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Deoxyribonucleic Acid Sequence Common to Staphylococcal and Streptococcal Plasmids Which Specify Erythromycin Resistance

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Plasmids from erythromycin-resistant *Staphylococcus aureus*, *Streptococcus sanguis*, and *Streptococcus faecalis* show deoxyribonucleic acid sequence homology. The homologous sequences can be localized to specific restriction endonuclease fragments, which in the case of *S. aureus* plasmid pI258 involves a single fragment from either *Eco*RI or *Hin*dIII digests known to contain the erythromycin resistance determinant. Complementary ribonucleic acid probes prepared from *S. aureus* plasmid pI258 and *S. sanguis* plasmid pAM77 also hybridize to specific fragments in restriction endonuclease digests of deoxyribonucleic acid from erythromycin-resistant *Streptococcus pyogenes* and *Streptococcus pneumoniae*. These studies suggest a common origin for a class of erythromycin resistance determinants in unrelated strains of pathogenic bacteria for which exchange of genetic material has not been demonstrated.

Erythromycin resistance in clinical isolates of pathogenic bacteria is generally associated with a chemical alteration of ribosome structure that, in addition, specifies coresistance to macrolide, as well as to lincosamide, and streptogramin B-type (MLS) antibiotics (29). A specific N^6-dimethylation of adenine in 23S rRNA constitutes the biochemical basis for MLS resistance, and we have recently extended these findings to *Streptomyces erythreus*, the organism used for production of erythromycin (10). Evidence for a common resistance mechanism in organisms not known to exchange genetic material suggests the possibility of DNA sequence homology. We report that such sequence homology can in fact be demonstrated in the comparison of plasmid DNAs from resistant clinical isolates of *Staphylococcus aureus*, *Streptococcus sanguis*, and *Streptococcus faecalis*, which in turn show sequence homology with DNA purified from isolates of resistant *Streptococcus pyogenes* and *Streptococcus pneumoniae*.

MATERIALS AND METHODS

Strains. The strains used and their derivation and relevant properties are listed in Table 1.

Growth of cells. *Bacillus subtilis*, *S. aureus*, and *S. faecalis* strains were grown in medium containing (grams per liter): tryptone, 5; yeast extract, 5; KH_2PO_4, 1; glucose, 2. Streptococci (except *S. faecalis*) were grown in Todd-Hewitt broth.

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DNA preparation. *S. aureus* RN453 cells were disrupted enzymatically with lysostaphin, and plasmid pI258 DNA was prepared as described (23). *B. subtilis* cells (4 g of paste from a 1-liter culture) were suspended in 15 ml of 0.05 M Tris-hydrochloride (pH 8.0)-25% (wt/wt) sucrose-0.05 M Na_2-EDTA. Lysozyme, 10 mg/ml in water (1.5 ml), was added, and the mixture was incubated at 37°C for 15 to 30 min. A 5-ml amount of 5 M NaCl and 2 ml of 10% sodium dodecyl sulfate were added in rapid succession. Cells were allowed to lyse at room temperature for 15 min, held on ice for an additional 30 min, and spun at 20,000 rpm for 15 min. Covalently closed circular (CCC) DNA was purified from the supernatant by dye-CsCl density gradient centrifugation (26). For some preparations, CCC DNA was concentrated by precipitation with polyethylene glycol 6000 (14). CCC DNA from *S. faecalis* was purified by the lysozyme-Sarcosyl-pronase method (4). Total cell DNA from the other streptococci was prepared as for *S. faecalis* except that the preparation was deproteinized by extraction with chloroform-isobutyl alcohol (24:1, vol/vol) immediately after lysis and digestion with pronase as described by Clewell et al. (4).

DNA sequence homology mapping. The method of Southern (27) was used for localization of DNA sequence homology; DNA to be used as "target" was digested with the restriction enzyme designated and fractionated by agarose gel electrophoresis, and the resultant fragments were transferred from the agarose slab to nitrocellulose sheets (type BA-85, Schleicher & Schuell) by the blotting method described.

cRNA probes. Complementary RNA (cRNA) probes were prepared as described previously with *Escherichia coli* RNA polymerase (30), using CCC pI258, pAM77, and beta DNA, respectively, as templates. In homologous combinations of probe and tar-

