam*HI and *Xho*I and passed through a Wizard DNA cleanup column. A ligation reaction containing pBluescript (KS+) and the purified *Bam*HI-*Xho*I fragment was carried out at 4°C overnight. Transformations were performed with competent DH5α cells, and transformants were plated on BHI broth supplemented with 50 μg of ampicillin per ml, 20 mM IPTG, and 80 μg of X-Gal per ml at 37°C overnight. Pale blue colonies were found to have the 3.8-kb fragment in pBluescript (KS+).

Subcloning of DNA sequences within the insert in pAES63 into pGEM-T. Plasmid AES63 was used as template DNA. Forward and reverse primers are described below. All reactions were performed in 10 mM Tris-HCl (pH 8.3)–50 mM KCl in a 20-μl reaction volume. The $MgCl_2$ concentration used was 1.5 mM. Nucleotide triphosphates were used at a concentration of 200 μM. *Taq* polymerase (0.5 U) was added to each reaction mixture. Control reactions in which only one primer was used at a time with and without template DNA and with and without *Taq* polymerase were performed. Polymerase reactions were amplified on a Perkin-Elmer GeneAmp PCR system 9600. A typical protocol consisted of a melting step of 2 min at 95°C, followed by 25 cycles of the following steps: a melting step at 94°C for 1 min, a primer annealing step at 53°C for 1 min, and an extension step at 72°C for 1 min. The protocol was terminated by a final incubation at 72°C for 10 min. The PCR product was electrophoresed, extracted from the gel, ligated to pGEM-T, transformed into DH5α cells, and plated on BHI broth supplemented with 100 μg of ampicillin per ml, 0.5 mM IPTG, and 80 μg of X-Gal per ml. White colonies were checked for the presence of an insert. The primers used to synthesize streptococcal inserts in plasmids were as follows: for pF1R3, 5′-CCTCTTGATCCTTCATGG-3′ and 5′-AAAGGTACCTATGTTAGGACG-3′; for pF5R5, 5′-GGCGGTTATATGATTACCTT-3′ and 5′-GAAGCAGATACCTTAGCAGC-3′; and for pF6R7, 5′-GGCTTACATCTGGGACATAAAG-3′ and 5′-GCACTATACCAAAAGAGCG-3′. These plasmids are described in Table 1.

Molecular cloning of *mefE* from *S. pneumoniae* 02J1052 into pGEM. A primer pair specific for *mef* (9) was used in PCRs with genomic DNA from a clinical strain, *S. pneumoniae* 02J1052, as the template. This produced the expected oligonucleotide of 1,759 bp containing *Pst*I and *Nsi*I recognition sites. The 1.7-kb PCR fragment containing *mefE* was directionally cloned into pGEM-5Zf(+) to produce pMR3.

Erythromycin inactivation assays. Inactivation assays were performed essentially by the method of Clancy et al. (9). Briefly, cells from exponential cultures of *E. coli* DH5α strains were incubated for 48 h with erythromycin (80 μg/ml) and samples were removed at various time points and centrifuged. Supernatant solutions from these samples were applied to filter paper discs in duplicate and placed on a lawn of a macrolide-susceptible test strain. Petri plates were incubated overnight, and the diameter of the zone of inhibition around each disc was measured. *E. coli* BM694(pAT63) (*ereA*) and BM694(pAT72) (*ereB*) (2) were used as positive controls, and *E. coli* DH5α(pGEM) was used as a negative control. In some experiments, exponential cultures of these same strains were lysed by passage through a French pressure cell and centrifuged and the supernatant solution was incubated for up to 40 h with 80 μg of erythromycin per ml in Tris buffer (pH 7.4). Samples from these preparations were removed at various times, spotted on paper discs, and assayed as described above.

