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Tiamulin Resistance Mutations in *Escherichia coli*

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Forty "two-step" and 13 "three-step" tiamulin-resistant mutants of *Escherichia coli* PR11 were isolated and tested for alteration of ribosomal proteins. Mutants with altered ribosomal proteins S10, S19, L3, and L4 were detected. The S19, L3, and L4 mutants were studied in detail. The L3 and L4 mutations did not segregate from the resistance character in transductional crosses and therefore seem to be responsible for the resistance. Extracts of these mutants also exhibited an increased in vitro resistance to tiamulin in the polyuridylic acid and phage R17 RNA-dependent polypeptide synthesis systems, and it was demonstrated that this was a property of the 50S subunit. In the case of the S19 mutant, genetic analysis showed segregation between resistance and the S19 alteration and therefore indicated that mutation of a protein other than S19 was responsible for the resistance phenotype. The isolated ribosomes of the S19, L3, and L4 mutants bound radioactive tiamulin with a considerably reduced strength when compared with those of wild-type cells. The association constants were lower by factors ranging from approximately 20 to 200. When heated in the presence of ammonium chloride, these ribosomes partially regained their avidity for tiamulin.

The antibiotic tiamulin, a semisynthetic derivative of pleuromutilin, inhibits protein synthesis on procaryotic ribosomes (12). Two binding sites have been found, one on the 50S subunit saturated at a 1:1 stoichiometry and a second that required an intact 70S ribosome (13). In the presence of the drug, the formation of the first bond of the nascent peptides is inhibited due to the formation of abortive initiation complexes (6, 11).

The development of resistance to tiamulin has been studied with laboratory strains of *Klebsiella pneumoniae* and *Staphylococcus aureus* (7). It was found that resistance emerges stepwise and that some of the mutations conferring resistance are ribosomal in nature (7, 12). It is the purpose of this paper to give a detailed genetic and biochemical analysis of such mutations.

MATERIALS AND METHODS

Organisms and media. The strains of *Escherichia coli* used in this study are listed in Table 1. The composition of the media employed was described previously (1).

Genetic procedures. Tiamulin-resistant mutants were isolated from strain PR11, a genetically well-marked strain of *E. coli*, on tryptone-glucose-yeast extract (TGYE)-rich medium plates by stepwise selection, using plate concentrations of 100, 250, and 500 μg/ml of tiamulin consecutively. Both spontaneous and ethyl methane sulfonate-induced mutants were isolated. Strain constructions and genetic mapping were carried out by P1*vir*-mediated transductions, using procedures described previously (16). Minimal inhibitory concentrations (MICs) on plates were determined (17) by streaking loopfuls of a suspension of cells (about 10^8 cells per ml) on TGYE plates containing the antibiotics at one of the following concentrations: tiamulin, 50, 100, 200, 400, or 800 μg/ml; chloramphenicol, 1, 2, 4, 8, or 16 μg/ml; erythromycin, 25, 50, 100, 200, or 400 μg/ml; lincomycin, 50, 100, 200, 400, or 800 μg/ml. The concentration of the antibiotic that prevented growth upon incubation at 37°C for 2 to 3 days was taken as the MIC.

Recombinants were analyzed for resistance in essentially the same way, but with a more narrow range of antibiotic concentrations; in the case of tiamulin these were 100, 150, 200, and 250 μg/ml.

Isolation of ribosomes and ribosomal subunits and electrophoretic analysis of ribosomal proteins. Isolation of ribosomes and ribosomal subunits was carried out by the method of Kühberger et al. (15). The preparation and electrophoretic separation of ribosomal proteins were performed by the procedure of Geyl et al. (9). The analyses of polyuridylic acid (polyU)-dependent polyphenylalanine synthesis and of phage R17 RNA-dependent polypeptide formation were carried out as described previously (15).