TABLE 1. *Bacterial strains used in this work*

Strain	Strain designation	Relevant plasmid	Plasmid mol wt ($\times 10^6$)	MLS phenotype[a]	Source (reference)
B. subtilis	BD170(pE194)	pE194	2.3	Inducible	D. Dubnau (30)
B. subtilis	BR151(pAM77)	pAM77	4.5	Inducible	Y. Yagi and D. B. Clewell (33)
S. faecalis	JH2-2β	beta	17.0	Constitutive	V. Hershfield (6, 12)
S. pyogenes	AC-1	pAC-1	17.0	Inducible	D. B. Clewell (3)
S. sanguis	A1	pAM77	4.5	Inducible	D. B. Clewell (33)
S. aureus	RN453	pI258	18.0	Constitutive	R. P. Novick (25)
S. pneumoniae	B1 (type 19A[b])	None known[c]		Inducible	R. Austrian
	B116 (type 19A[b])	None known		Sensitive	R. Austrian
	B363 (type 19A[b])	None known[c]		Inducible	R. Austrian

[a] Determined by disk sensitivity method.
[b] According to the Danish nomenclature (see reference 16).
[c] Resistance determinant may reside in chromosome (see text).

get DNA, hybridization to all DNA bands visualized by fluorescence was found.

Determination of m_2^6A. N^6-dimethyladenine (m_2^6A) in 23S rRNA was determined as described previously (17, 18). Cells were grown in [8-^{3}H]adenine, and after purification of 23S rRNA, the amount of m_2^6A relative to A was determined by depurination with acid and heat, followed by separation of adenine plus methylated adenine bases as a group by column chromatography on Dowex 50 and a final separation of adenine, N^6-methyladenine, and m_2^6A from each other by paper chromatography.

RESULTS

Plasmids pI258 and pAM77, purified as CCC DNA from erythromycin-resistant *S. aureus* and *S. sanguis*, respectively, were tested for sequence homology by using the DNA blot method of Southern (27). Plasmid DNAs were digested with restriction endonuclease *Hin*dIII and fractionated by agarose ethidium bromide gel electrophoresis, and the resultant fractionated digest was transferred to nitrocellulose membrane filters, for use as DNA "target." The respective CCC plasmid DNA preparations were used in parallel as templates for the synthesis of cRNA probes labeled with α-[^{32}P]ATP. After hybridization and autoradiography, the results shown in Fig. 1 were obtained.

The distribution of radioactivity in the homologous hybridization reactions (Fig. 1a and d) coincided with the distribution of fluorescence in the agarose gel fractionation. The two heterologous combinations (Fig. 1b and c) show reciprocal hybridization localized to only a single band in each fractionation. Thus, the site(s) of homology appears to be localized.

We examined the localized homology in pI258 digested with several restriction endonucleases, using pI258 cRNA as homologous probe and pAM77 cRNA as heterologous probe. pI258 DNA preparations digested with restriction endonucleases *Eco*RI, *Hin*dIII, *Sma*I, and *Hae*III, respectively, were used as target in the hybridizations. The results are shown in Fig. 2. In each of the homologous combinations, a pattern of hybridization which paralleled the distribution of fluorescent bands in the gel is seen; in each of the heterologous combinations, hybridization localized primarily to a single band is seen. For *Eco*RI, it is the A-fragment which shows homology with the pAM77 probe; the erythromycin R-determinant has been localized to this A-fragment by deletion mapping studies of Novick et al. (25). For the *Hin*dIII digest of pI258 DNA, hydridization against the heterologous pAM77 cRNA probe showed homology localized to the B-fragment. In recombinant DNA studies with the *Hin*dIII fragments of pI258 DNA, Wilson and Baldwin (31) found that clones obtained by selection for erythromycin resistance contain this B-fragment.

Historically, erythromycin-resistant strains of *S. aureus* were the first MLS-resistant organisms found (2). The phenotype was subsequently reported in streptococci belonging to Lancefield groups A, B, and D (3–9, 13, 15, 19–22, 28, 32); for some of these strains, plasmids associated with the resistant phenotype were reported. One plasmid, beta, found originally in *S. faecalis*, has been the object of intensive genetic and biochemical investigation. Genetic studies by Hershfield (12) and LeBlanc et al. (19, 20) have shown conjugative transmission of erythromycin resistance determinants under laboratory conditions. Yagi et al. (32) have estimated that the extent of sequence homology between erythromycin resistance plasmids of *S. faecalis* DS-5 and *S. pyogenes* AC-1 was approximately 95% based on DNA-DNA hybridization on membrane filters. We therefore included in the hybridization experiments target DNA from other streptococci and probes pre-

