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Plasmid-Mediated Resistance to Lincomycin by Inactivation in *Staphylococcus haemolyticus*

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Staphylococcus haemolyticus **BM4610 was resistant to high levels of lincomycin and susceptible to macrolides, clindamycin, and streptogramins. This resistance phenotype, not previously reported for a human clinical isolate, was due to inactivation of the antibiotic. The gene conferring resistance to lincomycin in strain BM4610 was carried by a 2.5-kilobase plasmid, pIP855, which was cloned in *Escherichia coli*. Plasmid pIP855 caused inactivation of both lincomycin and clindamycin in *S. haemolyticus* and in *E. coli* but conferred detectable resistance to lincomycin only in *S. haemolyticus* and to clindamycin only in *E. coli*.**

Resistance of *Staphylococcus* spp. to lincosamide antibiotics is nearly always associated with coresistance to macrolides and streptogramin B-type antibiotics, the so-called MLS phenotype (7, 10a). The mechanism of this broad resistance involves modification of the intracellular target of the antibiotics by N^6 dimethylation of adenine in 23S rRNA (33). Recently, two non-MLS types of resistance toward lincomycin have been described in staphylococci of animal origin (14). In the first one, detected in *Staphylococcus aureus* and *Staphylococcus intermedius* (21), the cells inactivate both lincomycin and clindamycin but resist high levels of lincomycin only. The second type of resistance was observed in *S. aureus* and *Staphylococcus hyicus* (15). The strains exhibit low-level resistance to lincomycin and streptogramin A-type antibiotics without apparent inactivation. The biochemical mechanisms and the genetic basis of these resistances have not been studied. El Solh et al. (18) have also described strains of *S. aureus* resistant to lincomycin and streptogramin A-type antibiotics. The bacteria degrade streptogramin A-type antibiotics but not lincosamides. In this study, we report resistance to lincomycin by inactivation in *Staphylococcus haemolyticus*, a phenotype not previously described in a clinical isolate. The plasmid gene encoding the inactivating activity was cloned and expressed in *Escherichia coli*.

MATERIALS AND METHODS

Bacterial strains and plasmids. Coagulase-negative *S. haemolyticus* BM4610 was isolated in 1984 from a human urine sample and was identified by the method of Schleifer and Kloos (26). *E. coli* DB10 (12) is a fusidic acid and macrolide–lincosamide–streptogramin-susceptible derivative of strain PR7 (31). *E. coli* HB101 harboring plasmid pBR329 (Tra$^-$ Mob$^-$ Ap Cm Tc) (11) and *S. aureus* 209P were from our laboratory collection.

Media. Brain heart infusion broth and agar (Difco Laboratories) were used. Disk sensitivity tests were done on Mueller-Hinton agar (Institut Pasteur Production). All incubations were at 37°C.

Antibiotic susceptibility. The agar-disk diffusion test was used. Disks containing pristinamycin I (40 μg), pristinamycin II (20 μg), cadmium nitrate (2×10^{-4} μmol), mercuric nitrate (2×10^{-4} μmol), and sodium arsenate (2×10^{-3} μmol) were prepared. The method of Steers et al. (28) was used to determine the MICs of the antibiotics.

Curing of antibiotic resistance. Curing of antibiotic resistance traits with ethidium bromide (6), metronizadole (16), and novobiocin (24) was performed as previously described.

Enzymic and inactivation assays. Penicillinase was detected with nitrocefin (29). To assay for aminoglycoside-modifying enzymes, staphylococcal extracts were prepared (9), and the enzymes were assayed by the phosphocellulose paper-binding technique (20). Inactivation of lincosamides was screened by the Gots test (19) with *Micrococcus luteus* ATCC 9341 as indicator organism and concentrations of 0.05 and 0.1 μg of clindamycin and lincomycin per ml in the culture medium, respectively. Kinetics of inactivation of lincosamides by resting cells were determined in liquid medium. Cells from 150 ml of an overnight broth culture were harvested, washed once in 0.01 M phosphate buffer (pH 7.0), suspended in 2.5 ml of the same buffer containing 20 μg of antibiotic per ml, and incubated for various periods at 37°C. The pH of this suspension, which remained constant, was monitored, and inactivation of lincosamides was followed by a microbiological technique (8).

Preparation of plasmid DNA. Isolation of staphylococcal plasmid DNA (10) and large-scale isolation of pBR329 and derivative plasmid DNA (13) were as previously described. Staphylococcal plasmids were separated by electrophoresis in horizontal slab gels (20 by 20 by 0.7 cm) containing 0.8% low-temperature gelling agarose, and pIP855 DNA was purified as previously described (23).

