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R Plasmids in *Streptococcus agalactiae* (Group B)

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Two plasmids determining resistance to tetracycline (RIP500) and to chloramphenicol, erythromycin, lincomycin, and pristinamycin I (RIP501) were isolated from a strain of *Streptococcus agalactiae*. The frequency-of-resistance loss is very low for RIP500 ($<3 \times 10^4$) but higher for RIP501 (the efficiency was dependent upon the curing agents and incubation temperature and varied between 0.5 and 96%). Derivatives susceptible to all drugs were also obtained. RIP500 and RIP501 have similar molecular weights (17.9×10^6 and 20×10^6, respectively) and represent different percentages of total deoxyribonucleic acid (0.4 and 4%, respectively). The number of copies of RIP500 and RIP501 per cell is different, and these plasmids are likely replicated under different kinds of control (stringent and/or relaxed). No plasmid deoxyribonucleic acid was found in a derivative of strain B96 susceptible to all drugs.

Increased frequency of the appearance of drug resistance strains in Lancefield group A (12, 27), B (16), and D (29) streptococci has been reported in the last years, especially for erythromycin, lincomycin, and tetracycline antibiotics. Since the initial report of Courvalin et al. of two R plasmids in a group D *Streptococcus faecalis* (8), the existence of plasmids in other strains of streptococci has been demonstrated in *S. mutans* (13), *S. pyogenes* (3, 23), group N (7, 22), and *S. faecium* (6). In all these strains, cryptic (7, 13), metabolic (6, 7, 22), and R plasmids (3, 4, 21) were described. Jacob et al. (20) and Dunny and Clewell (14) reported more recently the capacity of plasmids to promote transfer during mixed incubation in broth in *S. faecalis*.

Evidence of R plasmids in group B streptococci has not yet been reported. This study deals with the genetic and physical properties of two R plasmids (designated RIP500 and RIP501) carried by *S. agalactiae* strain B-49813/75, referred to as strain B96 in this study.

MATERIALS AND METHODS

Bacteria and plasmids. *S. agalactiae* strain B-49813 (designated here as B96), isolated from a clinical sputum specimen, was used in this investigation. The identification on horse blood agar was verified by Fuller's serological test (18). This strain is resistant to tetracycline (T), chloramphenicol (C), macrolide antibiotics (including erythromycin [E], oleandomycin [O], and spiramycin [Sr]), lincomycin (L), and pristinamycin I (PI), a streptogramin B-type antibiotic similar to vernamycin Bα (3). The R plasmids described in this study are: RIP500, mediating resistance to T; and RIP501, resistant to C, E, L, and PI. The wild strain is referred to as B96 (RIP500 + RIP501) (see Table 1). T169, a tetracycline resistance plasmid from *Staphylococcus aureus* strain RN1304 (26), was used as the control in this study.

Media and materials. Brain heart infusion broth (Difco) was used as nutrient broth; tryptic soy agar (Difco) with 5% horse serum and Mueller-Hinton agar (Institut Pasteur) with 5% horse blood were used as nutrient agar. Davis minimal broth (11) supplemented with 2% protease peptone no. 3 (Difco) was used as the semisynthetic medium. Drugs used were as follows: tetracycline (Rhône-Poulenc, France), chloramphenicol (Roussel, France), erythromycin (Abbott, England), lincomycin (The Upjohn Co), and rifampin (Lepetit, France). Reagents used were: acridine orange (ESBE, France), acriflavine (Serlabo, France), ethidium bromide (Boots Drug Co), cesium chloride (ultrapur 2039, Merck), [^{3}H]-thymidine (44 Ci/mmol) and [^{14}C]thymidine (57 mCi/mmol) (Commissariat à l'Energie Atomique, France), Sarkosyl (NL97, Geigy Chemical Corp.), and lysozyme (Sigma).

MICs. The minimum inhibitory concentrations (MICs) were determined by the spot technique (10), using a Steers multiple inoculator apparatus (28). Eighty to 100 colony-forming units were plated onto nutrient agar plates containing doubling concentrations of an antibiotic, starting from 0.015 μg/ml. Antibiotics used and their MICs are listed in Table 1. The MIC was recorded as the lowest concentration of antibiotic preventing colony formation after 18 h of incubation. Resistance to O, S, clindamycin (Cl), virginiamycin, and PI and PII was determined with susceptibility disks.