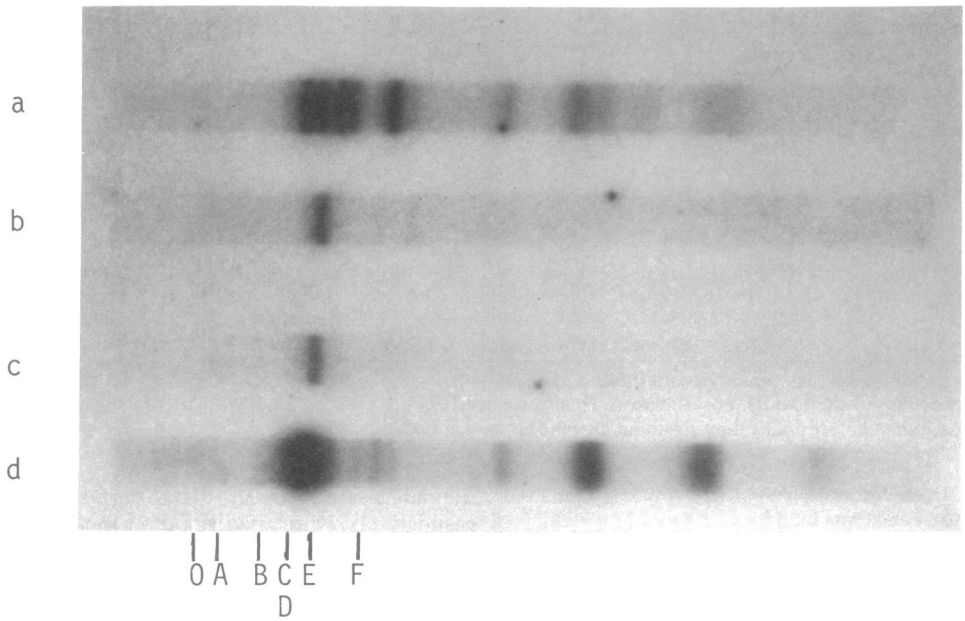


FIG. 1. *DNA sequence homology between plasmid pI258 from S. aureus and pAM77 from S. sanguis. The combinations of target DNA and ^{32}P-labeled cRNA probe, respectively, were: (a) pI258 (HindIII), pI258; (b) pI258 (HindIII), pAM77; (c) pAM77 (HindIII), pI258; (d) pAM77 (HindIII), pAM77. Electrophoretic migration was from left to right beginning at the origin (O). Molecular weight markers, comprising the EcoRI digestion fragments of lambda phage DNA, formed five bands (A, B, C & D, E, and F) as indicated, corresponding to molecular weights of 13.6 × 10^6, 4.7 × 10^6, 3.7 × 10^6, 2.5 × 10^6, and 2.1 × 10^6, respectively.*

pared from pI258 and pAM77 CCC DNA, as well as cRNA probes transcribed from beta plasmid DNA. Autoradiograms of the hybridizations, using homologous combinations of target and probe, are shown in Fig. 3a, f, and i.

The *S. faecalis* and *S. pyogenes Hin*dIII target DNAs show strong similarity when hybridized against beta cRNA (Fig. 3f and i, respectively), with the exception of some high-molecular-weight bands in the *S. faecalis* target DNA which may represent a cryptic plasmid in the preparation. In the heterologous target-probe combinations (Fig. 3b, c; d, e; and g, h) one strongly hybridizing band is seen which corresponds to the second largest major fragment in the *Hin*dIII digest of the plasmid.

In the hybridizations described above, pAM77 cRNA was prepared with pAM77 CCC DNA obtained from transformed *B. subtilis* cells. pAM77 isolation was facilitated in this manner since lysis of *S. sanguis* cells with lysozyme was found to be much less efficient under conditions used for lysis of *B. subtilis*. To determine whether any changes in pAM77 after introduction into *B. subtilis* could account for some of our findings, cRNA transcribed with pAM77 isolated from *B. subtilis* was hybridized against target DNAs consisting of the *Hin*dIII digest of total *S. sanguis* A1 DNA and of CCC pAM77 DNA, both run in parallel. The results of the hybridization (Fig. 4) show essentially indistinguishable patterns consisting of four predominant bands in both samples.

S. pneumoniae resistant to erythromycin and lincomycin was first described by Dixon and Lipinski (8). Recently, erythromycin- and clindamycin-resistant pneumococci have been isolated from patients in South Africa (16). Examination of these newly isolated, multiply resistant strains by disk sensitivity on blood agar reveals the characteristic patterns of erythromycin-induced MLS resistance. Mayer and Ploscowe (Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, D26, p. 36) sought but were unable to find evidence for a plasmid in these strains. In view of the cross-homology found in the experiments reported above, two resistant pneumococcal strains (B1 and B363) were examined by hybridizing pI258 and pAM77 cRNA's against pneumococcal DNAs used as target. Erythromycin-sensitive strain B116 was included as a control in this comparison. By disk test, both B1 and B363 showed the erythromycin-inducible MLS resistance phenotype (data not shown). The re-