[^{14}C]erythromycin accumulation assays. These assays were performed by the method of Clancy et al. (9). Briefly, *E. coli* DH5α strains were grown to a turbidity corresponding to ~10^7 viable cells per ml and resuspended in MHC broth-PMBN-[*N-methyl*-^{14}C]erythromycin. These cultures were grown in a shaking water bath at 37°C for 30 min. Samples (5 ml) were removed at 0, 5, 12, 20, and 30 min; filtered through a Whatman GF/C filter; dried; and placed in scintillation vials. Samples were counted in an optimized ^{14}C channel in a scintillation spectrometer for 15 min. CCCP, DNP, and arsenate were added to some cultures during the assay to final concentrations of 100 μM, 1 mM, and 50 mM, respectively.

For *S. agalactiae* accumulation experiments, cultures were incubated in MHC, as described above, without PMBN. Cultures were preincubated with 100 μM CCCP, 10 mM arsenate, or 1 mM DNP for 10 min where appropriate.

Nucleotide sequence accession number. The *mreA* gene sequence has been deposited in the GenBank database under accession no. U92073.

RESULTS

Phenotypic analysis of *S. agalactiae* COH31 γ/δ. In several experiments, attempts to amplify genomic DNA from *S. agalactiae* COH31 γ/δ with a pair of oligonucleotides that corresponded to conserved amino acid motifs in known Erm methylases (3) or *mefAE* (9) were unsuccessful under conditions in which DNAs from streptococcal strains containing *erm* and *mef* determinants did support the synthesis of consensus DNA regions (data not shown). COH31 γ/δ was resistant to erythromycin (MIC, 3.9 μg/ml), other 14-membered macrolides, the 15-membered macrolide azithromycin, compounds of the lincosamide class (MIC of clindamycin, 0.49 μg/ml), and the two 16-membered macrolides spiramycin and tylosin (MICs of 500 and 62.5 μg/ml, respectively). This strain was comparable to other *S. agalactiae* strains in its MICs of streptogramin A, streptogramin B, ciprofloxacin, vancomycin, gentamicin, ampicillin, ceftriaxone, and chloramphenicol (data not shown). As noted above, in PCR assays, it lacked *erm* and *mef* genes. Taken together, these results suggested that the resistance of COH31 γ/δ to 14-, 15-, and 16-membered macrolides was due to the presence of a novel macrolide resistance gene. This observation was confirmed by subsequent subcloning and DNA sequencing experiments (see below).

Cloning of macrolide resistance determinant. A sample of pBluescript (SK−) phagemids was excised from the vector, transformed into DH5α, and plated on Luria-Bertani–ampicillin–X-Gal–IPTG plates. Three hundred white colonies were stabbed into BHI-erythromycin (75 μg/ml), BHI-azithromycin (6.5 μg/ml), and BHI-spiramycin (600 μg/ml) agar petri plates