Binding studies. Ribosomes for binding studies were prepared by growing *E. coli* cells in tryptic soy broth (Merck) to the late exponential phase. Bacteria from 3l cultures were washed with 10 mM Tris-hydrochloride (pH 7.8)–10 mM magnesium acetate–60 mM KCl–6 mM β-mercaptoethanol (buffer A) and suspended in 15 ml of the same buffer. The suspension was exposed at 0°C to five sonication pulses, each lasting for 45 s, with a Branson Sonifier. The broken cells were centrifuged for 20 min at 40,000 × *g* at 4°C, and the supernatant

TABLE 1. Strains of *E. coli* used in this study

Strain	Genotype	Reference or source
K10	Hfr (Cavalli) *rel-1 tonA22* $T2^r$	3
A19	F^+ *thi met rna* λ^+	8
PR11	F^- *ara leu lacY purE trp his argG malA xyl mtl ilv metA(B) thi spc-13 aroE353 rpsL*	14
BR273	Hfr (Cavalli) *rel-1 pheA valS*(Ts) $T2^r$ *tonA22 rpsH rpmD*	10
TS10-21	Like PR11, "two-step" tiamulin resistant	This work
TS15-23	Like PR11, "two-step" tiamulin resistant	This work
TS14-13-10	Like PR11, "three-step" tiamulin resistant	This work

was recentrifuged for 15 h at 110,000 × *g* in an ultracentrifuge through 28 ml of a sucrose cushion. This solution contained 1.1 M sucrose, 20 mM Tris-hydrochloride (pH 7.8), 0.5 M ammonium chloride, 10 mM magnesium acetate, and 0.5 mM EDTA. The clear ribosomal pellets were dissolved in buffer A to give an absorbance at 260 nm (A_{260}) of 191 to 205, equivalent to a concentration of 4.7 to 5.0 μM. Binding of radioactive tiamulin hydrochloride to ribosomes was done by equilibrium dialysis in 100-μl vessels at 4°C as described previously (13). The ^3H-labeled drug, with a specific activity of 179.5 mCi/mmol, was prepared by F. Battig of the Sandoz Forschungsinstitut in Vienna. The ribosomes for these experiments were used either without pretreatment or after the addition of ammonium chloride to give a final concentration of 200 mM and subsequent heating to 40°C for 10 min.

RESULTS

Isolation of mutants resistant to tiamulin. Since *E. coli* exhibits a high intrinsic resistance to tiamulin, it was necessary to define the most appropriate medium for the isolation of mutants. Figure 1 shows the effects of different medium constituents on growth inhibition by tiamulin and shows that NaCl antagonized the inhibitory effect, whereas K_2HPO_4 enhanced it. With 200 μg of tiamulin per ml, there was slow but still exponential growth; the antibiotic was easily washed from the cells, and a short time after drug withdrawal, growth attained the pre-exposure rate.

For mutant isolation, rich medium TGYE was chosen. As already reported for *S. aureus* and *K. pneumoniae* (7), it was not possible also to isolate high-level resistant mutants of *E. coli* in a single selection step, neither spontaneously nor after mutagenesis with ethyl methane sulfonate. Tiamulin-resistant mutants appeared, however, on plates containing the antibiotic in concentrations between 100 and 150 μg/ml, which is about two- to threefold above the concentration preventing colony formation of wild-type cells. The frequency was between 20 and 50 colonies per 2 × 10^8 cells plated. Many of these single-step mutants were unstable or no longer viable upon restreaking on plates containing the antibiotic at the concentration previously used for isolation. We analyzed 50 stable isolates for resistance to tiamulin in the polyU-dependent polyphenylalanine synthesis; none of them, however, exhibited any increased in vitro resistance.

It has been reported that mutants with ribosomal resistance to several ribosome-targeted antibiotics can only be obtained by stepwise exposure to increasing drug concentrations (5, 18). We have therefore isolated a total of 40 independent mutants resistant to 250 μg of tiamulin per ml from strain PR11. Spontaneous mutants resistant against 100 μg of tiamulin per ml underwent mutagenesis with ethyl methane sulfonate and high-level resistant cells selected on plates with 250 μg of tiamulin per ml (frequency in the second step, 5 to 20 colonies per 2 × 10^8 cells plated). None of the single-step mutants could grow on tiamulin concentrations higher than 200 μg/ml; the two-step mutants were able to form colonies up to tiamulin concentrations between 300 and 600 μg/ml, depending on the mutant. In addition to these 40 two-step mutants, 13 mutants were isolated after