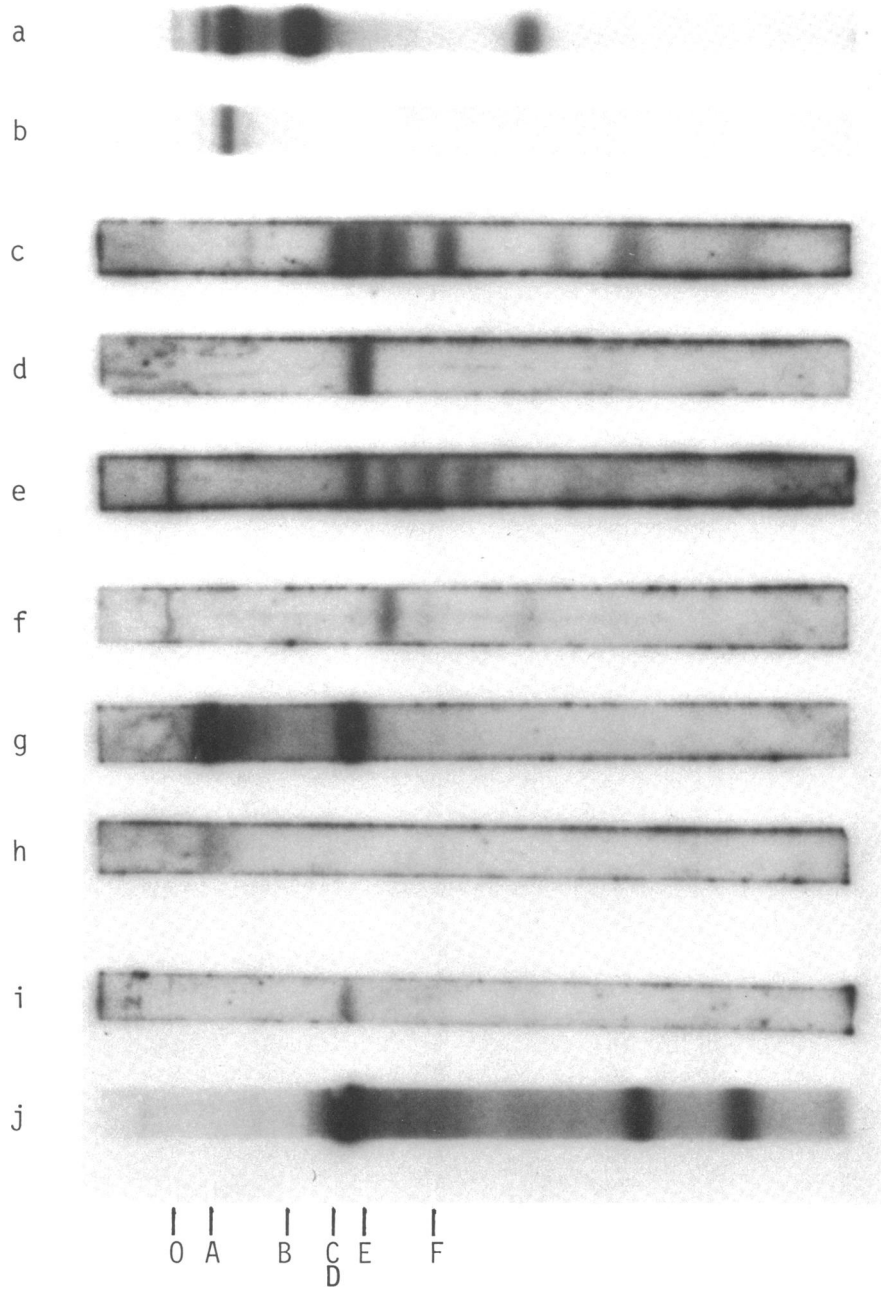


FIG. 2. *Localization of pAM77-pI258 DNA sequence homology in specific fragments obtained by digestion of pI258 with four restriction endonucleases. The respective combinations of target DNA and ^{32}P-labeled cRNA probe were: (a) pI258 (EcoRI), pI258; (b) pI258 (EcoRI), pAM77; (c) pI258 (HindIII), pI258; (d) pI258 (HindIII), pAM77; (e) pI258 (HaeIII), pI258; (f) pI258 (HaeIII), pAM77; (g) pI258 (Sma), pI258; (h) pI258 (Sma), pAM77; (i) pAM77 (HindIII), pI258; (j) pAM77 (HindIII), pAM77. (See legend to Fig. 1 regarding molecular weight markers.)*

sults (Fig. 5) suggest that the homology indeed exists for both probes to DNA of high molecular weight in the *Hin*dIII digest and that this homology can be localized to discrete smaller fragments in the *Alu*I digest. Since positive hybridization to both the pAM77 and pI258 probes was localized to a fragment which in pI258 is known to carry erythromycin resistance, we infer that

