Genetic Study of Plasmid-Associated Zonal Resistance to Lincomycin in *Streptococcus pyogenes*

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The phenomenon of zonal resistance to lincomycin, which is characteristic of most clinical isolates with lincomycin resistance in *Streptococcus pyogenes*, has been studied. These strains grow within a defined concentration range of lincomycin (~60 to 200 µg/ml), or at lincomycin concentrations below the minimal inhibitory concentration for susceptible strains. It is shown that the zonal growth phenomenon is a stable phenotype and results from induction of resistance only within the zonal concentration range of lincomycin. These strains also possess inducible resistance to erythromycin which is nonzonal in character. One-step mutations to constitutive resistance have been isolated which are of two types: constitutive for lincomycin or for erythromycin, but not for both. Those strains with constitutive erythromycin resistance retain their zonal resistance for lincomycin. Mutants doubly constitutive for both lincomycin and erythromycin can be obtained by a second mutational step from either of the singly constitutive mutants. Satellite deoxyribonucleic acid has been shown to be present in the zonal resistant strains. A plasmid, pSM10419, of 14.9 megadaltons, has been isolated from one of the doubly constitutive mutants and used to jointly transform *Streptococcus sanguis* strain Challis to constitutive resistance to both lincomycin and erythromycin. From this, a multicopy plasmid of reduced size, pSM10 (5.4 megadaltons), which retains its resistance phenotype, has been isolated and mapped with restriction endonucleases *Hind*III (three sites), *Eco*RI (one site), *Kpn*I (one site), and *Hpa*I (one site). The staphylococcal plasmid pC221 (2.9 megadaltons; chloramphenicol resistant) has been fused to pSM10 at the *Eco*RI site resulting in a chimeric plasmid, pSM10221 (8.3 megadaltons), which retains resistance to chloramphenicol, erythromycin, and lincomycin. pSM10 is therefore suggestive as an effective cloning vehicle for the genus *Streptococcus*.

Streptococcal strains of clinical origin which are resistant to lincomycin (Lm^r) can be divided into two phenotypic groups according to their growth behavior (13, 14). In one group, the MLS resistance phenotype group (resistance to macrolides, lincosaminides, and streptogramin B group antibiotics), growth on all of these antibiotics is uniform up to their respective minimal inhibitory concentrations (MICs). In the second group, the zonal resistance phenotype group, which represents the majority of the clinical Lm^r isolates of *Streptococcus pyogenes* (14), the growth behavior in the presence of lincomycin differs. These strains grow within a sharply defined zonal range of lincomycin concentrations (~60 to 200 µg/ml), with no growth above or below this range; they can, of course, grow at lincomycin concentrations below the MIC for susceptible strains. Their growth response to erythromycin is the same as that of the MLS resistance phenotype (21).

Plasmid deoxyribonucleic acid (DNA) is associated with the MLS resistance phenotype (4, 8-10, 16, 20, 22), and the biochemical basis for resistance is known to result from methylation of specific adenine residues in 23S ribosomal ribonucleic acid (RNA) (31). Both MLS and zonal resistances are inducibly controlled.

The present paper is concerned with the genetic and physical bases of the zonal resistance phenomenon with lincomycin. In the course of this effort we have also isolated a plasmid, pSM10, whose properties suggest its usefulness as an effective cloning vehicle for the streptococci.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used and the characterized plasmids contained therein are listed in Table 1.

Media. Serum broth (SB) and serum agar (SA), whose compositions have been described elsewhere (19), were used for quantitative antibiotic susceptibil-