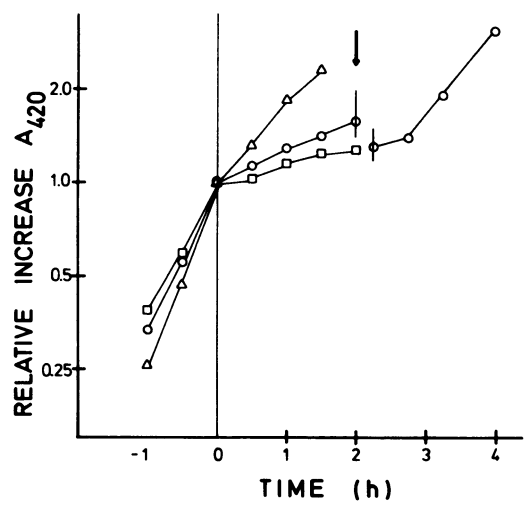

FIG. 1. Growth inhibition by tiamulin (200 μg/ml). The antibiotic was added at time zero. Symbols: (○) TGYE; (△) TGYE plus 2% NaCl; (□) TGYE plus 20 mM K_2HPO_4. All media were adjusted to pH 7.0. At the time indicated by the arrow, the cells were centrifuged from the TGYE culture and suspended in prewarmed (37°C) fresh medium lacking tiamulin.

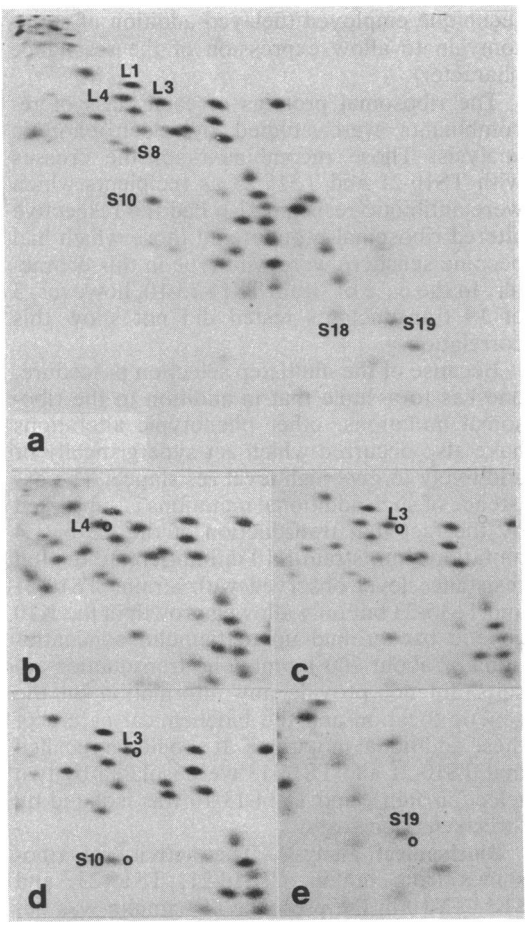

FIG. 2. Two-dimensional electropherograms of ribosomal proteins from *Escherichia coli* strains (a) PR11, (total 70S); (b) TS15-23 (section); (c) TS10-21 (section); (d) TE13-23 (section); and (e) TS14-13-10 (section). The positions of the relevant wild-type proteins are indicated by circles. The more negatively charged protein L4 of mutant TS15-23 can be recognized relative to a line connecting the centers of the L1 and S8 spots.

additional mutagenesis of a single two-step mutant and exposure to 500 μg of tiamulin per ml. (This particular two-step [parental] mutant used had a wild-type electrophoretic pattern of ribosomal proteins).

Electrophoretic analysis of ribosomal proteins. Ribosomal proteins were isolated from the 53 high-level tiamulin-resistant mutants and tested for any alteration by two-dimensional polyacrylamide gel electrophoresis. Several changes were detected. The electrophoretic migration of the altered ribosomal proteins from some of the mutants is shown in Fig. 2. There were three mutants with an altered large subunit protein L3, two mutants with an altered L4, and one mutant with two alterations, one in L3 and one in the small subunit polypeptide S10, among the 40 "two-step" selected mutants. Among the "three-step" resistant strains, there was one mutant with an alteration in ribosomal protein S19. Most of the mutants exhibited an increased resistance to other protein synthesis inhibitors acting at the 50S subunit (Table 2). It was not tested whether this cross-resistance is caused by ribosomal mutations or by some other not identified mutations.

