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Mechanisms of R Factor R931 and Chromosomal Tetracycline Resistance in *Pseudomonas aeruginosa*

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The mechanism of tetracycline resistance mediated by R931 (a *Pseudomonas aeruginosa* R factor not yet successfully transferred to *Escherichia coli* recipients) was examined. In strain 931 (R931) (minimal inhibitory concentration [MIC] 200 μg/ml) significant tetracycline uptake did not occur until 100 μg of tetracycline per ml was included in uptake studies. The introduction of R931 into strain 280 resulted in a significant decline in ^{3}H-tetracycline uptake. In both strains 931 (R931) and 280 (R931), a further reduction in tetracycline uptake resulted from pre-incubation with 1 μg of tetracycline per ml. Tetracycline resistance in R$^-$ *P. aeruginosa* strains 1731, 1885, and 494, considered to be of chromosomal origin, was associated with a lack of tetracycline uptake until the MIC of the strain was obtained. No evidence of tetracycline inactivation or ribosomal resistance was detected in R$^-$ or R$^+$ strains. The MIC for R$^-$ strains was generally about 25 μg/ml and that for R$^+$ strains was 75 to 200 μg/ml.

The R factor 931 is an example of a group of R factors detected in strains of *Pseudomonas aeruginosa*, which are unusual in that they fail to transfer to *Escherichia coli* or *Proteus mirabilis* but act as a naturally occurring, high-frequency transfer system when *P. aeruginosa* recipients are used in mating (4). Thus R931 appears to be of unique origin relative to previously studied R factors. The mechanisms of R931-mediated streptomycin and tetracycline resistance are of considerable interest for purposes of comparison with those mediated by other plasmids (6, 9). Such information could be used as clues to the origin of r-determinants carried on diverse R factors (5). It may also be of use in design of drugs effective against R factor-mediated antimicrobial resistance. The mechanism of R931-mediated streptomycin resistance has been shown to be phosphorylation of streptomycin (3). The mode of plasmid or chromosomal tetracycline resistance is unknown in *P. aeruginosa*. The results of an examination of such tetracycline resistance are reported in this paper.

MATERIALS AND METHODS

Organisms. The characteristics and sources of bacterial strains are as previously described (3, 10). The strains used are listed in Table 1 except for strain 1330. The latter is an R$^-$ (R factor negative) tetracycline-resistant strain. Strains with a minimal inhibitory concentration (MIC) of more than 10 μg of tetracycline per ml are considered resistant. R$^-$ resistant strains (1731, 1885, 494, and 1330) are considered chromosomally resistant.

Media. Media used were Trypticase soy (TS) broth and agar (TSA) (BBL).

MIC determinations. A conventional tube dilution method described previously (10) was used.

Tetracycline uptake determination. The procedure used for tetracycline uptake was as previously described for the study of streptomycin uptake (10) except that ^{3}H-tetracycline (3.2 mg/mCi, New England Nuclear Corp.) was substituted for streptomycin. Uptake values for tetracycline have been corrected for nonspecific binding of tetracycline and are given as ng/10^9 cells.

Examination for inactivation of tetracycline. *P. aeruginosa* strains 931 (R931) and 494 were exposed, respectively, to 30 and 15 μg of tetracycline (containing 10^7 dpm of ^{3}H-tetracycline) per ml under the following conditions. Bacteria were inoculated into 20 ml of TS broth in a 125-ml flask to an initial density of 0.1 A_{550} units (Beckman DBG spectrophotometer). The mixture was incubated in a shaking water bath at 37 C for 3 h. The final density was adjusted to 0.4 A_{550} units with TS broth. The cells were rapidly chilled in an ice-water bath and centrifuged at 4 C at 2,000 × *g* for 15 min, and the pellet was washed twice with 25 ml of cold TS broth. The final pellet, containing about

10^{10} cells, was extracted with 2 ml of 0.1 N HCl-methanol (1:3) for 5 min at 25 C. The mixture was centrifuged at 2,000 × *g* (25 C) for 15 min, and the pellet was removed and re-extracted two additional times. The extraction process removed more than 95% of the radioactivity associated with the bacteria. The extract was concentrated by vacuum and chromatographed on silica gel-impregnated paper treated with 2% disodium ethylenediaminetetraacetic acid (EDTA) by using *n*-butanol-methanol-10% citric acid (4:1:2). The paper was dried, cut into 1-cm fractions, suspended in 5 ml of Omnifluor (Beckman Scientific Co.), and counted in a Beckman model LS-250 scintillation counter. Control preparations of ^{3}H-tetracycline treated as above were similarly chromatographed. The R_f value for ^{3}H-tetracycline was 0.60.

Bioassay for filtrates of cultures treated with tetracycline for 3 h were carried out as previously described (10). Fractions of the filtrate were also chromatographed as described above.

A 25-μliter drop containing about 1 μg of tetracycline in distilled water, extracted from strain 931, was applied to TSA inoculated with *Staphylococcus aureus* NCTC-6571 as for Kirby-Bauer antimicrobial testing (2). A 1-μg amount of standard tetracycline exposed to a bacterial suspension as described (but at 4 C) and otherwise identically extracted was applied separately to the same TSA as described above. Plates were incubated at 37 C for 18 h, and the zones of inhibition were determined (6 mm).