TABLE 1. *Bacterial strains and plasmids used in this study*

Strain	Species	Description ^a	Source or reference
777, 6517, 9486 10416	<i>S. pyogenes</i>	Clinical isolates with zonal Lm resistance	13, 14
SM10417	<i>S. pyogenes</i>	Mutant of 10416 with constitutive Lm resistance	This paper
SM10418	<i>S. pyogenes</i>	Mutant of 10416 with constitutive Em resistance	This paper
SM10419	<i>S. pyogenes</i>	Mutant of SM10417 with constitutive resistance to both Lm and Em	This paper
SM6518	<i>S. pyogenes</i>	Mutant of 6517 with constitutive Em resistance	This paper
SM6519	<i>S. pyogenes</i>	Mutant of SM6518 with constitutive resistance to both Lm and Em	This paper
Challis-6	<i>S. sanguis</i>	Plasmid-free transformable strain	J. Ranhand
Challis (pSM10419)	<i>S. sanguis</i>	Challis-6 transformant containing plasmid pSM10419 (Em ^r Lm ^r ; 14.9 Md) originating from SM10419	This paper
Challis (pSM10)	<i>S. sanguis</i>	Challis-6 containing plasmid pSM10 (Em ^r Lm ^r ; 5.4 Md) derived from pSM10419	This paper
8325-4 (pC221)	<i>S. aureus</i>	Containing plasmid pC221 (Cm ^r ; 2.9 Md)	33
Challis (pSM10221)	<i>S. sanguis</i>	Challis-6 containing the plasmid chimera pSM10221 (Em ^r Lm ^r Cm ^r ; 8.3 Md) constructed by fusion of pSM10 and pC221 at <i>Eco</i> RI cleavage sites	This paper
SM60 (pEL1)	<i>S. pyogenes</i>	SM60 transductant containing plasmid pEL1 (MLS resistant; 21 Md; formerly ERL1)	6, 22

^a Abbreviations: Lm, lincomycin; Em, erythromycin; Cm, chloramphenicol.

ity tests, and SB was also used for growing cells to be labeled with radioactive thymidine. Cultures of *Streptococcus sanguis* for use in transformation experiments were grown in media identical to those used by LeBlanc and Hassell (18).

Determination of growth and inducibility of resistance. Overnight broth cultures of the test strains were diluted 1:5 with SB or SB containing 0.05 µg of either erythromycin (Sigma) or lincomycin (Upjohn) per ml and grown for 3 h at 37°C to about 10⁹ colony-forming units (CFU)/ml. The resultant cultures were washed and diluted 10³-fold with SB, and 0.1-ml portions were inoculated into tubes of 1 ml of SB with serial doubling concentrations of erythromycin or lincomycin. After incubation at 37°C for 24 h, appropriate dilutions were spread on antibiotic-free SA plates. In cases where antibiotic that carried over onto the plates during dilution could be expected to influence the CFU, the cells were first centrifuged, washed, and suspended in phosphate buffer before plating. Plates were incubated for 48 h at 37°C, and MIC values were determined from the antibiotic concentration in the last tube with no visible growth.

Isolation of extrachromosomal DNA. *S. pyogenes* cells were labeled with 10 µCi of [³H]thymidine (10 Ci/mmol; ICN Pharmaceuticals) or 1 µCi of [¹⁴C]thymidine (43 mCi/mmol; New England Nuclear) per ml. DNA was extracted from 25 or 50 ml of stationary-phase cells by the phage-associated lysis-pronase-dodecyl sulfate procedure and subjected to dye-CsCl-buoyant density-gradient centrifugation as previously reported (22). Challis cells were grown in the presence of 20 mM DL-threonine before being lysed by the lysozyme-pronase-dodecyl sulfate method as described by LeBlanc and Hassell (18). *Staphylococcus aureus* cells were lysed by lysostaphin (Schwarz/Mann), and covalently closed circular (CCC) DNA was isolated as described by Stiffler et al. (26). For extracting large quantities of plasmid DNA from Chal-

lis strains, the alkali denaturation-neutralization procedure of Currier and Nester (11) was used with modification (5). Fractions of the density gradients comprising the desired DNA peaks were pooled, ethidium bromide was removed by isopropanol extraction, and the DNA was dialyzed against TES buffer [30 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 8.0, 5 mM ethylenediaminetetraacetate (EDTA), 50 mM NaCl]. DNA concentrations were estimated from absorbance readings at 260 nm.