Enzymes. Restriction endonucleases *Eco*RI, *Hin*dIII, and *Pst*I, T4 ligase, and calf alkaline phosphatase (Boehringer GmbH) were used according to manufacturer recommendations. Lysozyme and lysostaphin were from Sigma Chemical Co. RNase A (bovine pancreas) was from Calbiochem-Behring.

Chemicals. The antibiotics were provided by the following companies: clindamycin and lincomycin, The Upjohn Co.; oleandomycin, Pfizer Inc.; erythromycin, Roussel-Uclaf; spiramycin, Specia; josamycin, Spret-Mauchant; midecamycin, Clin-Midy; pristinamycin I and II, Rhône-Poulenc.

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TABLE 1. MICs of various macrolide, lincosamide, and streptogramin antibiotics against bacterial strains

Organism	MICs (μg/ml)								
	Ery[a]	Ole	Spi	Jos	Lin	Cli	PriI	PriII	Pri
S. aureus 209P	0.12	0.5	0.5	0.5	0.5	0.06	4	1	0.25
S. haemolyticus BM4610	0.12	0.5	0.5	0.5	64	0.12	8	1	0.25
S. haemolyticus BM4610-1	0.12	0.5	0.5	0.5	0.25	0.06	8	1	0.25
E. coli DB10	2	8	8	4	8	0.5	>128	2	2
E. coli DB10(pAT22)	2	8	8	4	16	4	>128	2	2

[a] Abbreviations: Ery, erythromycin; Ole, oleandomycin; Spi, spiramycin; Jos, josamycin; Lin, lincomycin; Cli, clindamycin; PriI, pristinamycin factor I; PriII, pristinamycin factor II; Pri, pristinamycin.

Sarcosyl (sodium lauryl sarcosinate) was provided by Colgate-Palmolive.

RESULTS

Antibiotic resistance characters of *S. haemolyticus* BM4610. Strain BM4610 was resistant to penicillin, kanamycin, fosfomycin, cadmium, and arsenate and to high levels of lincomycin. Resistance to penicillin was associated with the production of a beta-lactamase because nitrocefin was hydrolyzed. Resistance to kanamycin and structurally related antibiotics was due to synthesis of a 3′-aminoglycoside phosphotransferase of type III (9).

In curing experiments with novobiocin, high-level resistance to lincomycin was lost at low frequency (approximately 1% of 1,200 colonies tested). This character was also lost spontaneously, and one clone (strain BM4610-1) was studied further. The MICs of macrolide, lincosamide, and streptogramin antibiotics for the parental strain and BM4610-1 are shown in Table 1.

Plasmid content of BM4610 and its derivative BM4610-1. The plasmid DNA from strain BM4610 and its cured derivative BM4610-1 was purified by ultracentrifugation and analyzed by agarose gel electrophoresis before and after digestion with *Eco*RI endonuclease (Fig. 1). Comparative analysis of the phenotypes with the plasmid content and the *Eco*RI-generated fragment patterns of plasmid DNA in the strains led us to conclude that the wild-type strain BM4610 harbored four plasmids. Plasmid pIP855, which was absent in strain BM4610-1, encoded high-level resistance to lincomycin; it had a molecular size of 2.5 kilobases (kb) and a single *Eco*RI recognition site. The other plasmids had molecular sizes of 34, 4.1, and 1.8 kb.

Molecular cloning of the staphylococcal lincomycin R plasmid pIP855 in *E. coli*. Plasmid pIP855 DNA digested with *Eco*RI endonuclease and pBR329 DNA cleaved with *Eco*RI and dephosphorylated were mixed, ligated, and introduced into *E. coli* DB10 by transformation (22), and clones were selected on ampicillin (100 μg/ml). The plasmid content of transformants resistant to ampicillin and tetracycline, susceptible to chloramphenicol, and able to inactivate lincomycin (see below) was analyzed by agarose gel electrophoresis of crude bacterial lysates (5). One of the transformants harboring the smallest plasmid relative to pBR329 was studied further; its hybrid plasmid was called pAT22. Plasmid pAT22 DNA was purified and analyzed by agarose gel electrophoresis after digestion with *Eco*RI endonuclease (Fig. 1). It appeared that pAT22 consisted of pBR329 fused to pIP855.