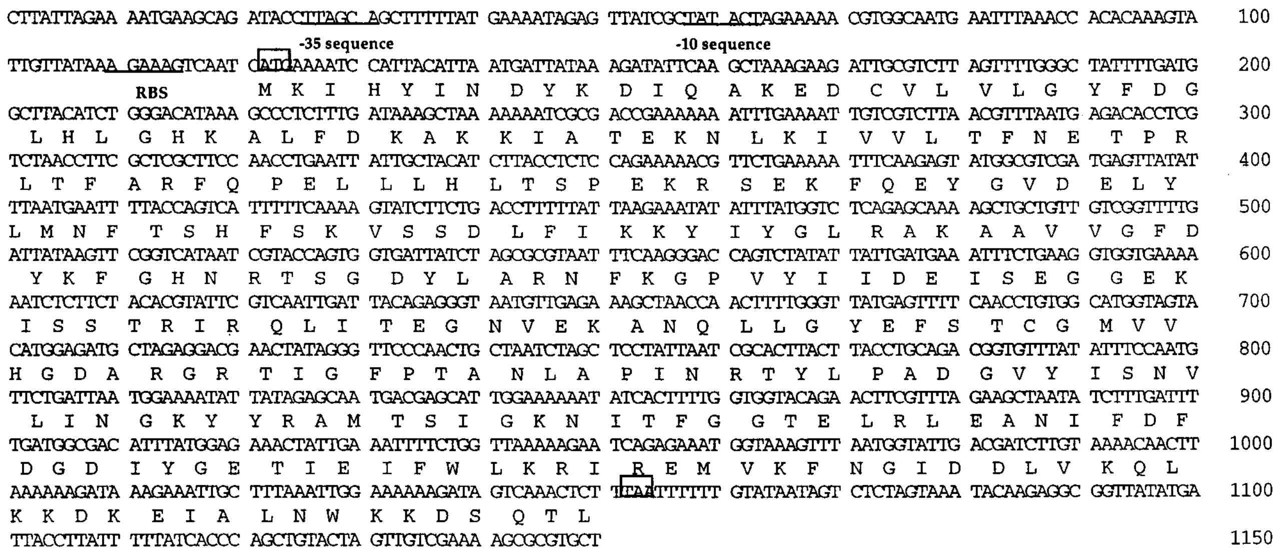

FIG. 1. Nucleotide and amino acid sequences of *mreA*. Possible −10 and −35 promoter sequences, ribosome binding site (RBS) (underlined), and initiation and termination codons (boxed) are shown.

and incubated for 40 to 48 h at 37°C. PMBN was not utilized in these experiments. One clone, DH5α(pAES63), grew on all of these antibiotic plates, but strains containing the vector alone did not. Subclones of pAES63 produced by ligation of PCR-synthesized gene segments to pGEM or pBluescript vectors were transformed into *E. coli* DH5α (Table 1). The functional regions required for the expression of azithromycin, erythromycin, and spiramycin resistance were present on plasmids pAES63, pAES63.I, and pF1R3, whereas pF5R5 was erythromycin and azithromycin resistant but encoded decreased resistance to spiramycin. An analysis of the nucleotide sequence of the streptococcal insert in pF5R5 revealed a single open reading frame.

Sequence analysis of *mreA*. The nucleotide sequence of this gene is shown in Fig. 1. A nonconsensus −35 sequence, TTA GCA, may be present from nucleotides 26 to 31; a nonconsensus −10 sequence, TATACT, is located between nucleotides 58 and 63. Comparable levels of macrolide resistance were noted in pAES63 and pAES63.I, which differ only in their opposite orientations in the vector. This suggests that *mreA* utilizes its own promoter and is not dependent for expression on promoter sequences in the vector. A possible ribosome binding site, AGAAAG, is located 7 bases in front of the archetypal initiation codon, ATG. The putative 310-amino-acid sequence, with a predicted molecular mass of 35.4 kDa, terminates with an ochre codon, TAA. A hydrophilicity plot by the Kyte-Doolittle algorithm suggests that this gene encodes a hydrophilic protein with interspersed hydrophobic and amphipathic sequences (data not shown).

By BLASTX and FASTA, no known macrolide resistance gene was localized to this fragment. Instead, by GAP analysis, this open reading frame has 31% identity and 40% homology to an unknown protein, protein X, from *E. coli*. The function of this protein, which resides in the *ileS* (isoleucyl-tRNA ligase)-*lsp* (prolipoprotein signal peptidase) operon (24), is unknown. Homologs of this gene are also found in *Haemophilus influenzae*, *Corynebacterium ammoniagenes*, and *Saccharomyces cerevisiae*. The penicillin acylase preprotein sequence from *Kluyvera citrophila* (5), a secreted protein (hevein) from the rubber tree (6), and a c-*src* oncogene homolog from *Drosophila melanogaster* also show sequence similarities to *mreA*.

There are no obvious influx or efflux pumps among these homologs. Instead, several of them have known or putative tyrosine kinase, threonine/serine kinase, or other functions in which a substrate is phosphorylated and ATP or CTP donates the phosphate group. Several of these *mreA* homologs are specialized precursor sequences of enzymes or structural proteins which appear in mature form on the cell surface, in the periplasm of a gram-negative bacterium, or in the external medium.