Sucrose-gradient centrifugation. Neutral 5 to 20% sucrose gradients were prepared, centrifuged (Beckman L-2, SW50.1 rotor, 36,000 rpm, 2 h, 20°C), and processed essentially as previously described (22). Alkaline 5 to 20% sucrose gradients were prepared containing 1 M NaCl, 0.3 M NaOH, and 2 mM EDTA. DNA samples were predenatured for 10 min at room temperature before being layered on top of the gradients and centrifuged at 46,000 rpm for 30 min at 20°C. All solutions used for preparing the gradients were filter sterilized, and sucrose solutions were autoclaved. Plasmid ColE1, whose CCC form was assigned an S value of 23(8), was labeled with [¹⁴C]thymidine as the reference DNA.

Transformation. For transforming *S. sanguis* Challis with the total cell DNA from zonal resistance strains, DNA was prepared by a phenol method (22) and dissolved in sterile 25 mM sodium phosphate buffer, pH 7.5, at about 500 µg/ml. Competent Challis cells prepared by the method of LeBlanc and Hassell (18) by growing a 1:200-diluted overnight culture in transformation medium for 90 min at 37°C were incubated for 20 min with high concentrations of DNA (~30 µg/ml). Transformation was terminated by addition of deoxyribonuclease (100 µg of beef pancreas deoxyribonuclease I per ml; Sigma), and incubation was continued for 3 h before diluted samples were plated on brain heart infusion agar (Difco) for determining the recipient cell titer and on the same medium

containing 0.5 μg of erythromycin per ml for selecting transformants. Results were recorded after incubating the plates for 72 h at 35°C.

For transforming strain Challis with native or enzymatically treated plasmid DNA, 1-ml samples of the competent culture were exposed to 20- to 250- μl volumes of 10 mM Tris-hydrochloride, pH 7.5, containing 1 to 2 μg of DNA. The transformation mixtures were incubated for 150 min at 37°C and then plated on SA for making viable counts and on the same medium containing 10 μg of erythromycin or chloramphenicol, or both, for selecting transformants. After incubation for 48 h at 37°C, transformant colonies to be studied further were isolated and purified by two repeated single-colony isolations.

Enzymatic treatment of plasmid DNA and agarose gel electrophoresis. Restriction endonucleases and T4 polynucleotide ligase were prepared in Jena, GDR, according to established procedures (15, 25). Plasmid DNA samples (0.5 to 1 μg) contained in 10 to 50 μl of the appropriate buffers (15) were exposed to 2 to 5 U of a restriction endonuclease and incubated at 37°C for 1 to 3 h, depending on the enzyme employed. When digestions by two endonucleases were performed, the enzyme requiring the higher salt concentration was used last. The reactions were terminated by the addition of 0.1 volume of a stop solution containing 20% (vol/vol) glycerol, 200 mM EDTA, and 0.01% bromophenol blue. Plasmid DNA fragments were resolved on 1 or 1.2% agarose (Sigma) vertical slab gels (180 by 145 by 2.5 mm) (27) by electrophoresing for 12 to 16 h at 30 V. The gels were stained in aqueous ethidium bromide solution (1 $\mu\text{g}/\text{ml}$), destained in water, and visualized under short-wave-length ultraviolet light. Size estimates of the plasmid fragments were based on the migration rate relative to endonuclease-generated fragments of λ (24), pBR322, and pBR325 DNAs (7, 28), assuming a logarithmic relationship between molecular mass and electrophoretic mobility and an average mass of 641 daltons for a nucleotide pair.

For ligation of *Eco*RI-cleaved plasmid DNAs, the endonuclease reactions were stopped by heating at 65°C for 5 min, and equimolar mixtures of the appropriate cleaved DNAs were treated with DNA ligase as described (5). The ligated preparations (1 to 2 μg of DNA in 250 μl) were dialyzed against 10 mM Tris-hydrochloride, pH 7.5, and used to transform the Challis strain as described above. The experiment, involving the splicing together of pSM10 and pC221, was carried out in Jena after having been reviewed and approved by the Staatliche Hygieneinspektion.

Electron microscopy. Electron microscopy of plasmid DNA was carried out by the aqueous technique of Davis et al. (12), using endonuclease-cleaved λ DNA fragments as molecular weight standards.