All 53 mutants were able to grow aerobically at the expense of succinate as carbon source, indicating that they were not deficient in electron-transport mediated membrane energization. Mutants deficient in electron transport processes are frequently encountered among strains resistant to antibiotics which are accumulated by active transport (1).

Genetic analysis. Three mutants were chosen for further analysis, strains TS15-23 (altered protein L4), TS10-21 (altered protein L3), and TS14-13-10 (altered protein S19). Phage P1 lysates were prepared from the tiamulin-resistant strains and used to transduce streptomycin resistance into *E. coli* K10. The cotransduction frequency of tiamulin resistance and spectinomycin resistance with the selected marker (streptomycin resistance, Sm^r) was determined, and selected transductants were tested for coinheritance of tiamulin resistance and the ribosomal protein alteration. For mutants TS10-21 and TS15-23, the cotransduction frequencies (data not shown) indicate a map position for the tiamulin resistance marker either close to the spectinomycin resistance locus (*rpsE*) or between the *rpsE* and *aroE* markers (see Fig. 3). In the case of strain TS14-13-10, the data were in favor of a location of the tiamulin marker between *rpsE* and the streptomycin resistance locus (*rpsL*). Nine transductants from each cross were tested by electrophoresis, and there was a complete correlation between the antibiotic phenotype and the state of the ribosomal protein.

Since transductions in which Sm^r was select-

TABLE 2. MICs of tiamulin-resistant strains to ribosomal 50S inhibitors

Mutant	Altered proteins	MIC (μg/ml) of:			
		Tiamulin	Chloramphenicol	Erythromycin	Lincomycin
TS10-21	L3	400	4	100	200
TS15-23	L4	800	8	>400	400
TE13-23	L3, S10	400	2	25	300
TS14-13-10	S19	800	2	100	300
PR11 (parental)		100	4	25	200

TABLE 3. Transductional analysis of tiamulin resistance mutations[a]

Transduction	Distribution of unselected markers	%	Protein alteration
A19 × TS15-23	Spc^r Tia^r Sm^r	18	8/8 mut
	Spc^s Tia^s Sm^s	30	2/2 wt
	Spc^s Tia^r Sm^r	9	3/3 mut
	Spc^s Tia^s Sm^r	36	6/6 wt
	Spc^s Tia^r Sm^s	3	2/2 mut
	Spc^r Tia^s Sm^r	1	1/1 wt
	Spc^r Tia^r Sm^s	1	1/1 mut
	Spc^r Tia^s Sm^s	2	2/2 wt
BR273 × TS10-21	Spc^r Tia^r Sm^r	16	4/4 mut
	Spc^s Tia^s Sm^s	41	3/3 wt
	Spc^s Tia^r Sm^r	8	3/3 mut
	Spc^s Tia^s Sm^r	31	3/3 wt
	Spc^r Tia^r Sm^s	1	1/1 mut
	Spc^s Tia^r Sm^s	2	2/2 mut
	Spc^r Tia^s Sm^r	1	1/1 wt
BR273 × TS14-13-10	Spc^r Tia^r Sm^r	32	4/4 mut
	Spc^s Tia^s Sm^s	32	3/3 wt
	Spc^s Tia^r Sm^r	4	1/1 mut
	Spc^s Tia^s Sm^r	20	4/4 wt
	Spc^r Tia^r Sm^s	4	2/2 mut
	Spc^s Tia^r Sm^s	2	2/1 mut; 1 wt[b]
	Spc^r Tia^s Sm^r	5	2/1 mut[b]; 1 wt
	Spc^r Tia^s Sm^s	1	1/1 mut[b]

[a] Selection was for $aroE^+$; 200 transductants each were analyzed. Spc^s, Spc^r, Sm^s, Sm^r, Tia^s, and Tia^r denote sensitivity and resistance to spectinomycin, streptomycin, and tiamulin, respectively. The first entry gives the total number of transductants of this class tested by electrophoretic analysis, the second entry the number thereof being wild-type (wt) or mutationally altered (mut). In the crosses with strain BR273 (which harbors mutations in ribosomal proteins S8 and L30), the distribution of these mutations among recombinants was in accord with published data (4) and supported the location of the tiamulin resistance marker discussed.