Curing procedures. Curing by high temperature was as follows. An 18-h broth culture was diluted 1:10 in broth and incubated without aeration for 18 h at 42°C. Control cultures were grown at 37°C. For curing by chemical agents (acridine orange, acriflavine, or ethidium bromide), 1 to 3 mg of the chemicals was placed on nutrient agar (tryptic soy

agar plus 5% horse serum) inoculated with a diluted (1:200) 6-h broth culture. After 18 h of incubation at 37°C, three zones were observed: (i) total inhibition, (ii) partial inhibition, and (iii) noninhibition. A loopful from the border of the second zone was subcultured in nutrient broth for 18 h. Master plates were prepared from this broth, as well as from those incubated either at 42 or 37°C and replica-plated for loss of T, C, E, or L resistance. Curing by rifampin was obtained by using nutrient agar plates containing 10, 25, 50, and 100 μg of rifampin per ml, and the plates were inoculated with a 6-h broth culture, 20-fold concentrated by centrifugation. After 18 to 36 h of incubation at 37°C, they were directly replica-plated as described above.

Plasmid deoxyribonucleic acid (DNA) isolation and sedimentation analysis. Streptococci were grown in 20 ml of semisynthetic broth containing 4% (wt/vol) glucose (optical density = 25 Klett units) at 37°C. Bacteria were labeled at optical density = 50 Klett units with 5 μCi of [*methyl*-^{3}H]thymidine or 100 μCi of [*methyl*-^{14}C]thymidine per ml. At optical density = 110 Klett units the cells were harvested and washed in cold tris(hydroxymethyl)aminomethane (0.05 M), ethylenediaminetetraacetic acid (0.001 M), pH 8 (TES buffer). The cells were treated for 1 h at 37°C with lysozyme (final concentration, 400 μg/ml in TES buffer) and for 30 min at 37°C with Sarkosyl (final concentration, 0.5%). These crude lysates (8 ml) were then centrifuged in the presence of cesium chloride (8 g) and ethidium bromide (0.4 ml of a stock solution containing 10 mg/ml). After 48 h at 42,000 rpm (20°C) in a 50 Ti rotor (L2-65 B Beckman ultracentrifuge), gradients were fractionatated by drop collection and counted. Plasmid T169 from *Staphylococcus* strain RN1304 was isolated as described by Novick and Bouanchaud (26). Sedimentation analysis in a neutral sucrose grdient of plasmid molecules was carried out as described recently (19).

RESULTS

Antibiotic resistance of *S. agalactiae* strains. The resistance pattern of 856 group B streptococci isolated from separate patients in the last 5 months of 1975 in Louis Pasteur Hospital, Strasbourg (France), was determined by using the routine susceptibility disk method. A total of 852 strains had the usual susceptibility spectrum of *S. agalactiae*, but four strains were resistant to T, C, E, Sr, O, L, Cl, and PI. One of these resistant strains, referred to as B96, was chosen for further studies. The MICs of different antibiotics are shown in Table 1.

Stability of drug resistance in strain B96. Spontaneous loss of drug resistance after 6 months of storage in nutrient broth at 4°C, or after lyophilization of the strain, was tested by the replica-plating technique (Table 2). As can be seen, no loss of drug resistance was observed, except in a lyophilized sample, in which only C, E, L, and PI markers were lost at low frequency (<0.5%). The results of several "curing" experiments using different agents are summarized in Table 2. One important feature is the detection of a rather large number of clones resistant to T and one clone (from a total of 3,376 clones examined) resistant only to C, E, L, and PI. The lack of loss of T was recently reported by Clewell et al. (4) and was in contrast to the efficiency of curing (31%) obtained by Courvalin et al. (8). Thus, to obtain susceptible clones of B96, the C E L PI-resistant strain was treated by different curing procedures. We obtained an efficiency of 1% with rifampin (25 μg/ml) and 1.2% at the elevated incubation temperature (42°C; Table 2). These results suggested that the resistance markers in B96 strain were plasmid borne and that this strain carried two plasmids, referred to as RIP500 and RIP501, coding for T and C E L PI, respectively. When the wild strain B96 (RIP500 + RIP501) was grown at 42°C, the frequency of colony-forming units of plasmid RIP501 (C E L PI) "cured" was 0.5, 48.5, and 96% in three independent experiments. The RIP500 (T) plasmid was never cured at high temperature. No loss of any resistance markers was obtained with acriflavine or ethidium bromide.