Amino acid incorporation studies. The method used for incorporation of amino acids was as previously described (10). The specific activity of the ^{14}C-valine was 50 μCi/μmol.

Detection of R factors. Methods for R factor detection were as described previously (3).

RESULTS

Tetracycline uptake studies. Tetracycline uptake has been shown to be an energy-requiring system in *E. coli* (6) and *S. aureus* (9). This was confirmed to be true also in *P. aeruginosa* (Fig. 1). Thus, in the absence of 0.025% NaN_3, there is an increase, with time, in the amount of ^{3}H-tetracycline associated with strain 280; in the presence of 0.025% NaN_3 at 4 C the tetracycline accumulation by strain 280 is eliminated. A control study was carried out to confirm that tetracycline does not leak from the bacterium at the low temperature used in washing procedures as reported for α-methyl glucoside in *P. aeruginosa* (M. Midgley, Proc. Biochem. Soc., p. 2, 1972). Less than 2% of the labeled tetracycline accumulated by strain 280 at 20 or 100 μg of tetracycline per ml under conditions described is lost upon subsequent storage at 4 C in TS broth for 30 min. However, storage at 37 C for 30 min under the same circumstances results in a loss of 95% per ml of accumulated label when the uptake is carried out at 10 μg of tetracycline per ml; the loss decreases to 75%

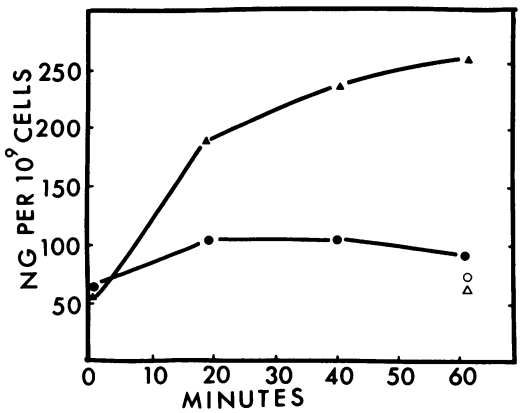

FIG. 1. *Kinetics of uptake of tetracycline by strains 280 and 494 in TS broth at a tetracycline concentration of 10 μg/ml. Symbols: ▲, strain 280; ●, strain 494, △, strain 280 with NaN_3 (0.025%) at 0 to 4 C; ○, strain 494 with NaN_3 at 0 to 4 C.*

when the uptake tetracycline concentration is 100 μg/ml. Thus, the cell-associated radioactivity obtained with 0.025% NaN_3 at 4 C under our experimental conditions represents background levels which appear to be nonspecific binding of tetracycline to the bacterium.

Figure 1 also illustrates the tetracycline uptake pattern at a concentration of 10 μg/ml with representative susceptible and resistant strains. It may be observed that the amount of tetracycline associated with strain 494 increases only slightly with time and that the total amount of tetracycline associated with that strain is approximately equal to that of strain 280 examined at 4 C in the presence of 0.025% NaN_3.

The tetracycline uptake of several strains of *P. aeruginosa* at various concentrations of tetracycline is seen in Table 1. Several points are apparent from that table. In general, the concentration of tetracycline used in uptake studies must approximate the MIC of a R^- (R factor negative)-resistant strain before tetracycline uptake is equivalent to that of susceptible strains 280 and 2379 at 10 μg of tetracycline per ml. When the concentration of tetracycline used in uptake studies exceeds a strain's MIC, the tetracycline uptake increases very rapidly, as is seen with strain 494. A second point is the marked reduction in tetracycline uptake produced by acquisition of the *Pseudomonas* R factor, 931. Thus strain 280 (R931), obtained by mating strains 931 (R931) and 280, displays about one-quarter the uptake shown by strain 280 at 10 μg of tetracycline per ml.

Data from Table 1 also establishes the preincubation of resistant R^+ (R factor containing) strains with subinhibitory tetracycline concen-

TABLE 1. *Uptake of tetracycline by strains of P. aeruginosa at various tetracycline concentrations*

Strain	MIC (μg/ml)	Uptake[a] at tetracycline concn of:						
		10[b]	17.5	20	25	50	100	200
R⁻280	10	*206.3*	—	—	—	—	—	—
2379	10	*238.8*	—	—	—	—	—	—
1731	22.5	29.9	—	*163.7*		—	—	—
1885	25	25.4	68.1	—	*352*	—	—	—
1885 pre-treated[c]	—	—	—	—	*302*	—	—	—
494	25	30.0	—	—	*298*	2,114.4	—	—
R⁺280 (R931)	75	56.3			100	*245*	—	—
280 (R931) pre-treated	—					*140.4*	—	—
931 (R931)	200	11.6	—	—	—	19.7	1,612.8	
931 (R931) pre-treated	—	—	—	—	—	—	175	*1,336*

[a] Uptake expressed in ng per 10^9 cells. Values italicized represent tetracycline uptake at or about the MIC.
[b] Tetracycline concentration in micrograms per milliliter.
[c] Pre-treated strains were exposed to 1 μg of tetracycline per ml for 3 h at 37 C prior to uptake determinations.

trations reduces tetracycline uptake. The most marked example is strain 931, but reduction also occurs with 280 (R931). In these R^+ strains, the correlation of MIC and tetracycline uptake is best shown by the uptake values obtained when strains are pre-incubated with low subinhibitory tetracycline concentrations. A negligible effect of preincubation is seen with the R^- strain 1885 in which the tetracycline resistance is presumed to be chromosomal.