[b] Recombinants from strain TS14-13-10 which showed segregation between the S19 alteration and tiamulin resistance.

ed gave ambiguous results, further transductions were made in which $aroE^+$ was selected (Table 3). The tiamulin-resistant strains (which are *aroE*) were used as recipients, and cotransduction of *aroE* with resistance to spectinomycin, tiamulin, and streptomycin was analyzed. In this case, the linkage of the tiamulin resistance markers was in agreement with the known map position of the L3 and L4 cistrons (Fig. 3), indicating that the cotransduction statistics of the previous crosses (Sm^r selection) were biased, possibly because of (i) reduced viability of some types of transductants or (ii) the selection technique employed (delayed addition of streptomycin to allow expression of the resistance character).

The ribosomal proteins of each class of recombinants were studied by electrophoretic analysis. Those recombinants of the crosses with TS10-21 and TS15-23 as recipients which were antibiotic resistant also had the respective altered ribosomal protein, and those which had become sensitive were wild type in this character. In the case of strain TS14-13-10, however, 3 of 19 transductants tested did not show this correlation.

Because of the multistep selection procedure, one has to assume that in addition to the ribosomal mutations, other phenotypic alterations have also occurred which act synergistically or additively to give high-level resistance. The existence of such additional mutations is supported by the fact that transduction of the L3 or L4 mutations into strain K10 did not create the full resistance level observed with strains TS10-21 and TS15-23 but only allowed growth in the K10 genetic background up to tiamulin concentrations of about 200 μg/ml. The transduction results did not provide any information on the genetic location and the biochemical nature of these additional mutations. It should be recalled that TS10-21 and TS15-13 were isolated by two selection steps, and TS14-13-10 was isolated by three selection steps.

Biochemical analysis. The activity of ribosomes from mutants TS10-21, TS15-23, and TS14-13-10 in the presence of tiamulin was determined to further confirm the ribosomal nature of the resistance mutations and to attribute it to alterations of either the 30S or the 50S subunit. Figure 4 shows the effect of different tiamulin concentrations on polyU-dependent polyphenylalanine synthesis. In the presence of 8 mM Mg^{2+}, the polypeptide synthesis by the L3 and L4 mutant extracts was distinctly less sensitive to tiamulin inhibition. At 12 mM Mg^{2+}, however, the difference nearly disappeared for the L4 mutant, and the L3 extracts were repeatedly

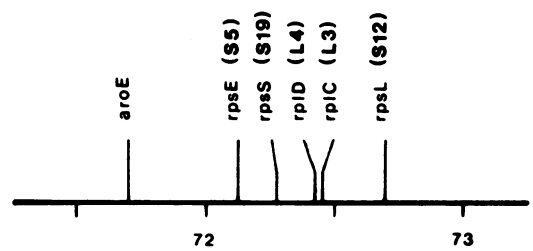

FIG. 3. Map of the *E. coli aroE-rpsL* chromosome segment (4), giving the relative position of markers used in this study.

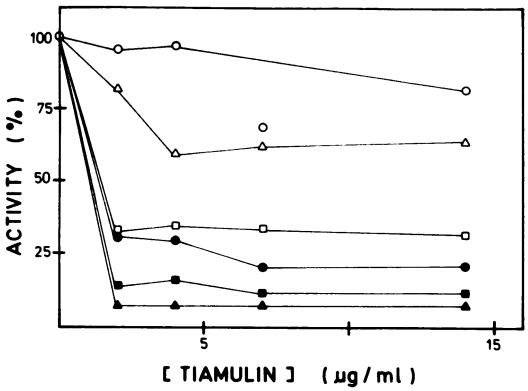

FIG. 4. Effect of tiamulin on polyU-dependent polyphenylalanine synthesis in the presence of 8 mM Mg^{2+} (open symbols) or 12 mM Mg^{2+} (closed symbols). Symbols: (○,●) TS15-23; (△,▲) TS10-21; (□,■) PR11. Ten A_{260} units of a 30,000 × *g* supernatant extract were used.

found to be more sensitive than wild-type extracts.