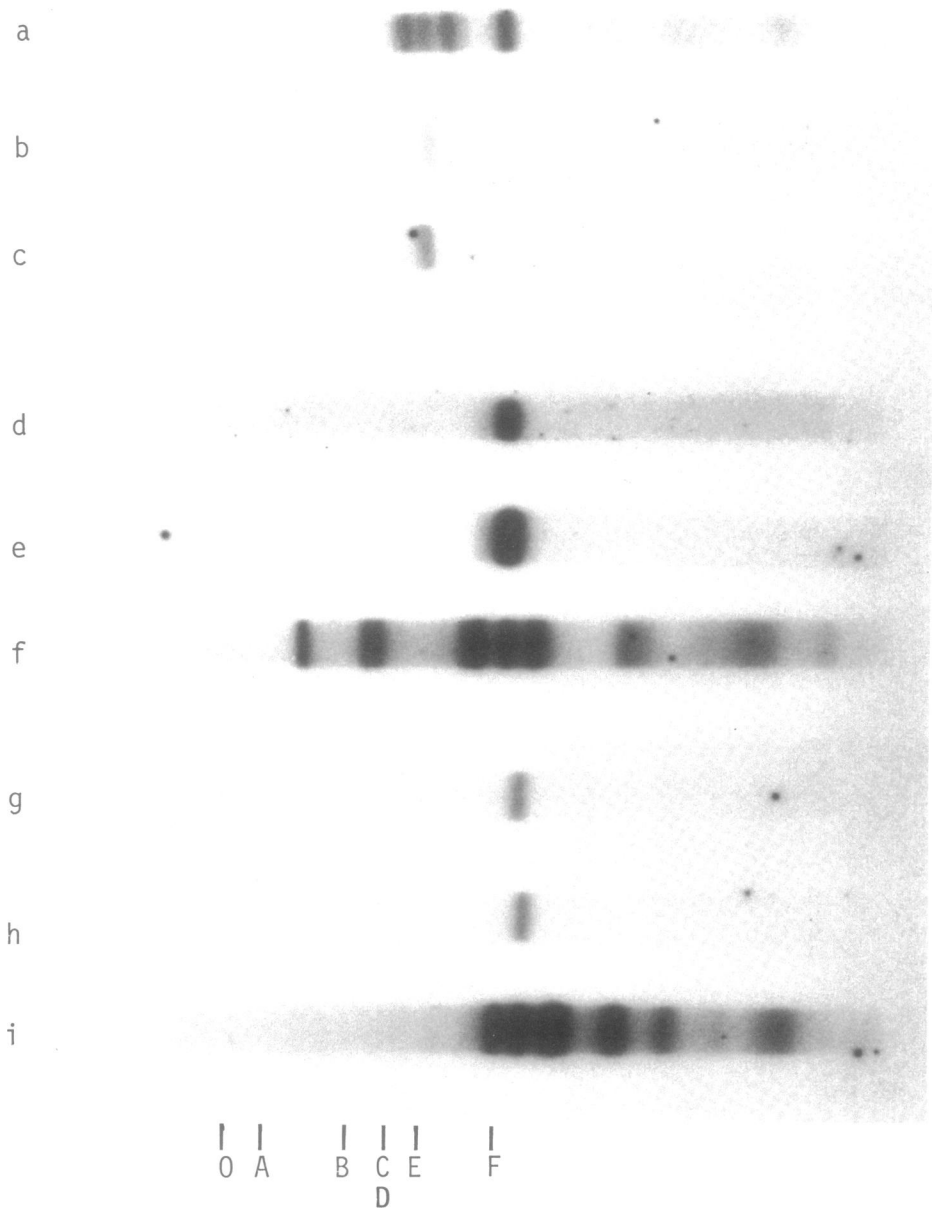


FIG. 3. *Localization of pI258 beta and pI258 S. pyogenes DNA sequence homology in specific fragments obtained by digestion of beta and S. pyogenes DNA with HindIII. pI258 DNA was included in the comparison both as target and as template for cRNA probe preparation. The respective combinations of target DNA and ^{32}P-labeled cRNA probe were: (a) pI258 (HindIII), pI258; (b) pI258 (HindIII), pAM77; (c) pI258 (HindIII), beta; (d) beta (HindIII), pI258; (e) beta (HindIII), pAM77; (f) beta (HindIII), beta; (g) S. pyogenes AC-1 (HindIII), pI258; (h) S. pyogenes AC-1 (HindIII), pAM77; (i) S. pyogenes AC-1 (HindIII), beta. (See legend to Fig. 1 regarding molecular weight markers.)*

it is the erythromycin resistance determinant in the pneumococcal DNA that is likewise responsible for the hybridization.