Mechanism of resistance to lincosamides mediated by plasmid pIP855 and expression in *E. coli*. *S. haemolyticus* BM4610 and its derivative BM4610-1 and *E. coli* strain DB10 and transformants were examined for lincosamide inactivation by microbiological techniques (Fig. 2 and 3). Strains harboring plasmids pIP855 or pAT22 were found to inactivate lincomycin and clindamycin but none of the other commercially available macrolide or streptogramin antibiotics. The inactivating activity was more efficient against clindamycin as substrate than against lincomycin when present in *S. haemolyticus* (half-time decay of 85 or 160 min, respectively) or in *E. coli* (half-time decay of 15 or 110 min, respectively) (Fig. 3). However, surprisingly, resistance was conferred to only lincomycin in *S. haemolyticus* and to only clindamycin in *E. coli* (Table 1). The inactivating activity was found in growing (data not shown) and resting (Fig. 3) cells.

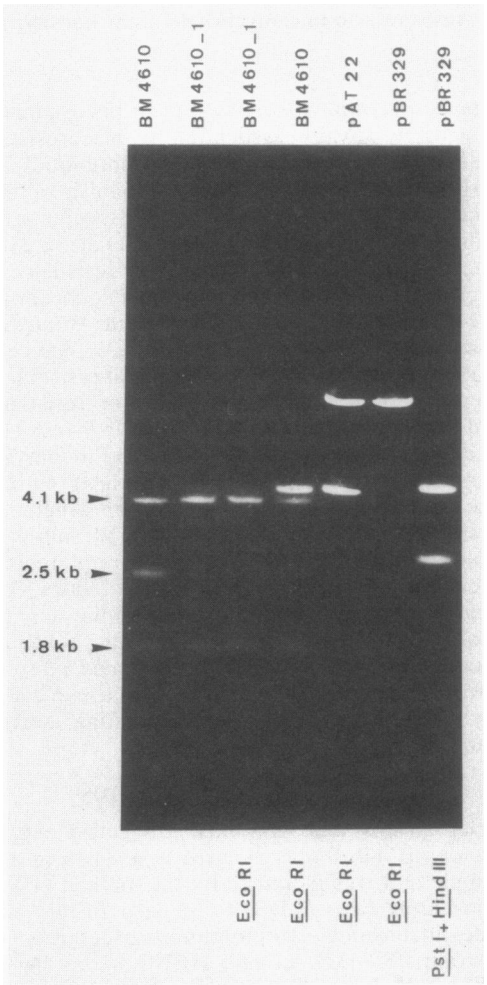

FIG. 1. Analysis of plasmid DNA by agarose gel electrophoresis. Plasmid DNA (1 to 2 μg) was digested with the restriction endonucleases indicated at the bottom. Fragments obtained by digestion of bacteriophage λ *c*I857 DNA with *Hin*dIII (25) and plasmids pIP803 (2 kb), pUC8 (2,678 base pairs), pBR329 (4,150 base pairs), pBR322 (4,362 base pairs), pII147 (31.5 kb), and Rsa (39.5 kb) were used as molecular size standards. Electrophoresis was done in a 0.8% agarose horizontal slab gel (18 by 13 by 0.4 cm) as previously described (27). The sizes of the plasmids are indicated at the left. The band corresponding to the 34-kb plasmid is not visible in this particular analysis.

DISCUSSION

S. haemolyticus BM4610 was a human clinical isolate resistant to lincomycin only, an unusual phenotype for macrolide, lincosamide, and streptogramin resistance. This resistance was due to the inactivation of the antibiotic (Fig. 2). The resistance gene was borne by a 2.5-kb plasmid, pIP855. Enterobacteria, including *E. coli*, are intrinsically resistant to low levels of MLS antibiotics by cellular impermeability (30). Plasmid pIP855 was cloned in its entirety in an *E. coli* mutant susceptible to MLS antibiotics (Fig. 1).

In the original gram-positive host and in the new gram-negative host, both lincomycin and clindamycin were inactivated. In the two types of organisms, clindamycin was a much better substrate for inactivation (Fig. 3), and its bactericidal activity was abolished (data not shown). However, the inactivating activity conferred detectable resistance to lincomycin only in *S. haemolyticus* and to clindamycin only in *E. coli* (Table 1). The reason(s) for the difference in phenotypic expression of the R determinant in the two backgrounds remains unexplained.

Since the detection of BM4610 we have isolated from patients strains of *S. aureus*, *Staphylococcus epidermidis*, and *Staphylococcus cohnii* and a strain of *Streptococcus faecium* which also resist lincomycin only by inactivation. Similar gram-positive cocci of animal origin have been recently reported (14, 17). We are sequencing the gene responsible for lincomycin inactivation carried by plasmid pIP855, and the distribution of this new resistance gene, in strains resistant to lincomycin, will be studied by colony hybridization by using an intragenic probe.

