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Chelocardin-Inducible Resistance in *Escherichia coli* Bearing R Plasmids

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Two plasmid-linked tetracycline resistance characters, *tet* A and *tet* B, were distinguishable in part, according to the level of resistance they conferred to minocycline (<3 μg/ml for *tet* A; >6 μg/ml for *tet* B). *Escherichia coli* K-12 strains that harbored the *tet* B character were also resistant to tetracycline but susceptible to chelocardin. In such *tet* B strains, subinhibitory concentrations of tetracycline could induce resistance to chelocardin as well as to otherwise inhibitory concentrations of tetracyclines. Chelocardin itself was ineffective as an inducer and therefore could be used to select constitutively resistant mutants. *E. coli* K-12 strains harboring the *tet* A character were also resistant to tetracycline and susceptible to chelocardin; tetracycline did not induce resistance to chelocardin in these strains.

Two classes of tetracycline resistance characters mediated by R plasmids in *Shigella flexneri* have been postulated by Kabins and Cohen (10) on the basis that some isolates gave an unusual pattern of growth around tetracycline disks. This pattern, referred to as B^+, was characterized by a zone of partial inhibition and an inner zone of growth concentric with the disk. Scavizzi (15) differentiated two characters mediated by plasmids, *tet* A and *tet* B, according to the minocycline resistance levels in enteric bacteria. *Escherichia coli* K-12 harboring the *tet* B resistance character originating from different R plasmids is inhibited by up to 6 μg of minocycline per ml; when tested by the disk method, the pattern of growth around the disk is similar to the B^+ phenomenon.

Plasmid-linked tetracycline resistance in *E. coli* is inducible by preexposure to subinhibitory concentrations of tetracycline (7, 9). The increase in resistance level to tetracycline appears to be much higher in *E. coli* harboring the *tet* A character than in *E. coli tet* B. Minocycline resistance is poorly inducible by tetracycline in *E. coli* harboring R plasmids mediating either character.

β-Chelocardin (14) is a tetracycline-like antibiotic binding to 30S ribosomal subunits as does tetracycline (12). Chelocardin is active against some tetracycline-resistant strains (14). The present paper deals with studies performed to evaluate the activity of chelocardin on *E. coli* K-12 bearing R plasmids that belong to different compatibility groups (4, 5) and code for *tet* A and *tet* B characters. Furthermore, in preliminary studies on chelocardin activity by the liquid dilution method, there appeared a secondary growth in tubes containing inhibitory concentrations of chelocardin only with *tet* B strains and but not with *tet* A strains. This secondary growth was due to bacteria stably resistant to chelocardin, and this character was subsequently transferred between *E. coli* K-12 strains.

These observations suggested that these bacteria might be constitutive mutants resistant to chelocardin and that, at least in *tet* B *E. coli* K-12, chelocardin resistance might be induced by other tetracyclines, as in *Staphylococcus aureus* harboring R plasmids mediating inducible Ero resistance (17).

In this report we describe studies of the effects of tetracycline-chelocardin combinations, the kinetics of induction by tetracycline, and the rate of mutation to chelocardin resistance of *tet* A and *tet* B *E. coli* K-12.

MATERIALS AND METHODS

Antibiotics. Chelocardin (potency 816 μg/mg) was supplied by Abbott Laboratories, Queensborough, England, oxytetracycline (905 μg/mg) and doxycycline (860 μg/mg) by Pfizer Laboratories, Massy, France, demethylchlortetracycline (1,000 μg/mg) and tetracycline (1,000 μg/mg) by Specia Laboratories, Paris, and minocycline (807 μg/mg) by Lederle Laboratories, Pearl River, N.Y. In serial dilutions, working solutions were corrected according to these potencies.

Bacterial strains. We used *E. coli* K-12 J5 $F^-pro^-met^-lac^+$ (from N. Datta) resistant to nalidixic acid bearing the following R plasmids coding for *tet* A or *tet* B characters (15) and belonging to

different compatibility groups according to the denomination of Datta (5) and Chabbert et al. (4): R IP7 (*str kan sul tet* A); R IP15 fi$^+$ group F$_{II}$ (*str chl sul tet* A); R IP111 fi$^-$ group N (*tet* A); R IP24 fi$^+$ group F$_{II}$ (*tet* B); R IP71a fi$^-$ group 9 (*amp chl sul tet* B); and R IP69 fi$^-$ group 7-M (*amp kan tet* B). (Symbols are as follows: amp, resistance to ampicillin; str, to streptomycin; kan, to kanamycin; chl, to chloramphenicol, sul, to sulphonamide, tet, to tetracycline.)