To date, we have documented the association of MLS resistance with the presence of m_2^6A in rRNA from both *S. aureus* (17, 18) and *S. erythreus* (11). The relative amounts of adenine and two of its methylated derivatives in 23S

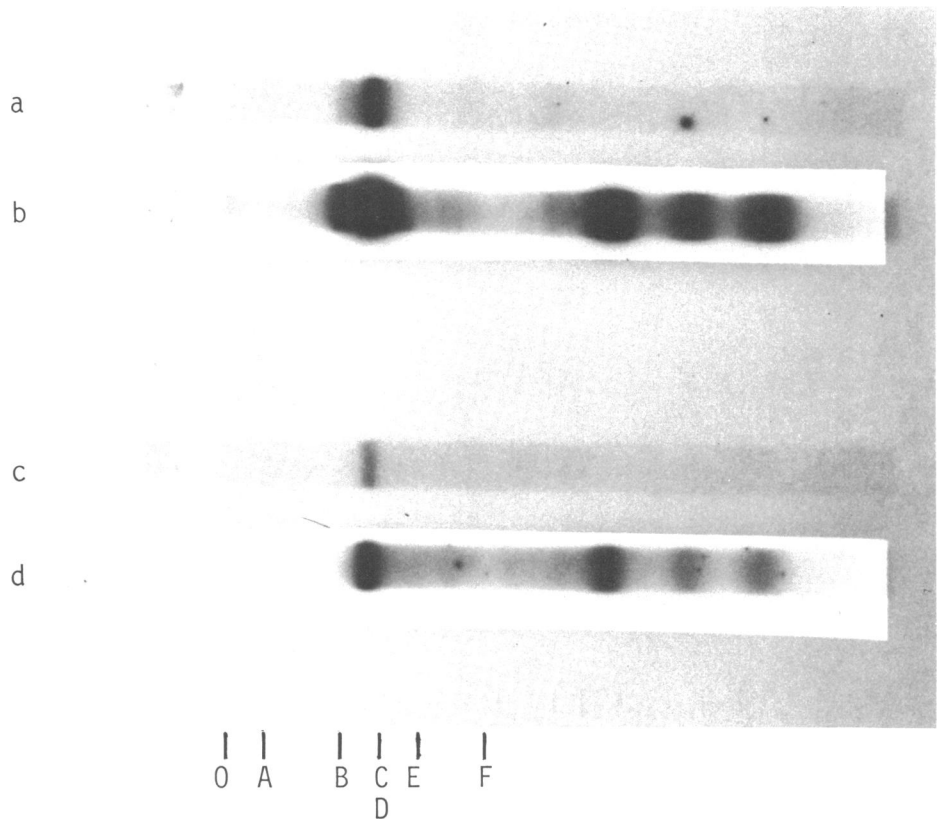


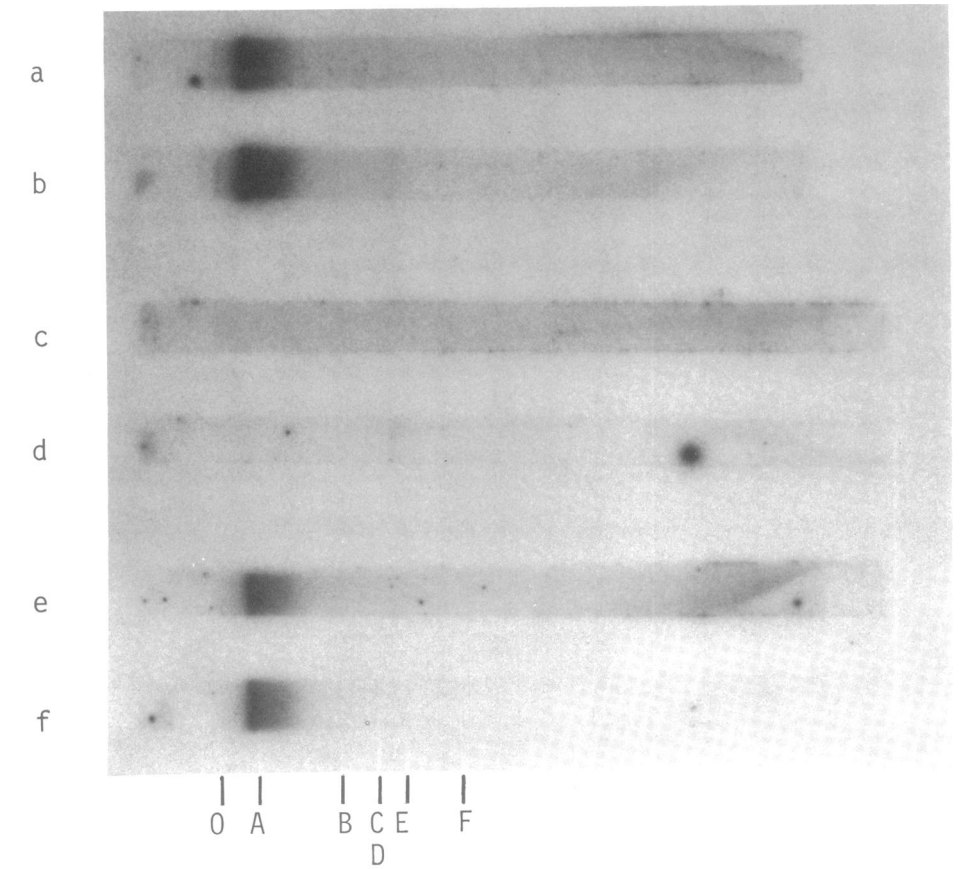
FIG. 4. *Comparison of sequence homology of pAM77 prepared in B. subtilis with pAM77 in the S. sanguis DNA preparation. Total S. sanguis A1 DNA containing pAM77 and pAM77 DNA prepared as CCC DNA from transformed B. subtilis cells digested with HindIII were used as drivers. The respective combinations of target DNA and ^{32}P-labeled cRNA probe were: (a) pAM77 (HindIII), pI258; (b) pAM77 (HindIII), pAM77; (c) S. sanguis A1 DNA (HindIII), pI258; (d) S. sanguis A1 DNA (HindIII), pAM77. (See legend to Fig. 1 regarding molecular weight markers.)*

