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Molecular Studies and Possible Relatedness Between R Plasmids from Groups B and D Streptococci

NÉVINE EL-SOLH, D. H. BOUANCHAUD,* THÉA HORODNICEANU, AGNÈS ROUSSEL, AND Y. A. CHABBERT

Service de Bactériologie Médicale, Institut Pasteur, 75724 Paris, France

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Resistance plasmids isolated from *Streptococcus agalactiae* (group B) and *S. faecalis* (group D) have been compared in regard to resistance markers, molecular weight, and DNA-DNA homology. Three of them (pIP501, pIP612, and pIP613) have been found to confer identical (or very similar) resistance patterns (erythromycin, lincomycin, and streptogramin B, respectively) and to have similar molecular weights (19.8×10^6, 22.7×10^6, and 17.6×10^6, respectively) and a high level of DNA-DNA homology in hybridization experiments (90 to 100%). These results are compatible with the view that these plasmids may derive from one common ancestor, and/or that they can be transferred between unrelated *Streptococcus* strains belonging to the same or different groups.

Strains of *Streptococcus* resistant to erythromycin (Em), lincomycin (Lm), and tetracycline (Tc) have recently appeared with increased frequency mainly in serological groups A (6, 7, 21, 24), B (8), and D (9, 18, 20, 22, 25, 27). Resistance to Em, however, is rare among group B streptococci (9). This observation has been confirmed by results obtained in our laboratory: only 1 of 94 strains of *Streptococcus agalactiae* isolated in different regions of France has been shown to be resistant to this drug, but 16 of 43 strains of *S. faecalis* and *S. faecium* were found to be resistant to Em.

Genetic and physical studies of groups A, B, and D streptococci have allowed the isolation and characterization of plasmids coding for antibiotic resistance (2-5, 9, 13, 14, 16-19). Comparison of nucleotide sequences of these plasmids by DNA-DNA hybridization makes possible study of their relatedness, evolution, and epidemiology. Such studies were carried out by Yagi et al., who compared two plasmids isolated from groups A and D streptococci (28).

We report here on the genetic and physical properties and on the phylogenic relationships of R plasmids isolated from groups B and D streptococci (*S. agalactiae* and *S. faecalis*).

MATERIALS AND METHODS

Bacterial strains and plasmids. These are described in Table 1.

Culture media. The culture media used were brain heart infusion (BHI) broth (Difco), tryptic soy agar (Difco) supplemented with 5% horse serum, and, for studies of antibiotic sensitivity, Mueller-Hinton agar (Pasteur Institute) supplemented with 5% horse blood.

Anitibiotics and reagents. These have been previously described (13).

Technique for genetic and physical studies. Techniques of cure, isolation of plasmid DNA, and determination of molecular weight (MW) were as previously described (13), with the following modifications: (i) BHI medium was supplemented with DL-threonine (20 mM) (1), and (ii) the bacteria were washed, frozen, and then thawed slowly in order to make the cell walls fragile before lysozyme treatment.

Preparation of radioactive plasmid DNA. Twenty milliliters of BHI + DL-threonine were inoculated with 1 to 3 ml of an 18-h culture to obtain a turbidity of 15 Klett units (KU, green filter no. 54) using a Klett-Summerson colorimeter. At 60 KU, the culture was labeled by addition of 10 μCi of [methyl-^{3}H]thymidine per ml (42 mCi/mmol, Commissariat à l'Energie Atomique, France). When the turbidity reached 130 KU, the culture was stopped, and lysis of washed bacteria was performed as described above. Isolation (see Fig. 1), purification, sonic treatment, and precipitation of radioactive plasmid DNA (DNA*) were as described by Roussel and Chabbert (23). This preparation gave plasmid DNA with specific activity ranging from 2.5×10^5 to 3.5×10^5 cpm/μg.