The MICs of antibiotics for all derivatives are shown in Table 1. The same pattern of susceptibility for C, E, L, and T was obtained for another strain of *S. agalactiae* (B19) used as the reference strain in this study. One of the cured cells of strain B96 was further examined for

TABLE 1. *MICs of antibiotics used*

Bacterial strain[a]	Plasmids	Resistance markers	MIC (μg/ml) for:				
			T	C	E	L	PI[b]
B96	RIP500 + RIP501	T C E L PI	64	16	4,000	256	R
B96	RIP500	T	64	2	0.03	0.03	S
B96	RIP501	C E L PI	0.25	32	4,000	256	R
B96		Cured	0.25	2	0.03	0.03	S
B19			0.25	2	0.03	0.03	S

[a] B19 is a susceptible, reference group B strain.
[b] Tested with sensitivity disks. S, Sensitive; R, resistant.

TABLE 2. *Curing of RIP500 and RIP501 by different agents*

Plasmids	Treatment[a]	No. of colonies tested	No. of resistant colonies				Efficiency of curing (%)
			T	C	E	L	
RIP500 + RIP501 (T C E L PI)	37°C	180[b]	180	180	180	180	0
		420[c]	420	418	418	418	0.5
		200[d]	200	199	199	199	0.5
	42°C	64[d]	64	33	33	33	48.5
		340[d]	340	14	14	14	96
	AO	67	66	62	62	62	1.5 for T[e] 7.4 for C E L PI
RIP500 (T)	37°C	133	133				0
	42°C	163	163				0
	AO	460	460				0
	Rif	33	33				0
RIP501 (C E L PI)	37°C	200		200	200	200	0
	42°C	98		97	97	97	1
	AO	250		250	250	250	0
	Rif	78		77	77	77	1.2

[a] AO, Acridine orange; Rif, rifampin.
[b] After 6 months of storage at 4°C.
[c] After lyophilization of the wild strain B96.
[d] Three independent experiments.
[e] A single tetracycline-susceptible clone was obtained from a total of 3,376 clones screened.

spontaneous reversion to high-level resistance on plates containing 8 μg of C and 25 μg of E, L, or T per ml. An inoculum of about 2.2×10^9 cells was spread on the plates; no reversion to C, E, L, or T resistance was observed. From the data shown in Table 2 we could conclude that resistance markers were stable in strain B96, and that curing agents have poor activity (if any) on plasmids RIP500 and RIP501. The same stability has been reported by Jacob and Hobbs in an *S. faecalis* strain (21) and by Clewell and Franke in an *S. pyogenes* strain (3).

Determination of chloramphenicol inactivation. Inactivation of chloramphenicol was demonstrated by using the satellitism test as described by Chabbert and Debruge (2) in *Staphylococcus* strains. Spots of B96 (RIP501) and of B96 (RIP500) were inoculated on nutritive agar containing spores of *Bacillus subtilis* strain ATCC 6633 and 2 μg of C per ml. After 18 h of incubation at 37°C, a dense halo of *B. subtilis* is visible around the spot of B96 (RIP501) but not around that of B96 (RIP500). This observation suggests that an inhibitor of chloramphenicol diffused in the agar from the B96 (RIP501) spot, presumably a chloramphenicol acetyltransferase, which will be described elsewhere.