[^{14}C]erythromycin accumulation assays. The ability of exponential cells of *E. coli* DH5α containing various cloned determinants in pGEM to accumulate [^{14}C]erythromycin when they were permeabilized with the cationic peptide PMBN (22) was assessed at various times during a 30-min incubation period and compared to that of equal numbers of DH5α cells containing pGEM alone. All strains containing cloned efflux determinants accumulated significantly less erythromycin than did strains containing the vector (Fig. 2).

The addition of arsenate, DNP, or CCCP to replicate cultures of DH5α(pF5R5) expressing the cloned *mreA* gene caused this strain to accumulate significantly more erythromycin (Fig. 3A). These compounds had a similar effect on the MefE pump encoded on pMR3 (Fig. 3B).

Studies of [^{14}C]erythromycin accumulation in the original *S. agalactiae* strain, COH31 γ/δ, confirmed that an energy-dependent macrolide efflux mechanism was present. At 8, 15, and 30 min, this strain accumulated significantly lower levels of [^{14}C]erythromycin than did replicate cultures of a macrolide-susceptible *S. agalactiae* strain, 02B1027. Cells of this strain also accumulated significantly less erythromycin than did comparable cultures incubated with 100 μM CCCP, 10 mM arsenate, and 1 mM DNP. These results suggested that the expression of this macrolide resistance phenotype in the original strain required a proton gradient between the inside and outside of the cell in order to function. Pretreatment of a macrolide-susceptible *S. agalactiae* strain with CCCP or uncouplers of oxidative phosphorylation caused no significant differences in the accumulation of erythromycin by cells of that strain (data not shown).

Erythromycin inactivation assays. Assays which assessed a diminution in the bioactivity of erythromycin incubated for up

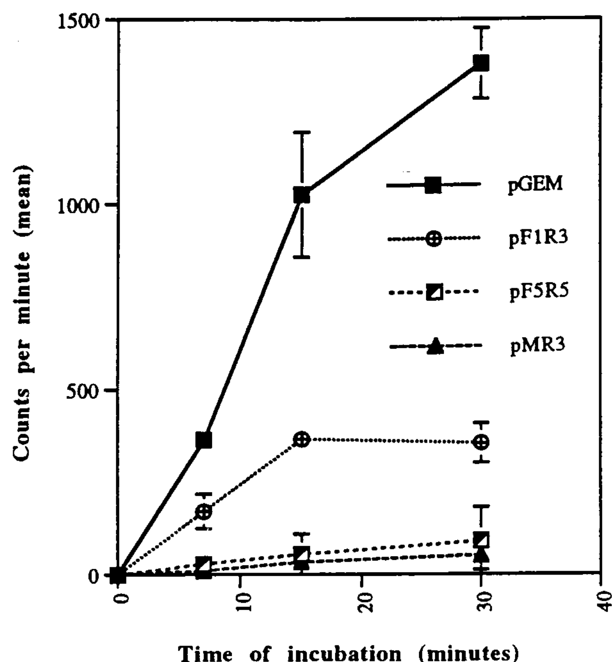

FIG. 2. Intracellular accumulations of [*N-methyl*-^{14}C]erythromycin by *E. coli* DH5α strains containing *mreA* and *mefE* determinants in pGEM.

to 48 h with *E. coli* strains expressing cloned determinants from the pGEM vector provided no consistent evidence for the ability of MreA to chemically inactivate erythromycin. The positive control strains, *E. coli* BM694(pAT63) expressing *ereA* and *E. coli* BM694(pAT72) expressing *ereB*, did decrease the bioactivity of erythromycin under the same conditions. In separate experiments, lysed cultures of DH5α(pGEM) and DH5α (pF5R5) were incubated with erythromycin to assess the possible presence of an intracellular macrolide-inactivating enzyme. No evidence of a consistent decrease in the activity of erythromycin incubated with such samples was obtained (data not shown).