RESULTS

Inducible and constitutive expression of resistance in zonal resistance strains. On solid medium, zonal resistance strains grew as a zone within a range of high lincomycin concentrations while being inhibited at intermediate concentrations of this drug (Fig. 1). Substrains

FIG. 1. Demonstration of the zone phenomenon on solid medium. An SA plate was flooded with 2 ml of a 1:10-diluted overnight culture of strain 6517, the excess fluid was removed, and holes in the agar were filled with 500 μg (left) or 1,000 μg (right) of lincomycin. The plate was incubated overnight at 37°C before photography.

from single-colony isolates of cultures grown within the zonal range continued to exhibit the zonal resistance growth pattern when retested. Thus, strain heterogeneity or mutant growth could not account for the zonal resistance phenomenon. Figure 2A to C illustrates the growth response of zonal resistance strain 10416 to erythromycin and lincomycin after pregrowth in antibiotic-free broth or broth containing subinhibitory concentrations of either drug. Pregrowing 10416 in erythromycin induced growth in intermediate lincomycin concentrations and also increased the MIC of erythromycin at least 10-fold (Fig. 2C). The continued presence of erythromycin was not required for maintaining the induced state for growth in intermediate lincomycin concentrations. However, lincomycin at low concentrations did not induce resistance to intermediate lincomycin concentrations and also failed to increase the MIC of erythromycin (Fig. 2B). Strain 10416 thus showed erythromycin-inducible resistance to lincomycin and erythromycin and the ability of uninduced cells to grow in the zonal range of lincomycin might reflect in situ inducibility by zonal, and only zonal, concentrations of lincomycin. Cells taken from cultures grown at the optimal zonal lincomycin concentration (125 $\mu\text{g}/\text{ml}$) and immediately used as inoculum for the tube dilution test with lincomycin again gave rise to the zone phenomenon, suggesting that the putative lincomycin-induced state of resistance could not be sustained in the absence of zonal lincomycin levels.

Attempts were then made to isolate constitutive mutants. Mutant strain SM10417 (Fig. 2D to F) was derived from one of several colonies that arose after plating an uninduced overnight SB culture of 10416 on SA containing an intermediate concentration of lincomycin (4 $\mu\text{g}/\text{ml}$).

FIG. 2. Colony-forming ability of zonal resistance strain 10416 and derived constitutive mutants. Cultures were pregrown for 3 h in SB or SB supplemented with 0.05 μg of either lincomycin or erythromycin per ml. After washing, 10^5 CFU were inoculated into 1-ml SB tubes containing lincomycin or erythromycin in the concentration range shown in the abscissa. The cultures were incubated for 24 h before being plated on SA for the estimation of growth. Cultures grown in antibiotic-free SB contained about 10^9 CFU/ml. (A) Strain 10416 pregrown in plain SB; (B) 10416 pregrown in SB with 0.05 μg of lincomycin per ml; (C) 10416 pregrown in SB with 0.05 μg of erythromycin per ml; (D) SM10417 pregrown in plain SB; (E) SM10417 pregrown in SB with 0.05 μg of lincomycin per ml; (F) SM10417 pregrown in SB with 0.05 μg of erythromycin per ml; (G) SM10418 pregrown in plain SB; (H) SM10418 pregrown in SB with 0.05 μg of lincomycin per ml; (I) SM10418 pregrown in SB with 0.05 μg of erythromycin per ml; (J) SM10419 pregrown in plain SB; (K) SM10419 pregrown in SB with 0.05 μg of lincomycin per ml; (L) SM10419 pregrown in SB with 0.05 μg of erythromycin per ml.