Isolation of plasmid DNA. Figure 1 shows the results of dye buoyant density centrifugation of the DNA of strain B96 (wild type) and of different variants obtained in curing procedures. Double-label experiments were carried out with mixtures of a lysate of B96 resistant to C E L PI T, and labeled with [^{3}H]thymidine, and a lysate of a derivative susceptible to all drugs of B96 labeled with [^{14}C]thymidine. The percentage of plasmid DNA was, respectively, 4 and 0% of the total bulk DNA (Fig 1a). In another experiment, lysates of B96 resistant to C E L PI T ([^{3}H]thymidine labeled) and of B96 resistant only to T ([^{14}C]thymidine labeled) were analyzed. The results are illustrated in Fig. 1b. The percentage of plasmid DNA was, respectively, 3.8 and 0.4% of the total DNA. In a third experiment (data not shown), it was observed that plasmid DNA represented 3.5% of the total DNA in a variant of B96 resistant to C E L PI but susceptible to T. These findings indicate the presence of plasmids in this strain. The loss of plasmid DNA associated with the loss of resistance provides evidence that the determinants of resistance are plasmid borne. RIP500 (T) and RIP501 (C E L PI) represent approximately 15 and 85% of the total plasmid DNA, respectively.

Determination of molecular weight and size of RIP500 and RIP501. Samples of pure plasmid DNA, obtained from B96 (RIP500) and B96 (RIP501) and labeled, respectively, with [^{14}C]thymidine and [^{3}H]thymidine, were sedimented in sucrose gradients as described in Materials and Methods. Figure 2a shows the results of band sedimentation of RIP501 and

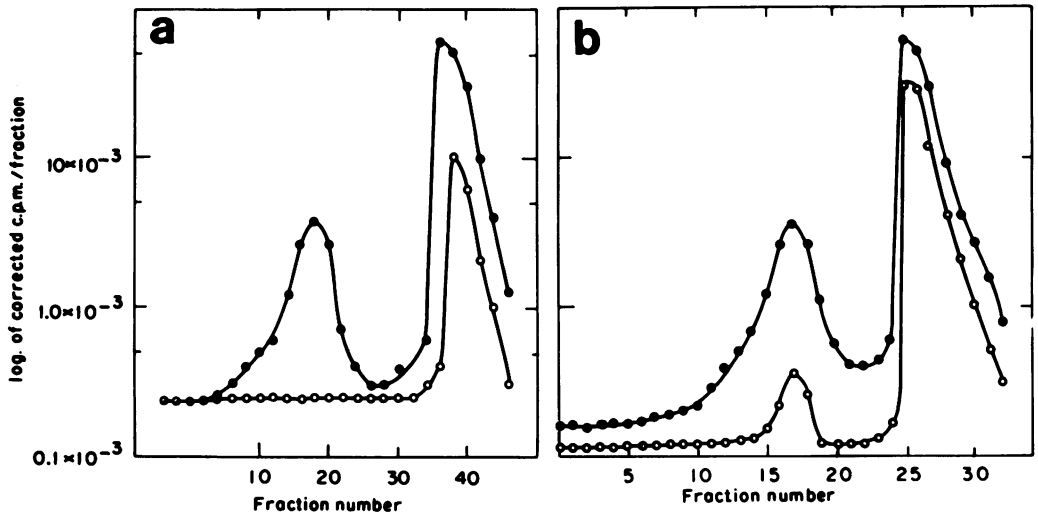

FIG. 1. *Isolation of plasmid DNA in dye buoyant density gradients. (a) Lysate of a mixture of wild strain B96 (RIP500 + RIP501) ([³H]thymidine, ●) and of a susceptible derivative of B96 ([¹⁴C]thymidine, ○). (b) Lysate of a mixture of B96 (RIP500 + RIP501) ([³H]thymidine, ●) and of B96 (RIP500) ([¹⁴C]thymidine, ○). Counts were corrected for double labeling and plotted on a log scale.*

T169 (used as a standard). The two peaks in fractions 24 and 32 represent form I (covalently closed circles of DNA) and form II (open circles) of RIP501 (C E L PI) plasmid. Using the value of S = 19.55 for T169 (26), the S value for RIP501 (form I) can be estimated to be 45.13, which corresponds to a molecular weight of 20 × 10^6, equivalent to 10 nm. The result of band sedimentation of RIP501 (^{3}H-labeled) and RIP500 (^{14}C-labeled) can be seen in Fig. 2b. The S value for RIP500 was calculated to be 43.3, which corresponds to a molecular weight of 17.9 × 10^6, or 9.07 nm. RIP500 (T) does not give rise to form II molecules, as was found for some plasmids of *S. aureus* (Bouanchaud and Novick, unpublished data) and from this point of view is strikingly different from RIP501 (C E L PI).