MIC determinations for isogenic *mreA*$^+$ and *mreA* mutant *E. coli* strains. Agar dilution MIC determinations of a number of standard antibiotics and hydrophobic agents were performed with *E. coli* DH5α(pF5R5) and DH5α(pGEM). No significant differences were observed in the MICs of rhodamine 6G, norfloxacin, tetraphenylphosphonium chloride, ethidium bromide, tetracycline, and minocycline. MICs of >200 μg/ml were noted for both strains with arsenate and acridine orange. Strains containing pF5R5 were four- to eightfold more resistant to acriflavine than were isogenic strains containing the vector (MICs of 50 and 6.25 μg/ml, respectively) (data not shown).

DISCUSSION

In this communication, we suggest the existence of another class of macrolide resistance genes in streptococci, distinct from both *erm* and *mef* gene families. The gene is called *mreA* (for macrolide resistance efflux) because *E. coli* strains containing the gene accumulate significantly less erythromycin than do isogenic strains containing the vector alone. Moreover, a comparable erythromycin efflux system is present in the original strain. The gene has no homology at either the gene or protein level to any known or postulated efflux or influx pump (14, 16) and is instead related to a hypothetical 35-kDa protein of unknown function. The *mreA* homolog from *Bacillus subtilis* is linked to *ribC*, the gene for riboflavin synthetase, although *mreA* has little or no homology to *ribC*. It is intriguing that mutations in the riboflavin synthetase gene in *B. subtilis* have