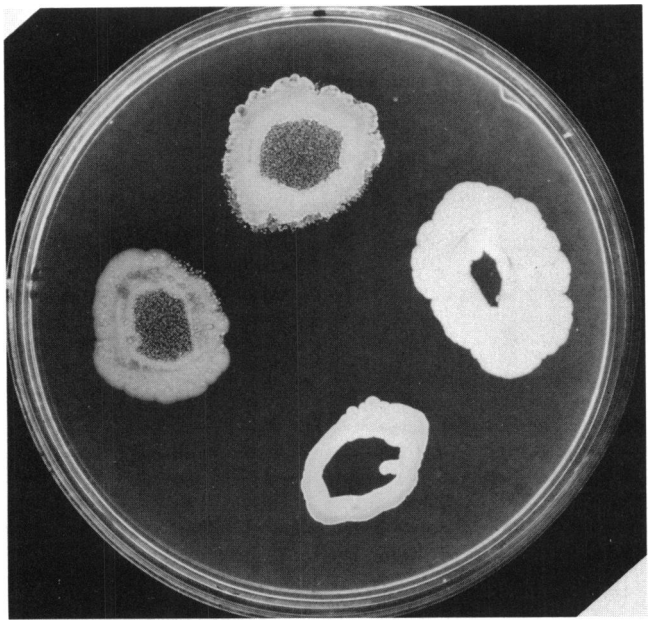

FIG. 2. Inactivation of lincomycin by *S. haemolyticus* BM4610 (top) and BM4610-1 (bottom) and by *E. coli* DB10(pAT22) (left) and DB10 (right) tested by the Gots technique (19). The agar contained a concentration of lincomycin (0.1 μg/ml) slightly higher than the MIC of the indicator organism (*M. luteus* ATCC 9341). The test strains were streaked on the surface of the plate. Inactivation of the antibiotic in the culture medium by the test organism allowed growth of the indicator in the surrounding medium. Similar results were obtained with clindamycin (0.05 μg/ml) in the plate.

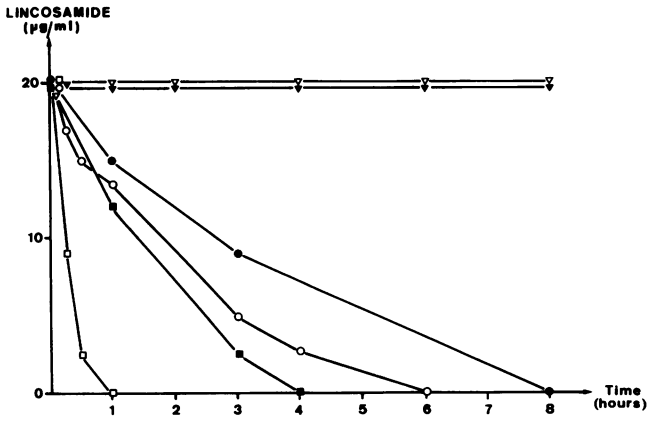

FIG. 3. Kinetics of inactivation of lincosamides by resting cells. Cells suspended in 0.01 M phosphate buffer (pH 7.0) containing 20 μg of lincomycin or clindamycin per ml were incubated at 37°C, and inactivation of the antibiotic was monitored by a microbiological technique (8). Symbols: ●, *S. haemolyticus* BM4610 and lincomycin; ■, BM4610 and clindamycin; ▼, *S. haemolyticus* BM4610-1 and lincomycin or clindamycin; ○, *E. coli* DB10(pAT22) and lincomycin; □, DB10(pAT22) and clindamycin; ▽, DB10 and lincomycin or clindamycin.

Microbial degradation of lincosamides by phosphorylation (1) or adenylyation (2) at the C-3 hydroxyl group has been detected in *Streptomyces* spp. As for the new character of resistance to high levels of erythromycin in *E. coli* that we recently reported (A. Andremont, G. Gerbaud, and P. Courvalin, submitted for publication), that an investigation is under way into the biochemical mechanism of resistance to lincomycin in *Staphylococcus* spp. by determination of the structure of the modified drug by physicochemical techniques (3). Comparison of this mechanism with those already reported in *Streptomyces* spp. will be of interest, especially because it has been proposed that clinical resistance to antibiotics determined by R plasmids could have originated in antibiotic-producing organisms (4, 32).

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