Determination of 50% inhibitory concentration (IC$_{50}$). In Mueller-Hinton agar (Institute Pasteur Production, Paris), the International Collaborative Study's reference method (6) of dilution was modified to obtain the following progression of antibiotic content (dilution factor 1.25): 1, 1.25, 1.6, 2, 2.5, 3.2, 4. . . . Petri dishes were inoculated by spreading 3 to 400 bacteria per plate. After 18 h of incubation, colonies were counted with a Fisher bacterial counter model 480. The percentage of colonies growing on each antibiotic concentration was plotted on probability-log paper, and the 50% end point was graphically calculated.

Studies of tetracycline combinations by diffusion techniques. Paper disks (Schleicher and Schüll 2668) containing 30 μg of different tetracyclines per disk were placed at a 20-mm distance on inoculated plates. Paper strips impregnated with a 1,500-μg/ml solution of each tetracycline were placed perpendicular to each other on inoculated plates (3).

Induction of resistance. A dilution technique in Mueller-Hinton broth was devised to combine 2, 4, or 8 μg of chelocardin per ml with 0.4, 0.8, 1.6, or 3.2 μg of tetracycline per ml. Tubes were inoculated with 10^6 bacteria per ml and incubated at 37 C. Every 30 min, optical density was determined in a Klett-Summerson photocolorimeter.

Experiments on kinetics of induction and deinduction were performed according to Weisblum et al. (17). Erlenmeyer flasks containing 10 ml of Mueller-Hinton broth and 2 μg (5×10^{-6} M) of tetracycline were inoculated with 10^7 uninduced cells. Every 10 min, a 0.1-ml sample was withdrawn and diluted 10^{-4}-fold. One-tenth milliliter was plated on agar containing 200 μg (5×10^{-4} M) of tetracycline and 4 μg (10^{-5} M) of chelocardin per ml. Deinduction was studied by diluting 10^{-4}-fold cells grown for 4 h in tetracycline media and plating dilutions on the same test plates.

Isolation of "constitutive" mutants. Gradient plates (16) containing 0 to 100 μg of chelocardin per ml were inoculated with 10^9 bacteria. The mutation rate to chelocardin resistance of *E. coli* K-12 J5 R IP69 (*amp kan tet* B) was determined by a modification of Matney's (13) technique, using cellophane membranes (3).

RESULTS

Comparative activity of tetracyclines on E. coli K-12 bearing R plasmids. For the seven tetracyclines studied, the range of the standard deviation expressed as a percentage of the mean (coefficient of variation) of the IC$_{50}$ in four independent determinations performed with *E. coli* K-12 R IP69 (*tet* B) was 7 to 20%. For tetracycline determinations the variation coefficient was 13%. IC$_{50}$ values for *E. coli* K-12 J5 and *E. coli* K-12 bearing the six plasmids studied are shown in Table 1 and Fig. 1. The IC$_{50}$ values of *E. coli* K-12 J5 for oxytetracycline, tetracycline, demethylchlortetracycline, doxycycline, minocycline, and chelocardin did not differ greatly. The range of these IC$_{50}$ values was between 0.62 μg/ml for chelocardin and 1.08 μg/ml for doxycycline. The IC$_{50}$ value for tetracycline was modified differently according to the *tet* A or *tet* B character by introducing different R plasmids in *E. coli* K-12 J5. After transfer of R IP111 (*tet* A), R IP7 (*tet* A), and R IP15 (*tet* A), the IC$_{50}$ values were, respectively, 21, 38, and 45 μg/ml. After transfer of R IP69 (*tet* B), R IP24 (*tet* B), and R IP71a (*tet* B), the IC$_{50}$ values were higher: 90, 95, and 260 μg/ml, respectively. The same grouping appeared for doxycycline and minocycline.

The IC$_{50}$ of *E. coli* K-12 J5 to chelocardin was 0.62 μg/ml. The values for *E. coli* K-12 R IP7 (*tet* A), R IP111 (*tet* A), and R IP15 (*tet* A), 0.64, 0.66, and 0.68 μg/ml, were not significantly different. Conversely, the group of R plasmids coding for the *tet* B character increased the IC$_{50}$ value of *E. coli* K-12 (1.02, 1.12, and 1.25 μg/ml).