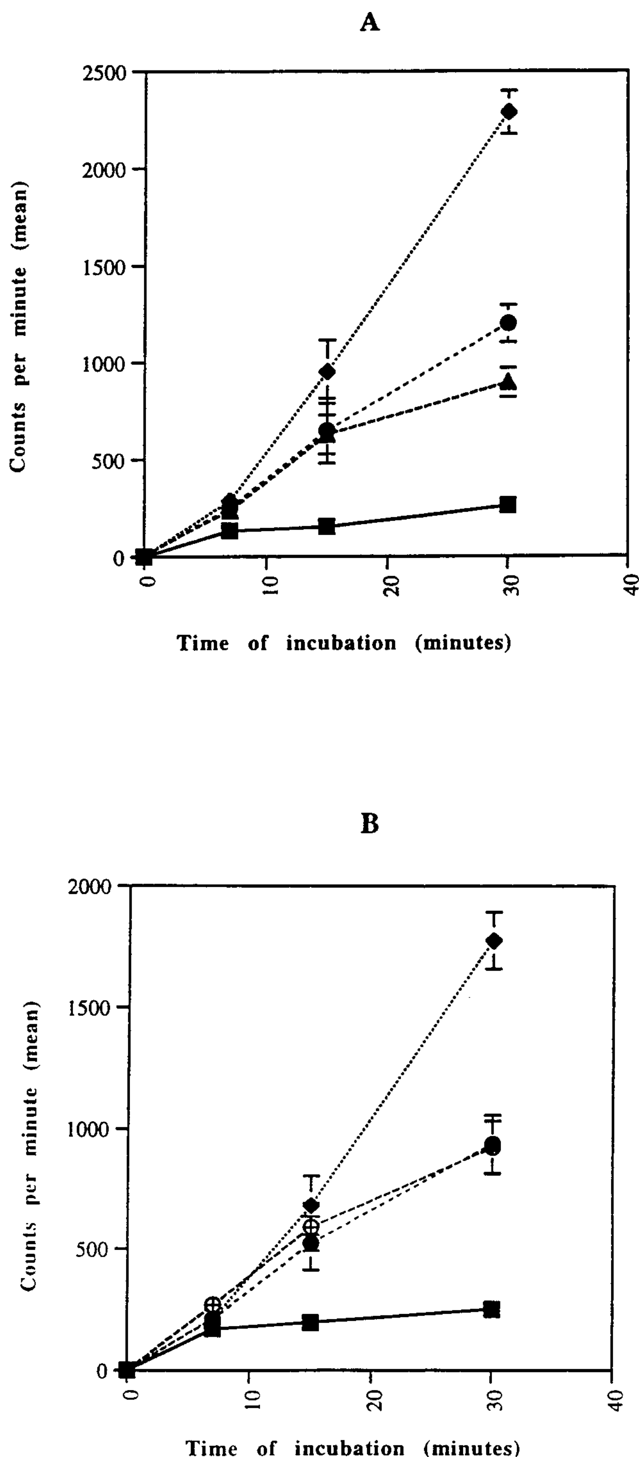

FIG. 3. Effects of DNP, CCCP, and arsenate on the accumulation of [*N-methyl*-^{14}C]erythromycin by *E. coli* DH5α strains. (A) DH5α(pF5R5) expressing *mreA* (■) with 100 μM CCCP (◆), 3 mM arsenate (●), or 1 mM DNP (▲) added. (B) DH5α(pMR3) expressing *mefE* (■) with 100 μM CCCP (◆), 50 mM arsenate (●), or 1 mM DNP (⊕) added.

previously been shown to be genetically linked to genes encoding acriflavine resistance and arsenate resistance (1, 7) since *E. coli* strains expressing *mreA* also show increased resistance to acriflavine.

The putative tertiary structure of MreA is probably not consistent with that of a protein which spans a biological membrane, as predicted by the Engelman-Steitz-Goldman algorithm (10), but it has short recurrent hydrophobic regions of approximately 10 amino acids that may associate transiently with the cell membrane or perhaps with specific membrane proteins. Further studies are needed to determine whether additional membrane-associated functionalities are required to effect macrolide efflux. If this is so, functionally equivalent structures would appear to be present in gram-positive and gram-negative bacteria, since our experiments suggest that *mreA* functions in both *E. coli* and *S. agalactiae*. The distant but extensive homologies to secreted proteins of some prokaryotic and eukaryotic organisms suggest that MreA plays a role in conveying the macrolide to the inner face of the membrane. The homologies of *mreA* to genes of the tyrosine kinase class, such as the c-*src* proto-oncogene of *D. melanogaster*, also support a role for MreA in interactions with membranes and perhaps in its participation in some sort of autophosphorylation mechanism to effect communication between the exterior and interior of the cell. Additional studies are required to confirm or exclude each of these hypotheses.

It is interesting that uncouplers of oxidative phosphorylation, such as DNP, CCCP, and arsenate, had very similar inhibitory effects on the erythromycin efflux pumps encoded by *mreA* and *mef* (Fig. 3). Such inhibitory effects are in fact strong evidence that the erythromycin efflux encoded by *mreA* is an energy-dependent process. A distinct erythromycin efflux mechanism that is encoded by *msrA* in *Staphylococcus aureus* has also previously been shown to be inhibited by arsenate and DNP (19). These experiments do not determine whether the efflux of erythromycin is achieved primarily by the proton motive force of the inner membrane or by hydrolysis of ATP. By their homologies to tetracycline antiporters, Mef pumps are hypothesized to use the former (9), whereas the Msr system, with its two ATP-binding motifs, is believed to use the latter (19).

Further studies are required to demonstrate unambiguously that *mreA* contributes to macrolide resistance in *S. agalactiae*. To do this, *mreA* must be introduced in single copy back into the chromosome of a susceptible strain or insertionally inactivated in the original strain and concomitant changes in macrolide resistance must be observed.

ACKNOWLEDGMENTS

We thank Craig Rubens (University of Washington School of Medicine) for sending us strain COH31 γ/δ, Margaret Anderson (Pfizer Central Research) for providing additional *S. agalactiae* strains, and George Karam (Pfizer Central Research) for useful discussions.

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