Ribosomal subunits were isolated from the parental strain PR11 and from mutants TS15-23, TS10-21, and TS14-13-10 and tested in all possible combinations for inhibition by tiamulin, in both the polyU- and the R17 phage RNA-dependent systems (Table 4). In each case, the initiating system (R17) was more affected by tiamulin than was the elongation (polyU) system. With TS10-21 and TS15-23, resistance was clearly associated with the 50S subunit. Ribosomes from strain TS14-13-10, however, were only slightly more resistant than those from strain PR11, and resistance was a property of the 50S subunit.

Binding studies. The ribosomes from the mutants bound tiamulin less strongly than did those of the wild type. Association constant (K_{ass}) values calculated from Scatchard plots (Fig. 5) were 5×10^4 M^{-1} for the L4 mutant TS15-23, 6×10^5 M^{-1} for the L3 mutant TS10-21, and 8×10^5 M^{-1} for the S19 mutant TS14-13-10. The estimation of a K_{ass} of 9.2×10^6 M^{-1} for the wild-type ribosomes from strain PR11 is close to the values published previously (13). The wild-type as well as the mutant ribosomes showed saturation of drug binding at an approximately 2:1 ratio of tiamulin over ribosomes. When wild-type ribosomes are depleted of ammonium ions, they suffer a dramatic loss in their binding capacity for tiamulin. This binding can be restored to normal values when the ribosomes are heated in the presence of 200 mM NH_4Cl (13).

When the mutant ribosomes were heated to 40°C for 10 min in the presence of 200 mM ammonium chloride, an increased binding for tiamulin ensued (Fig. 5). This effect was observed with all three classes of mutant ribosomes and led in two cases, TS14-13-10 and TS10-21, to binding constants of 5.4×10^6 and 4.6×10^6 M^{-1}, respectively, which are close to those measured with the wild-type ribosomes. Moreover, mutant ribosomes treated in such a way showed cooperative binding behavior. The ribosomes from the L4 mutant, TS15-23, could be also induced to increased tiamulin binding ($K_{ass} = 1.3 \times 10^6$) by the heating procedure, although in this case, the affinity was still lower by almost a factor of 10 as compared with wild-type ribosomes.

In addition to the results shown in Fig. 5, another, as yet genetically uncharacterized, tiamulin-resistant mutant derived from *E. coli* D10 was found to possess ribosomes with similar properties. They bound tiamulin with a K_{ass} of 2×10^5 M^{-1}, but the value increased to 4.3×10^6 M^{-1} after heating with NH_4Cl. Thus, the partial restoration of tiamulin binding by the heat treatment of resistant ribosomes may be a general phenomenon.

DISCUSSION

Like tetracycline (19) or the 2-deoxystreptamine aminoglycosides (5, 18), tiamulin is one of the ribosome-targeted antibiotics which does not

TABLE 4. Effect of tiamulin on polypeptide synthesis in the polyU- and phage R17 RNA-dependent systems[a]

Source of ribosome		Tiamulin (µg/ml)	Amino acid incorporation (cpm)	
30S	50S		R17	PolyU
PR11	PR11	0	3,010	30,000
PR11	PR11	0.8	590	12,050
PR11	TS15-23	0	2,420	24,900
PR11	TS15-23	0.8	1,350	21,850
TS15-23	PR11	0	2,860	29,250
TS15-23	PR11	0.8	470	8,310
TS15-23	TS15-23	0	3,110	24,400
TS15-23	TS15-23	0.8	1,270	18,090
PR11	TS10-21	0	3,900	31,500
PR11	TS10-21	0.8	1,470	20,810
TS10-21	PR11	0	3,580	32,240
TS10-21	PR11	0.8	740	12,850
TS10-21	TS10-21	0	3,850	32,440
TS10-21	TS10-21	0.8	1,220	20,380
PR11	TS14-13-10	0	3,750	26,320
PR11	TS14-13-10	0.8	1,060	14,000
TS14-13-10	PR11	0	3,410	31,860
TS14-13-10	PR11	0.8	540	14,110
TS14-13-10	TS14-13-10	0	3,600	26,550
TS14-13-10	TS14-13-10	0.8	1,070	16,340

[a] PolyU system: 3 A_{260} units, 30S, and 6 A_{260} units, 50S subunits. 8 mM Mg^{2+}. R17 system: 1 A_{260} unit, 30S, and 2 A_{260} units, 50S subunits. The 100,000 × *g* supernatant was from wild-type PR11 in each case.