SM10417 had lost the zonal pattern of growth in lincomycin and expressed resistance to intermediate lincomycin concentrations in the absence of pregrowth in erythromycin. Constitutive expression of resistance was restricted to lincomycin, however, since SM10417 remained erythromycin inducibly resistant to erythromycin. Strain SM10418 (Fig. 2G to I) was selected by plating 10416 on SA containing an inhibitory concentration of erythromycin (10 μ g/ml). This strain did not require induction by erythromycin to express high-level erythromycin resistance (MIC = 1 mg/ml), but remained zonally resistant to lincomycin in the uninduced state. Again, the zonal growth in lincomycin could be eliminated by prior erythromycin induction. Finally, strain SM10419 (Fig. 2J to L) was selected as a second-step mutant by plating SM10417 on SA containing 100 μ g of erythromycin per ml. SM10419 expressed constitutive resistance to both erythromycin and lincomycin, with the MICs of the two antibiotics being about 500 μ g/ml. This mutant analysis provided conclusive evidence that resistance to lincomycin and erythromycin in zonal resistance strain 10416 is of the inducible type and that constitutive resistance is expressed only to the antibiotic used for selection. Generalized constitutive resistance to both intermediate concentrations of lincomycin and high concentrations of erythromycin required two mutational events in a stepwise manner. In general, the resistance levels expressed in the constitutive state were somewhat higher than the corresponding levels attained by erythromycin induction (Fig. 2), indicating that the inducing ability of the drug was less than complete.

Although studied in less detail, investigation of the other zonal resistance strains included in this work (Table 1) confirmed the basic characteristics of the zone phenomenon established for strain 10416. A special feature of SM6518 was that acquisition of constitutive erythromycin resistance (MIC = 1 mg/ml) was accompanied by increased sensitivity to zonal lincomycin concentrations in the uninduced state (Fig. 3), so that in liquid medium, 48 h of incubation was necessary for the zone phenomenon to appear. Erythromycin induction, however, led to the same level of lincomycin resistance as shown by the induced wild type (MIC = 500 μ g/ml). Awareness of the possible existence of such strains may be helpful in assessing antibiotic susceptibility patterns in the clinical microbiology laboratory.

Plasmid DNA related to zonal resistance. All of the zonal resistance strains and their constitutive mutants listed in Table 1 harbored extrachromosomal DNA as judged by the for-

FIG. 3. Demonstration of increased sensitivity of SM6518 to zonal lincomycin concentrations and distortion of the lincomycin inhibition zone by inducing erythromycin concentrations. The holes were filled with lincomycin (1,000 μ g/ml; left) or erythromycin (10 μ g/ml; right). After incubation for 24 h at 37°C, the zone phenomenon was barely visible, but lincomycin resistance remained erythromycin inducible.

mation of a satellite DNA peak (~2% of total DNA) after ethidium bromide-CsCl density-gradient centrifugation of their total DNA. One troublesome aspect of these experiments was our inability to demonstrate the presence of CCC plasmid molecules after sedimenting the satellite DNA through neutral and alkaline sucrose gradients. This suggests that supercoiled DNA present in the zonal resistance strains could not be stably maintained in this form subsequent to dye-CsCl density-gradient centrifugation. Instability of CCC DNA extracted from streptococci has also been observed by others (18). Preliminary investigation of the satellite DNA from SM10419 by electron microscopy revealed the presence, among a majority of linear molecules, of only a minority component of open circular molecules of at least three size classes (A. E. Franke, personal communication). The extensive conversion of the satellite DNA to degraded, linear molecules, which are ineffective in transformation of the Challis strain (5), may explain our failure to obtain Em^r transformants with the satellite DNA from SM10419.

To identify a stable plasmid DNA species related to drug resistance, strain Challis was therefore used as the recipient for transformation to erythromycin resistance with unfractionated DNA from SM10419. When selection was made with 0.5 μ g of erythromycin per ml, 4×10^7 CFU of a competent culture exposed to 58 μ g of SM10419 DNA yielded 15 transformants, one of which was further investigated after having been subcultured fortnightly for 9 months on nonselective medium. This transformant behaved like the donor strain in exhibiting constitutive resistance to both erythromycin and lincomycin, with MICs of 1,000 and 250 μ g/ml, respectively. Its satellite DNA, amounting to 16 to 28% of the total DNA, contained a single,