Estimation of the number of copies of each plasmid. Assuming that plasmid and chromosomal DNA have the same radioactive specific activity and that the molecular weight of the *S. faecalis* chromosome is 1.47 × 10^9 (1), the relative number of copies of RIP500 and RIP501 per chromosomal genome equivalent (CGE) can be estimated from the molecular weight and the relative amount of each plasmid. The apparent number of copies is 0.3/CGE for RIP500 (T) and 3/CGE for RIP501 (C E L PI). A summary of the most important physical properties of RIP500 and RIP501, described above, is given in Table 3.

DISCUSSION

There is increasing interest in group B streptococci in the medical literature of the past decade, due to the multiple clinical infections caused by this organism: perinatal-puerperal infections, neonatal sepsis (with or without meningitis), pneumonia, empyema, peritonitis, urinary tract infections, arthritis, and endocarditis (15). Moreover, group B streptococci are usually very susceptible to several antibiotics and especially to penicillin G, which is the drug of choice in the treatment of *S. agalactiae* diseases. However, in penicillin-allergic patients, for whom penicillin is contraindicated, E or L is usually a satisfactory alternative. The appearance of strains resistant to E and L, reported by Eickhoff et al. (16) and Minck (unpublished data) in group B streptococci, and also the high frequency of T-resistant strains (approximately 80% of our recent *S. agalactiae* isolates) could make it difficult to provide an appropriate therapy.

Multiple-drug-resistant plasmids have recently been demonstrated only in group D (4, 8, 21) and A streptococci (3, 23), and at least two R plasmids carried by one strain were generally reported. The *S. agalactiae* strain (B96) described in this study also carries two R plasmids: RIP500 (T) and RIP501 (C E L PI). From this point of view, streptococci seem to be very different from staphylococci, in which the most

plasmids described so far encode only for one resistance marker, with the exception of the penicillin erythromycin plasmids reported by Novick (25).

It might be pointed out that an interesting feature of RIP501 is that it also carries the C-resistant marker, which was found only in a few strains of *S. pyogenes* (24).

The molecular weights of *Streptococcus* plasmids are very variable: from 10^6 for a cryptic plasmid in *S. lactis* (22) to 64.5×10^6 for a tetracycline resistance plasmid isolated from an *S. faecalis* strain (9). The molecular weights (and sizes) of RIP500 (T) and RIP501 (C E L PI) are very close, 17.9×10^6 (9.07 nm) and 20×10^6 (10 nm), respectively, but RIP500 (T) represents 0.4% and RIP501 (C E L PI) represents 4% of total DNA. The number of copies of *Streptococcus* plasmids is not easy to define, because of the uncertainty of the size of the bacterial genome. As observed in *Enterobacteriaceae*, smaller plasmids seem to be replicated under relaxed control, and larger plasmids under stringent control (5). The apparent number of copies is 0.3/CGE for RIP500 (T) and 3/CGE for RIP501 (C E L PI). These are minimum estimates, because a variable and, very likely, important proportion of plasmid DNA is lost in the assay procedures. For instance, the number of copies of RIP500 cannot be smaller than 1 to