rRNA from *S. faecalis* resistant strain DS-5 and sensitive strain DS-5C1 (derived from DS-5 by treatment with acridine orange [4]) have shown that the RNA sample from erythromycin-sensitive *S. faecalis* contained 0.05% relative to 1.5×10^5 cpm present in the adenine fraction, whereas the sample from resistant cells contained 0.18% of 2.76×10^5 total cpm found in the adenine fraction. These determinations correspond to 0.64 and 1.44 $m_2{}^6A$ residues per 23S rRNA, assuming that each 23S rRNA contains 800 adenine residues (11).

The results described above are consistent with a model of MLS resistance according to which nucleotide sequences associated with this form of resistance are present in strains from diverse sources not known to exchange genetic material. In particular, homology between *S. aureus* and several streptococcal resistance determinants associated with MLS resistance at the cellular level and $m_2{}^6A$ in 23S rRNA at the subcellular level suggests that the resistance determinant in these strains may have a common origin.

DISCUSSION

The MLS resistance phenotype has so far been described in clinical isolates of *S. aureus* and streptococci (groups A, B, and D) and in *S. erythreus*, the organism used for production of erythromycin. The association of this form of resistance with N^6-dimethylation of adenine in 23S rRNA has been documented previously (14, 15, 27, 28). Additional significant features of MLS resistance have been: (i) identification of plasmids which specify this resistant phenotype, notably, pI258 and pE194 in *S. aureus*, pAM77 in *S. sanguis*, beta in *S. faecalis*, and a beta-like plasmid, pAC-1, in *S. pyogenes*; (ii) transmission of beta from *S. faecalis* to a group F streptococcus by transformation (19), followed by facile conjugative transfer from the new host to *S. sanguis*, *S. mutans*, and *S. salivarius* (20). Moreover, pAM77 can be transformed into *B.*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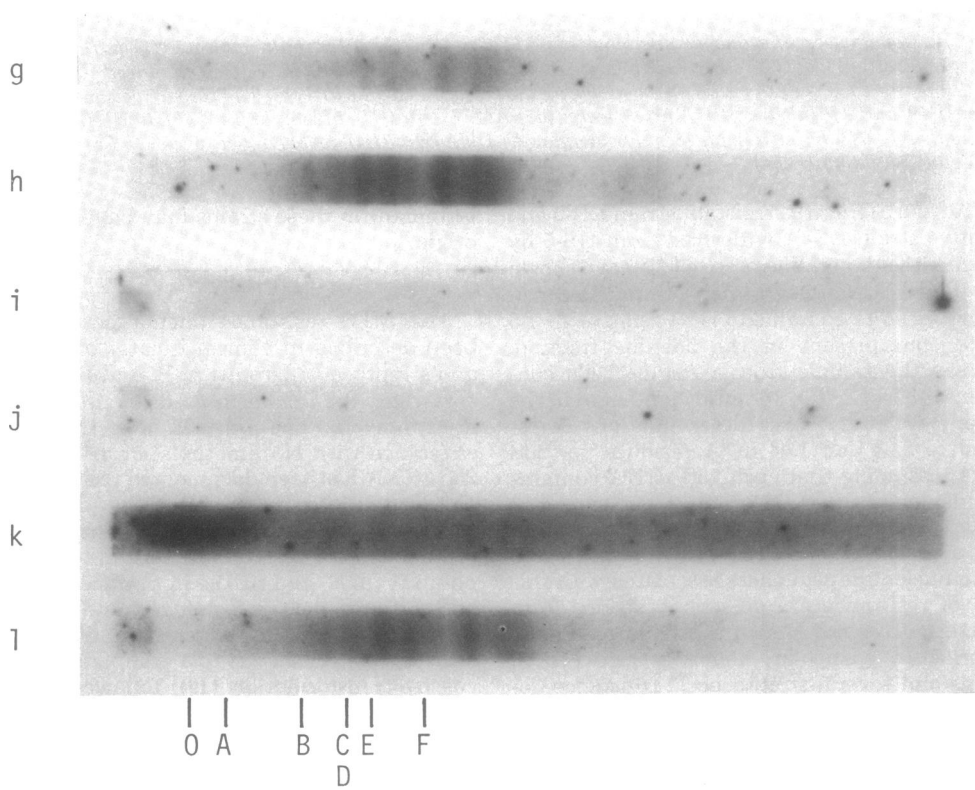
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subtilis (Y. Yagi and D. B. Clewell, personal communication).