alkali-stable plasmid species, pSM10419, whose CCC form and open circular form sedimented at, respectively, 39S and 26S in neutral sucrose gradients. A sedimentation coefficient of 39S for CCC DNA corresponds to a molecular mass of 14.1 megadaltons (Md) (3) which approximated the size of the plasmid as determined by summing the molecular masses of its restriction endonuclease cleavage fragments. Thus, *Hind*III digestion yielded 10 fragments ranging in size from 2.37 to 0.54 Md (Fig. 4). Of these, *Hind*III-BB', -EE', and -FF' were pairs of identical fragments resulting from long, nontandem inverted repeats as detected by electron microscopy of self-annealed molecules after denaturation and renaturation of plasmid DNA (6). *Eco*RI digestion of pSM10419 generated two fragments (7.8 and 6.7 Md), with cleavage occurring in *Hind*III-BB' (1.95 Md) to yield subfragments of 1.35 and 0.65 Md. On the basis of the molecular mass of the plasmid (14.9 Md, as determined by *Hind*III digestion), the size of the streptococcal chromosome (1,270 Md) (2), and the proportion of plasmid DNA, it may be calculated that pSM10419 is a multicopy plasmid with roughly 15 to 20 copies per *Challis* chromosome equivalent.

Characterization of pSM10, a deletion derivative of pSM10419. To derive from pSM10419 a plasmid of smaller size and with a reduced number of restriction endonuclease sites, we used an approach similar to that taken previously for reducing the size of another (MLS resistance) plasmid, pSM19035 (5). After cleaving pSM10419 with *Eco*RI and randomly rejoining the fragments with DNA ligase, the ligation mixture was used for transforming strain *Challis* to erythromycin resistance. Two transformants were chosen for further study. Of these, one carried a plasmid identical to pSM10419 as

judged by *Hind*III analysis. The other transformant harbored a plasmid, designated pSM10, with a mass of 5.4 Md as determined by agarose gel electrophoresis of native and endonuclease-cleaved DNA (Table 2). Of the three *Hind*III cleavage fragments of pSM10, *Hind*III-B and *Hind*III-C corresponded, respectively, to single complements of *Hind*III-BB' and *Hind*III-EE' of pSM10419, whereas the new *Hind*III-A fragment must represent the fusion product of non-deleted stretches of DNA flanking the *Hind*III-B-E/B'-E' segment of the parental plasmid. Single or double digestion of pSM10 with additional restriction endonucleases yielded the data listed in Table 2, which were used to construct the cleavage site map (see Fig. 6 below).

Construction of a hybrid plasmid between pSM10 and pC221. pC221 has single sites for *Eco*RI and *Hind*III (0.14 Md apart), into both of which DNA can be cloned without disrupting either plasmid replication or chloramphenicol resistance (33). Native pC221 DNA transformed competent strain *Challis* cultures (4×10^7 CFU/ml) to chloramphenicol resistance at a frequency of 458 transformants per 1 μ g of DNA, showing that the chloramphenicol acetyltransferase gene of the staphylococcal plasmid can be expressed in *S. sanguis*. To test whether or not DNA insertion into the *Eco*RI site of pSM10 inactivates expression of erythromycin resistance, pC221 DNA (1 μ g) and pSM10 DNA (1 μ g) were digested with *Eco*RI, mixed, and treated with DNA ligase. The ligation mixture was used to transform strain *Challis*. By using selection for erythromycin resistance alone, 4×10^7 CFU of a competent culture exposed to 2 μ g of ligated DNA yielded about 1.2×10^4 Em^r colonies, 96 of which were simultaneously Cm^r, as tested by replica plating. Selection for erythromycin plus chloramphenicol resistance under otherwise identical conditions yielded two doubly resistant colonies. No colonies were obtained if either the cells or the DNA was omitted. Plasmid DNA was extracted from one doubly resistant clone that appeared on a plate containing erythromycin and subjected to electron microscopy and genetic and restriction endonuclease analyses.

Contour length measurements showed that this clone carried a single plasmid, referred to as pSM10221, whose size was the sum of the sizes of the parental plasmids, namely, $4.25 \pm 0.18 \mu$ m, corresponding to 8.3 Md (12 molecules measured). *Eco*RI cleaved pSM10221 into two fragments (5.4 and 2.9 Md) having sizes that matched those of the *Eco*RI-cleaved parental DNAs (Fig. 5). As expected, *Hind*III digestion yielded four fragments (4.11, 2.16, 1.26, and 0.79 Md) (Fig. 5) whose sizes were consistent with

FIG. 4. Agarose slab gel electrophoretogram of pSM10419 digested with *Hind*III. Fragment sizes are given in megadaltons.