Preparation of "total" nonradioactive DNA. One hundred and fifty milliliters of BHI + DL-threonine were inoculated with 8 to 12 ml of an 18-h culture to obtain a turbidity of 15 KU. The culture was harvested at the end of exponential growth (130 to 140 KU). The bacteria were washed three times with 100 ml of TE buffer (5×10^{-2} M tris(hydroxymethyl)-aminomethane-10^{-3} M ethylenediaminetetraacetate, pH 8). The cells were frozen at −20°C for at least 12 h, then thawed slowly and resuspended in 10 ml of TE buffer containing 0.4 mg of lysozyme per ml (Sigma) and 0.05 mg of ribonuclease per ml (Calbiochem, bovine pancreas ribonuclease, salt free, A grade). After 1 h at 37°C, the cells were lysed by adding 1 ml of 5 M NaCl and 1 ml of 4% sodium sarcosinate solution. One

TABLE 1. *Bacterial strains and plasmids*

Streptococcus strains	Group, serotype, or species	Resistance markers	Origin	Plasmids	MW of plasmids	Reference
BM6101[a]	B, II	Cm Em Lm SgB Tc	Strasbourg 1975 (sputum)	pIP500 and pIP501		13
BM6102	B, II	Cm Em Lm SgB	BM6101	pIP501	19.8×10^6	13
BM6104	B, II		BM6102			13
BM6105	B, II	Cm Em Lm SgB	Strasbourg 1975 (throat)	pIP612	22.7×10^6	This paper
BM6106	B, II	Tc	BM6105			This paper
BM6201	D, *S. faecalis*	Em Lm SgB Tc	Paris 1963 (blood culture)	pIP613 and pIP614		5
BM6202	D, *S. faecalis*	Em Lm SgB	BM6201	pIP613	17.6×10^6	5
BM6203	D, *S. faecalis*	Tc	BM6201	pIP614	64.5×10^6	5
BM6204	D, *S. faecalis*		BM6201			5

[a] Previously referred to as B96 (13).

milliliter of a 1-mg/ml solution of Proteinase K (Merk 24568) preincubated for 2 h at 37°C was added, and the mixture was incubated another 2 h at 37°C to digest proteins. DNA was purified, deproteinized, and precipitated as previously described (23). DNA pellets were resuspended in 5 ml of 0.3 M NaOH and denaturated by incubation at 37°C for 3 h. The preparation was then neutralized and buffered by adding 1 ml of 2 M NaH_2PO_4. Distillated water and 20× SSC (3 M NaCl–0.3 M trisodium citrate, pH 7) were added to the total nonradioactive denatured DNA to obtain a final concentration of 2 μg/ml in 4× SSC. Purification of denatured DNA was controlled by UV light spectrophotometry, and DNA was assayed colorimetrically (26).

DNA-DNA hybridization of the various plasmids. The technique used was that of Kourilsky et al., modified by Roussel and Chabbert, for studying plasmid DNA-DNA hybridization (15, 23). Hybridization was performed on nitrocellulose microfilters (5-mm diameter) at low temperature (37°C for 18 h) in 50% formamide–2× SSC and under paraffin oil. Each hybridization experiment required five types of filters: (i) filters without DNA ("blank" filters), (ii) filters loaded with total DNA (plasmid and chromosomal) of the strain carrying the homologous plasmid ("R^+hom" filters), (iii) filters loaded with total DNA from the "cured" strain of the homologous plasmid ("R^-hom" filters), (iv) filters loaded with total DNA from the strain carring the heterologous plasmid ("R^+heter" filters), (v) filters loaded with total DNA from the cured strain with the heterologous plasmid ("R^-heter" filters). Each type was loaded with three different amounts of total cold DNA (0.5 to 1.5 μg) in order to obtain an excess of binding sites in at least two series (23). All filters were incubated with 0.0005 to 0.001 μg of purified, sonically treated, and denatured ^3H-labeled plasmid DNA.

RESULTS

Analysis of BM6105 and BM6106 strains. Incubation of the wild-type strain BM6105 at 42°C and treatment with ethidium bromide resulted in variants having lost four resistance markers (chloramphenicol [Cm]; Em, Lm, and streptogramin B [SgB]). At 42°C, the percentage of loss was 5.2 (10 of 193 colonies tested). After treatment with ethidium bromide, the percentage of loss was 2.5 (6 of 237). Loss of resistance to Tc never occurred (1,300 colonies tested). One of the cured colonies, BM6106 (see Table 1) was chosen for further studies. Equilibrium centrifugation of bacterial DNA in ethidium bromide-cesium chloride gradients revealed that strain BM6105 had a band of plasmid DNA (pIP612), whereas strain BM6106 did not (Fig. 1). Sedimentation analysis of pIP612 DNA molecules in neutral sucrose gradients demonstrated that the MW of this plasmid was close to 22.7×10^6 (Fig. 2).