The present studies allow us to extend the scope of these findings by demonstrating that the phenomena observed in the laboratory have probably occurred in nature and that a particular erythromycin R-determinant has a widespread distribution based on comparison of cellular phenotypes (antibiotic disk test), alteration of ribosome structure (presence of m_2^6A in 23S rRNA), and, most directly, demonstration of nucleotide sequence homology.

In the present study, we have characterized the probes synthesized from pAM77 and beta plasmids of streptococcal origin by hybridization against *S. aureus* plasmid pI258, about which more is known, especially the localization of the erythromycin resistance determinant in a specific restriction endonuclease digest fragment (Table 2). With these defined probes, we have identified nucleotide sequence homology with *S. pneumoniae*, using total unfractionated DNA from the latter as target. These recently isolated resistant pneumococcal strains are of particular epidemiological interest since they have emerged in recent outbreaks in South Africa caused by multiply resistant strains of *S. pneumoniae* (16). The apparent widespread distribution of erythromycin resistance determinant-associated sequences suggests that a transposon mechanism might be involved. It is of great interest that a transposon, Tn*551*, has been found by Novick and his colleagues (24) in pI258 that flanks the erythromycin resistance determinant.

Additional support for the notion of facile exchange of resistance determinants between staphylococci and streptococci (either directly or indirectly) come from parallel studies by T. White and J. Davies (personal communication), who have found that neomycin phosphotransferases from *S. aureus* and *S. faecalis* cross-react immunologically. More generally, the hypothesis proposed by Benveniste and Davies (1), that *Streptomyces*, which produce antibiotics, also serve as a source of the corresponding resistance determinants, finds support in previous studies from this laboratory on MLS resistance (10), as well as in the present studies insofar as a common genetic basis for resistance determinants in diverse bacterial strains is demonstrated.

TABLE 2. *Summary of positive hybridization demonstrated in this work*

Target DNA (on membrane filter)	Probe[a] (source of CCC DNA for cRNA prep)		
	pI258	pAM77	Beta
pI258	+	++	++
pAM77	++	+	++
Beta	++	++	+
S. pyogenes	+	+	+
S. pneumoniae	+	+	ND

[a] +, Positive hybridization for the probe-target combination specified; ++, positive reciprocal hybridization, namely, that the entry has a symmetrical (off-diagonal) counterpart. ND, Not done.

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FIG. 5. *Homology between S. pneumoniae and plasmids pI258 and pAM77. Total DNA from three strains of S. pneumoniae digested with HindIII and AluI, respectively, were used as target. (Strains B1 and B363 are erythromycin resistant, whereas strain B116 is erythromycin sensitive.) pI258 and pAM77 were used as templates for preparation of cRNA probes. The respective combinations of target DNA and ^{32}P-labeled cRNA probe were: (a) S. pneumoniae B1 (HindIII), pI258; (b) S. pneumoniae B1 (HindIII), pAM77; (c) S. pneumoniae B116 (HindIII), pI258; (d) S. pneumoniae B116 (HindIII), pAM77; (e) S. pneumoniae B363 (HindIII), pI258; (f) S. pneumoniae B363 (HindIII), pAM77; (g) S. pneumoniae B1 (AluI), pI258; (h) S. pneumoniae B1 (AluI), pAM77; (i) S. pneumoniae B116 (AluI), pI258; (j) S. pneumoniae B116 (AluI), pAM77; (k) S. pneumoniae B363 (AluI), pI258; (l) S. pneumoniae B363 (AluI), pAM77. (See legend to Fig. 1 regarding molecular weight markers.)*

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