TABLE 2. Complete fragments generated by digestion of pSM10 with restriction endonucleases

Frag- ment	Fragment size (Md)								
	<i>Eco</i> RI	<i>Hpa</i> I	<i>Kpn</i> I	<i>Eco</i> RI + <i>Hpa</i> I	<i>Eco</i> RI + <i>Kpn</i> I	<i>Hind</i> III	<i>Hind</i> III + <i>Hpa</i> I	<i>Hind</i> III + <i>Kpn</i> I	<i>Hind</i> III + <i>Eco</i> RI
A	5.40	5.40	5.40	3.50	3.45	2.16	2.16	2.16	2.16
B				1.80	1.85	1.95	1.95	1.95	1.35
C						1.26	1.20	1.25	1.26
D							0.10 ^a	0.05 ^a	0.65
Total	5.40	5.40	5.40	5.30	5.30	5.37	5.41	5.41	5.42

^a Deduced sizes of fragments undetected by agarose gel electrophoresis.

FIG. 5. Agarose slab gel electrophoretogram of pSM10221 and standard DNAs digested with endonucleases. Lane 1, pSM10221/*Hind*III; lane 2, pSM10221/*Eco*RI; lane 3, λ /*Eco*RI (marker sizes are 13.7, 4.74, 3.73, 3.48, 3.02, and 2.13 Md); lane 4, λ /*Hind*III (marker sizes are 14.4, 6.19, 4.16, 2.80, 1.57, 1.39, and 0.31 Md); lane 5, pBR322/*Hind*III + *Hind*III (partial digest; marker sizes are 2.80, 2.36, 2.09, 0.71, 0.40, and 0.31 Md). pSM10221 *Hind*III fragments are given in megadaltons.

the doubly resistant transformant carrying a composite plasmid rather than being transformed with separate molecules of pSM10 and PC221; although the 2.16- and 1.26-Md *Hind*III fragments of the plasmid chimera corresponded, respectively, to *Hind*III-A and *Hind*III-C of

pSM10, the other two fragments must represent recombinant DNA generated by fusion of the two plasmids within their *Eco*RI sites. The available *Eco*RI and *Hind*III cleavage site maps of the parental plasmids together with the data of Fig. 5 allowed the orientation of the constituent plasmids in the chimera, as shown in Fig. 6.

pSM10221 DNA, when used in a second round of transformation, could transform the Challis strain to both erythromycin and chloramphenicol resistance, with the two markers transferring at the same efficiency and exhibiting complete linkage. Thus, more than 1,000 transformants selected for erythromycin resistance were also Cm^r and, similarly, all Cm^r transformants tested (1,620) were also Em^r . Furthermore, the observed genotypes of the strains carrying the parental or recombinant plasmids were confirmed by the MIC values to erythromycin and chloramphenicol, which showed that the hybrid plasmid conferred resistance levels to the two drugs identical to those determined by the respective constituent plasmids (Table 3).

DISCUSSION

The induction and mutation studies establish that zonal resistance to lincomycin is inducible. The simplest explanation for the zone phenomenon therefore is that lincomycin concentrations of 60 to 200 $\mu\text{g}/\text{ml}$ induce resistance, whereas in lower concentrations, resistance remains repressed. The growth in very low lincomycin concentrations ($<0.1 \mu\text{g}/\text{ml}$) is due to intrinsic resistance to the antibiotic and therefore has nothing to do with the particular phenotype. In contrast to lincomycin, erythromycin can act as an inducer of resistance only at low, subinhibitory concentrations, whereas it inhibits protein synthesis at higher concentrations and is therefore unable to induce cells in situ. The inducing properties of the two drugs thus appear to be different in strains showing MLS and zonal resistance. In the former case, subinhibitory concentrations of both can induce cross-resistance to high concentrations of either, and in situ

FIG. 6. Restriction endonuclease cleavage site maps of pC221, pSM10, and the plasmid chimera pSM10221, constructed by fusion of the former two at EcoRI sites. The maps are calibrated in megadaltons, and the arbitrary origins of the circular coordinate systems are the EcoRI sites. In pSM10221 the thin and heavy lines correspond, respectively, to pC221 and pSM10. The position of the HindIII site in pC221 is that determined by Wilson and Baldwin (33).