Plasmid DNA-DNA hybridization. (i) Methodology and controls. Hybridization experiments carried out with three series of filters demonstrated that the amount of total DNA used (0.5, 1, and 1.5 μg/filter) was not a limiting factor under the conditions of reassociation used.

Evaluation of percentage of DNA-DNA reassociation between two plasmids required the preparation of "control" filters free of the DNA of the plasmid under analysis. Two sorts of control filters, R^-hom and R^-heter, were used. The percentage of reassociation "αi" was calculated as follows:

$$\alpha i = \frac{\text{Radioactivity bound to } R^+\text{heter filters} - \text{radioactivity bound to } R^-\text{heter filters}}{\text{Radioactivity bound to } R^+\text{hom filters} - \text{radioactivity bound to } R^-\text{hom filters}} \times 100$$

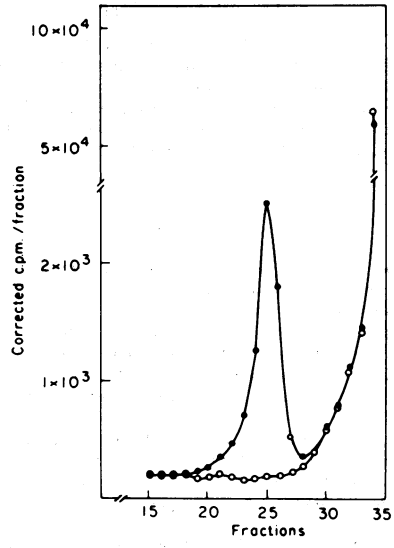

FIG. 1. *Isolation of plasmid DNA in dye buoyant density gradients: lysate of a mixture of wild strain BM6105 ([³H]thymidine, ●) and of a susceptible derivative BM6106 ([¹⁴C]thymidine, ○). Gradients were fractionated by drop collection, and 20-μl samples were spotted onto 3MM Whatman filters and counted for radioactivity. Counts were corrected for double labeling. Note change in scale.*

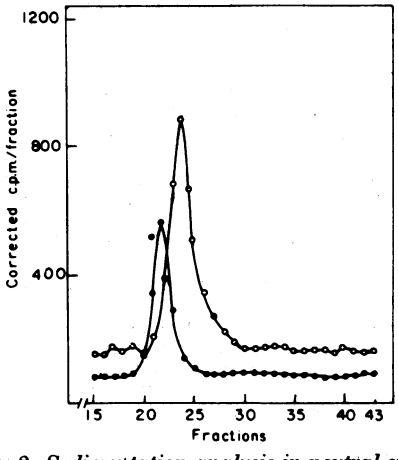

FIG. 2. *Sedimentation analysis in neutral sucrose gradient (10 to 30%) of pIP612 DNA ([³H]thymidine, ●) and of pIP613 DNA (5) (MW = 17.6 × 10⁶) ([¹⁴C]-thymidine, ○). After 2 h 30 min at 40,000 rpm in a SW65 rotor, gradients were fractionated by drop collection (0.125 ml per fraction) onto GFC Whatman filters and counted for radioactivity.*

The indicate values "αM" (Tables 2 and 3) were the mean of at least seven αi determinations obtained by hybridizing 4 μl from the same annealing mixture with seven (or more) filters of each kind.

No significant difference was observed between the amount of radioactivity retained by R⁻ and blank filters (data not shown). This lack of specific retention of radioactive plasmid DNA by control filters demonstrates that the preparations of plasmid DNA were completely free of chromosomal DNA and that there is no significant "mismatching." In addition, lack of retention reflects the absence of homology between nucleotide sequences of plasmids and of the bacterial chromosome.