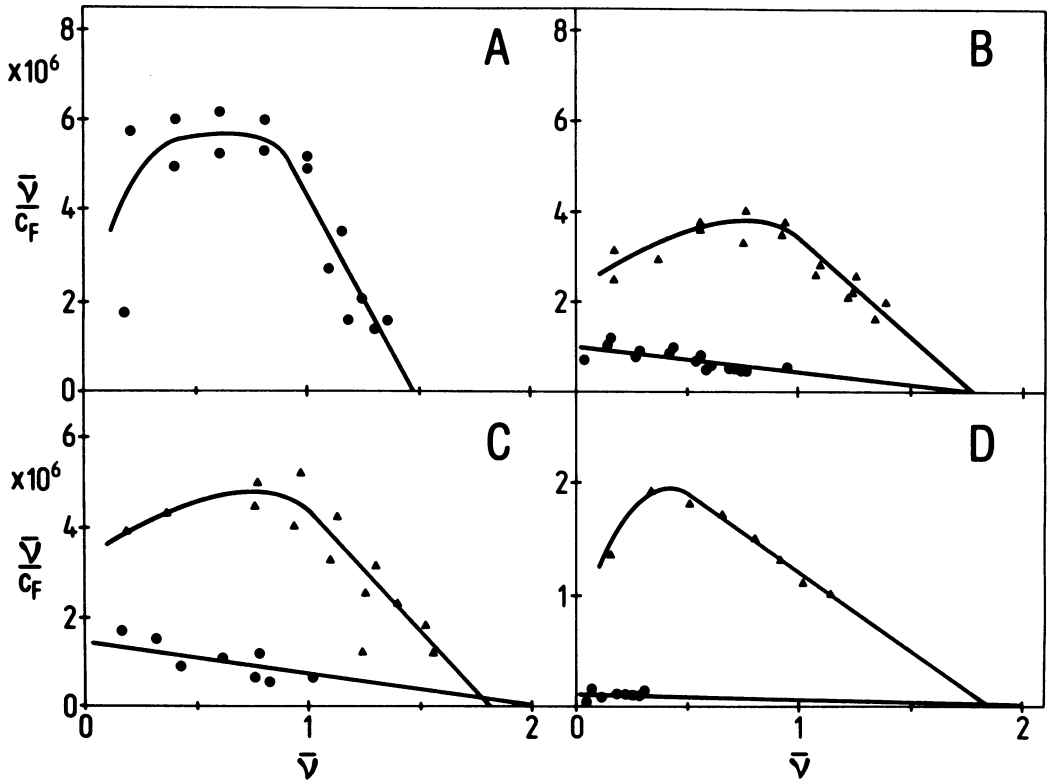

FIG. 5. Binding of [^{3}H]tiamulin to wild-type and mutant ribosomes. Scatchard plots, based on equilibrium dialysis experiments, are shown. (A) Ribosomes from PR11 (wild-type) cells; (B) ribosomes from TS10-21 (L3 mutant) cells, (C) ribosomes from TS14-13-10 (S19 mutant) cells; and (D) ribosomes from TS15-23 (L4 mutant) cells. Symbols: (●) ribosomes without pretreatment; (▲) ribosomes treated with 200 mM NH_4Cl at 40°C. Association constants given in the text were calculated from the tangential extrapolation of the straight part of the curves. $\bar{\nu} = C_b/C_r$, where C_b is the concentration of bound tiamulin and C_r is the concentration of the ribosomes, typically 4.8 μM. C_f is the concentration of free tiamulin.

permit the selection of single-step, high-level resistant mutants. This may be due to either one of the following reasons: (i) the existence of multiple binding sites for the drug, each of which has to be desensitized by a mutation to provide high level resistance, or (ii) the participation of several ribosomal components in the formation of the antibiotic binding site and the necessity to mutationally alter several of them to attain desensitization.