TABLE 3. Resistance levels of *S. aureus* 8325-4 and *S. sanguis* Challis carrying the indicated plasmids

Strain	MIC (μ g/ml)	
	Erythromycin	Chloramphenicol
Challis wild type	≤ 0.03	3.0
8325-4 (pC221)	0.125	25.0
Challis (pC221)	≤ 0.03	25.0
Challis (pSM10)	$> 1,000$	3.0
Challis (pSM10221)	$> 1,000$	25.0

inducibility seems possible at high concentrations of both antibiotics (17, 20). In staphylococci, where the MLS resistance phenotype is also known, erythromycin is among the most effective inducers, and lincomycin has no inducing activity (1, 32). It has been stated, however, that the usual specificities of induction seen in wild-type strains can be reversed by mutation (29), indicating that induction of MLS resistance is both drug and strain specific. Moreover, chemical modification of erythromycin can impair its inducing activity without affecting its inhibitory properties (1). The action of lincomycin in zonal resistance streptococci can be considered a phenomenon in which the inducing and inhibitory properties of the drug are reversibly dissociated in a concentration-dependent manner. Although it is unknown if MLS and zonal resistances are qualitatively of the same biochemical basis (i.e., methylation of 23S ribosomal RNA), the plasmids associated with them share much sequence homology (Boitsov, Golubkov, Iontova, Reichardt, Totolian, and Malke, unpublished data), suggesting that the biochemical mechanisms are similar and differ only quantitatively in the inducible control systems.

The existence of generalized and partially constitutive mutants of zonal resistance strains resembles the situation found in isolates of *S. aureus* with MLS resistance (32). In this species, however, it is not known whether more than one mutation is required for generalized constitutive expression of resistance. The same is true of MLS-resistant *S. pyogenes* isolates that are constitutively resistant to both erythromycin and lincomycin (17, 20). The fact that, in zonal resistance strains, generalized constitutive resistance requires two mutational events suggests that the biochemical mechanism of resistance to erythromycin and lincomycin involves more than one control system or that the putative repressor of resistance has distinct sites for erythromycin and lincomycin binding. In this connection, it should be mentioned that previous experiments failed to demonstrate separate genes for erythromycin and lincomycin in *S. pyogenes* strains with MLS resistance (23). Unfortunately, corresponding data are not available for zonal resistance strains, so that at the present state of knowledge, it seems premature to propose a model concerning the mechanism of regulation of resistance.

The genetic and physical data presented in this paper provide evidence that zonal resistance to lincomycin is related to plasmid pSM10419. The difficulties encountered in extracting CCC DNA from the original strains seem to be related to some peculiarities of the specific plasmid pool in zonal resistance strains because the pEL1 plasmid (22) introduced into strain 10416 by transduction could be reisolated very easily (Malke, unpublished results). Since *S. sanguis* strain Challis is well known to inflict gross alterations on plasmids during uptake or replication

(5), the ancestor of pSM10419 may differ structurally from the element obtained after transformation. Although we failed to trace pSM10419 back to a physically defined ancestor, the plasmid proved to be useful for deriving a plasmid of reduced size, pSM10, which is suitable as a cloning vehicle (Malke, Burman, and Holm, manuscript in preparation). pSM10 is a relatively small multicopy plasmid and contains unique cleavage sites for several endonucleases, some of which generate cohesive termini. Insertion into the single *EcoRI* site does not inactivate erythromycin resistance. Since the insert used is a functional replicon, temperature-sensitive mutants of either one of the constituent plasmids are required to learn which of the parental replicon functions are used by the hybrid plasmid. The pSM10221 chimera represents another example of heterospecific gene transfer and expression in procaryotes and may be used for developing a doubly selective cloning vehicle by searching for insertional inactivation (30) of drug resistance genes.

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