(ii) Comparison of nucleotide sequence of groups B and D streptococcal plasmids. Hybridization of plasmids pIP501 and pIP612 DNA isolated from group B streptococci showed that the percentage of reassociation between pIP501 and pIP612 is 100%, whereas that of the reciprocal reaction between pIP612 and pIP501 is significant ($P < 0.05$) and might be easily explained by the difference in molecular weights (15%) of pIP501 and pIP612 (Table 1).

Hybridization of pIP501 DNA with homologous DNA or with heterologous DNA from pIP613 and pIP614 plasmids (isolated from *S. faecalis*) shows that the percentage of reassociation of pIP501 DNA with pIP613 is 90% (Table 3). The percentage of reassociation between pIP501 and pIP614 DNA is 10%.

DISCUSSION

The multiresistant strains among members of the *Streptococcus* genus are rapidly increasing (6–9, 20–22, 24, 25, 27). Most of the strains have been shown to carry R plasmids (2–5, 9, 13, 14, 16–19). Molecular characterization of some of these plasmids allows the proposal of hypotheses about their phylogenic relationships and their spreading in groups B and D streptococci.

Hybridization of plasmid pIP501 (CmEm-

TABLE 2. *DNA-DNA homology between pIP501 and pIP612*

³H-labeled plasmid DNA	Unlabeled plasmid DNA[a]	
	pIP501	pIP612
pIP501	100[a] ∓ 3[b]	100 ∓ 4
pIP612	86 ∓ 2	100 ∓ 4

[a] αM, Mean of eight αi determinations.
[b] σm, Standard deviation of the mean.

TABLE 3. *Percentage of reassociation of ³H-labeled pIP501 DNA with pIP501, pIP612, pIP613, and pIP614 DNA*

Unlabeled plasmid DNA (αM and σm)[a]			
pIP501	pIP612	pIP613	pIP614
100 ± 25	100 ± 2	90 ± 2	10 ± 0.3

[a] Mean of eight independent determinations of αi and standard deviation of the mean (σm).

LmSgB), isolated from group B, with plasmid pIP613 (EmLmSgB), isolated from group D, shows that the DNA-DNA homology between these plasmids is very high (≥86%), whereas homology between pIP501 and pIP614 (Tc), isolated also from group D, is very low. Therefore, plasmids pIP613 and pIP614 which coexist in the same strain (BM6101) have very few nucleotide sequences in common. These results are similar to those of Yagi et al. (28), who found that the homology between two plasmids coexisting in the same strain was also very low. These facts support the hypothesis that, among streptococci as well as among gram-negative bacteria (10–12), most compatible plasmids have a very low degree of homology and may constitute distinct molecular species.

S. agalactiae strains BM6105 (group B, serotype II) and 6102 of the same serotype (isolated from another patient 3 months earlier) have similar resistance markers (13). Strain BM6105 carries plasmid pIP612, which has a high degree of molecular similarity (MW and DNA-DNA homology) to pIP501 from strain BM6102. The isolation of similarly resistant streptococcal group B strains from patients hospitalized at different times and in different wards might result either from the persistence and spread of the same strain or from the transfer in vivo of the same R plasmid between independent strains. Whatever the mode of appearance of plasmids pIP501 and pIP612, their molecular similarity might reflect a possible relatedness between them.

The same similarity (MW and 90% reassociation) was observed between plasmids pIP501 and pIP613 isolated from strains belonging to different serological groups (B and D) and isolated twelve years apart in different cities. Yagi et al. (28) also reported a 95% homology between two plasmids of the same molecular weight isolated from streptococci belonging to groups A and D. The molecular weights of pIP501, pIP612, and pIP613 are slightly different, but this does not result in any important difference in their homology. This high degree of homology suggests that the plasmids could have derived from the same ancestral DNA molecule and that minor changes in nucleotide sequences might be responsible for the appearance of three distinct but closely related plasmids. Taken together, these results support the hypothesis that very similar (if not identical) plasmids might be dispersed among different groups of streptococci. Conjugal transfer of R plasmids has already been demonstrated in group D streptococci (9, 14, 19). The transferability of R plasmids from group B strains is presently being studied.

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