The multistep nature of tiamulin resistance in laboratory strains complicates a straightforward genetic and biochemical analysis for several reasons. To begin with, ribosomal mutants are scarce among single-step resistant strains since there are other ways to confer this low level of resistance. In fact, it has been shown in the case of gentamicin, which resembles tiamulin in the generation of a multistep resistance property, that the majority of single-step mutations affect the permeability of cells and the uptake of the drug (1, 18). This can be the result of mutations at many different loci (1, 18); when the external antibiotic concentration is increased above the critical resistance level provided by these permeability mutations, an increased influx into the cells occurs, and only those organisms survive which have acquired a target site mutation (1, 5). The apparent scarcity of ribosomal mutants among the single-step tiamulin-resistant strains indicates that the same selection mechanisms may be operative. Furthermore, the low resistance level of each mutational step does not allow the direct selection of tiamulin-resistant recombinants in genetic crosses because of the high background of growth on plates. Finally, it is difficult to prove that a ribosomal mutation detected in a multiply mutated genetic background contributes to the resistance. Only reconstitution of functional subunits from the mutated protein of the resistant strain and the residual components of the sensitive wild type would provide definitive proof.

Despite these inherent difficulties, we believe

we have shown that alterations of the ribosomal proteins L3 and L4 are responsible for the occurrence of some ribosomal resistance phenomena in several strains. The fact that tiamulin resistance and the L3 or L4 mutation cosegregate in transductional crosses is a strong argument, although the number of transconjugates which could be analyzed by two-dimensional electrophoresis was not statistically large enough to provide absolute proof. Also in support of our assumption are the results of genetic mapping experiments. Tiamulin resistance mutations could be shown to map in accordance with the genetic loci for the ribosomal proteins L3 and L4. The fact that there were four L3 and two L4 mutants among 53 resistant strains analyzed argues against a purely statistical co-mutagenesis and thus lends further support to the theory that L3 and L4 alterations are responsible for tiamulin resistance. Additional support can be deduced from biochemical investigations. The functional analysis of reconstituted ribosomes showed that it is a component of the 50S subunit, which is responsible for tiamulin resistance of two in vitro protein-synthesizing systems. Moreover, many mutants of *E. coli* resistant to erythromycin possess cross-resistance to tiamulin (12; unpublished data), and, as with tiamulin resistance, an altered L4 protein may confer resistance to this antibiotic (20).

For the purpose of an easy genetic analysis, tiamulin-resistant mutants were isolated by using a strain containing *rpsL* and *rpsE* mutations. It has been shown that *rpsL* and *rpsE* mutations, when present together, may mask the expression of the erythromycin resistance mutations in the *rplD* (L4) cistron (2). This complication was not encountered in our experiments, since the transductional crosses listed in Table 3 generated recombinants with all possible combinations of these ribosomal markers.

The resistance of the ribosomes to tiamulin undoubtedly results from a reduced binding capacity for the antibiotic. As our experiments show, the two ribosomal binding sites for the drug were conserved in the ribosomes of all the mutants analyzed. Hence, it is clearly the avidity of the ribosomes rather than the number of binding sites that is reduced in the ribosomes extracted from the various mutant strains. It is interesting that whereas the ribosomes from the wild-type strain showed the expected cooperative binding behavior as expressed by the upward bend in the curve of the Scatchard plot, the data from the mutant ribosomes gave essentially straight lines. This seems to indicate that the mechanism of binding of tiamulin to the mutant ribosomes is different. Removal of ammonium ions from *E. coli* ribosomes destroys the tiamulin binding capacity. It can be reactivated by a brief exposure to heat in the presence of ammonium ions (13). This effect had been explained by the existence of two interchangeable configurations of the ribosomal tiamulin specific binding site. Untreated wild-type ribosomes were not stimulated to additional binding after being subjected to the renaturation procedure (13).

However, mutant ribosomes could be induced to bind tiamulin more strongly after the heat treatment. We explain these effects with the presence of mutationally altered ribosomal components, which, when assembled within the cell, give rise to particles which are functional in polypeptide synthesis but have lost much of their avidity for tiamulin, resulting in the resistance character. However, the exposure of such ribosomes to elevated temperatures in the presence of ammonium ions is instrumental in rearranging the ribosomal topography to a state resembling that of the wild-type particles. Possibly, the mutant ribosomes are assembled in a strained configuration which is relieved upon exposure to the conditions of renaturation. To our knowledge, this is the first example of the binding of an antibiotic to resistant ribosomes being enhanced by such a procedure. Whether the binding of other antibiotics to resistant ribosomes can be restored in a similar way remains to be determined.

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