Amino acid incorporation studies. There is no evidence to suggest that ribosomes are insensitive to tetracycline in any strain examined (Table 2). The presence of the R factor, R931, does not influence ribosomal susceptibility to tetracycline.

Inactivation of tetracycline. Several procedures were used to examine for inactivation of tetracycline by resistant strains. Bio-assays of cell-free supernatant fluids after 3 h of exposure at 37 C of strains 931 and 494 to 30 and 15 μg of tetracycline per ml, respectively, demonstrated that no detectable reduction in the bio-activity of tetracycline occurred. Tetracycline was extracted from resistant bacteria which had been exposed to tetracycline and subjected to paper (silica gel-impregnated) chromatography. The R_f value obtained for extracted tetracycline from strain 494 and the R factor-carrying strain 931 was 0.6. That value was identical to a tetracycline standard subjected to the same extraction and chromatography protocol. Extracted tetracycline from strain 931 retains biological activity similar to a tetracycline standard when examined by plate diffusion as described. Thus, there is no evidence of any altered form of tetracycline. In addition, from these data it seems unlikely that the explanation for differences in permeability between susceptible and resistant strains could be accounted for by the modification of tetracycline to a more rapidly excretable form.

DISCUSSION

The spectrum of susceptibility of *P. aeruginosa* to tetracycline covers a range of MIC values (10–100 μg/ml). Strains bearing transferable resistance markers for tetracycline are usually resistant to high concentrations (>100 μg/ml). However, such strains comprise less than 10% of the 50 strains tested. Most of our strains isolated from clinical specimens show tetracycline susceptibility values between 10 and 30 μg/ml. Strains which have an MIC of more than 10 μg/ml are considered clinically resistant to tetracycline.

TABLE 2. *Inhibition of amino acid incorporation by tetracycline in cell-free systems obtained from strains of P. aeruginosa*

Expt no.	Strains[a]		Incorporation of ^{14}C-valine (counts/min)[b]	
	S100	Ribosomes	Control	With tetracycline[c]
1	1731	1731	940	263
	1731	1330	1,096	191
	1731	931	860	110
2	931	1731	1,046	272
	931	931	1,368	174
3	280	280	1,752	226
	280	1330	830	162

[a] Bacterial strains used as sources of the ribosomes or S100 fractions (100,000 × *g* supernatant fraction) which were prepared as described previously (4).
[b] Background counts per minute were 100 to 125.
[c] Concentration of tetracycline was 20 μg/ml.

The work described in this paper indicates that there is a close correlation between the MIC of a strain and its uptake of tetracycline. The ribosomes obtained from representative strains in our series are all equally sensitive to tetracycline in the inhibition of amino acid incorporation. Incubation of labeled tetracycline with resistant strains did not result in detection of any tetracycline which posses reduced biological activity or altered chromatography characteristics. A susceptible strain, which acquired a transferable resistance marker for tetracycline [280(R931)] also shows decreased permeability to the drug. When strains of *P. aeruginosa* bearing a transferable factor for resistance to the tetracycline are grown in a low subinhibitory concentration of tetracycline (1 μg/ml), there is a marked fall in the accumulation of tetracycline by the cells. Since such adaptation phenomena occurred with very low concentrations of the drug and short pre-incubation times, the possibility that more resistant cells were being selected from a heterogeneous population of cells could be excluded. Thus it seems reasonable to assume that resistance to tetracycline in *P. aeruginosa* is due to diminished permeability to the drug. Similar observations have been reported for *E. coli* (6) and *S. aureus* (7, 8).

Despite the apparent origin of R931 in *P. aeruginosa*, the mechanisms of resistance to streptomycin and tetracycline are the same as those of R factor-mediated resistance in *E. coli* (6, 8). It seems possible that r determinants may be derived from a common source but that a distinct conjugation system has evolved in *P. aeruginosa*. It is also possible that there exists a limited number of methods by which R factor-mediated resistance can be expressed. It would be of interest to examine a variety of characteristics of the various streptomycin phosphotransferases to determine their degree of relatedness and thus the probability of common origin. In the case of R factor or episomal tetracycline resistance, there is evidence reported here and elsewhere (6, 9) that an inducible inhibition of tetracycline uptake occurs. Avtalion et al. (1) have reported serological evidence for such an inhibitor. Further work to examine serological cross-reactivity of the inhibitors from different sources would be of interest for the above reasons.

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