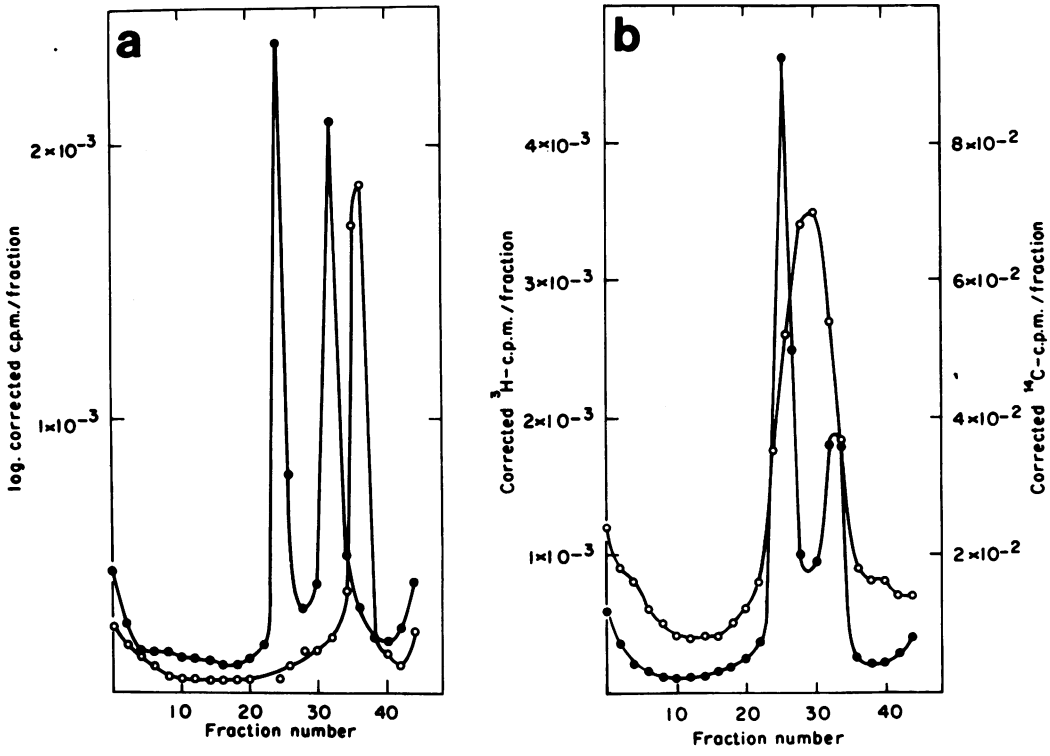

FIG. 2. *Sedimentation analysis of plasmid DNA in a neutral sucrose gradient. (a) RIP501 DNA ([³H]thymidine, ●) and T169 DNA ([¹⁴C]thymidine, ○). (b) RIP501 DNA ([³H]thymidine, ●) and RIP500 DNA ([¹⁴C]thymidine, ○). Note change in scale.*

TABLE 3. *Summary of the most important physical properties of RIP500 and RIP501*

Plasmids	Resistance marker	Mol. wt ($\times 10^{-6}$)	Size (nm)	% of total DNA	Minimum no. of copies per CGE	Form I (CCC) or II (OC)[a]
RIP500	T	17.9	9.07	0.4	0.3 (≥1)	CCC only
RIP501	C E L PI	20	10	3.8–4	≥3	CCC and OC

[a] CCC, Covalently closed circular; OC, open circular.

2/CGE, but a maximum of 1/3 (and often less) of RIP500 plasmid DNA is recovered in the density gradient. The molecular weight of RIP500 (T) is 17.9×10^6. Replicating plasmids of the same size have been found to be under stringent control in *S. faecalis* (4) and *S. pyogenes* (3). The DNA of RIP501 (C E L PI) represents 4% of the total DNA, but the data available so far do not allow a decision as to whether the number of copies of this plasmid is 9 to 10 times the number of copies of RIP500 or if the recovery of RIP501 DNA is better than the recovery of RIP500 DNA. However, the fact that RIP500 (T) seems to have less copies per CGE than RIP501 (C E L PI) does not agree with the difficulty in curing of the tetracycline plasmid. Clewell et al. (4) recently reported the same lack of success in curing a tetracycline plasmid, but the number of copies per CGE was larger.

The rather wide RIP500 band in the sucrose gradient is an unexplained but not uncommon feature of this type of experiment, as already shown by Freifelder for the F plasmid (17) and by Horodniceanu and Bouanchaud for the recombinant plasmid pIP218 (19). This fact contrasts with the sharp plasmid bands obtained with RIP501 DNA molecules.

Previous reports have demonstrated the presence of R plasmids in group A and D streptococci (3, 8); in the present study we demonstrate the presence of two R plasmids in group B streptococci. These reports suggest that the existence of R plasmids in other serological groups of human or animal origin is likely and is, perhaps, a more usual phenomenon. The similarity in these *Streptococcus* plasmids merits studies by DNA homology (as recently reported by Yagi et al. [30] for two E-resistant plasmids), to determine their possible interrelationships and define any genetic exchanges among these organisms.

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