"Antagonism" between tetracyclines and chelocardin. Combination of chelocardin with the tetracyclines oxytetracycline, tetracycline, demethylchlortetracycline, doxycycline, and minocycline was studied by the diffusion method on plates inoculated with *E. coli* K-12 susceptible to all tetracyclines and *E. coli* K-12 bearing R IP7 (*tet* A), R IP15 (*tet* A), and R IP111 (*tet* A). The inhibition zone near the chelocardin strip was not modified by strips of other tetracyclines (Fig. 2). Conversely, the inhibition zones due to chelocardin strips were greatly modified by others strips with *E. coli* K-12 bearing the *tet* B character from R IP69, R IP24, and R IP71a (Fig. 2). This phenomenon, previously termed "antagonism" (2), appeared only with combinations of chelocardin and other tetracyclines; it was never observed with all possible combinations of tetracycline, doxycycline, and minocycline.

On plates containing inhibitory concentrations of chelocardin (2, 4, or 8 μg/ml) inoculated with *E. coli* K-12 R IP69 (*tet* B), a 30-μg/ml tetracycline disk was placed at the time of inoculation or later (18 h). Further incubation showed a zone of secondary growth around the disk. If a zone colony was picked up and cultivated in antibiotic-free medium through five generations and plated again, it appeared susceptible to chelocardin. These facts suggest an

TABLE 1. *Fifty percent inhibitory concentrations*

E. coli strain	Antibiotic						Antagonism of chelocardin activity by tetracycline	Selection of resistant mutants to chelocardin
	Oxytetracycline	Tetracycline	Demethylchlortetracycline	Doxycycline	Minocycline	Chelocardin		
K-12 J5 *nal*	0.89	0.77	0.75	1.08	0.97	0.62	0	0
R IP7 *tet* A	96	38	15	6	0.79	0.64	0	0
R IP15 *tet* A	126	45	18	8	2.91	0.68	0	0
R IP111 *tet* A	42	21	14	6	2.82	0.66	0	0
R IP24 *tet* B–	279	95	29	24	6.5	1.10	+	+
R IP71a *tet* B	396	260	85	42	14	1.25	+	+
R IP69 *tet* B	261	90	27	25	6.5	1.02	+	+

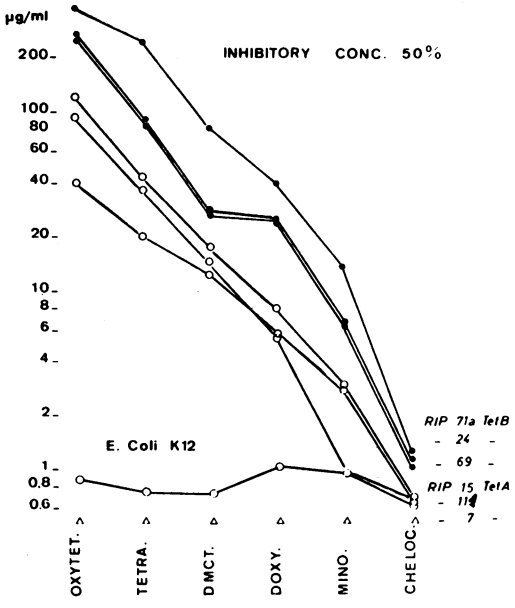

FIG. 1. *IC_{50} values of oxytetracycline (oxytet), tetracycline (tetra), demethylchlortetracycline (DMTC), doxycycline (doxy), minocycline (mino), and chelocardin (cheloc) for E. coli K-12 and E. coli K-12 harboring the following R plasmids: R IP71a (tet B), R IP124 (tet B), R IP69 (tet B), R IP15 (tet A), R IP111 (tet A), and R IP7 (tet A).*

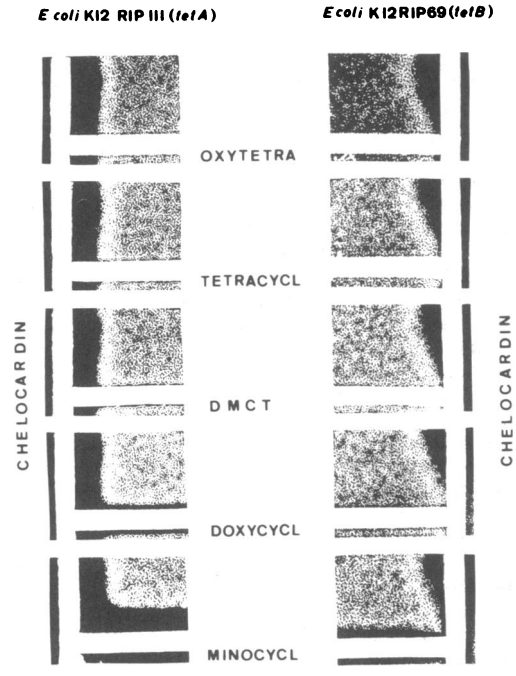

FIG. 2. *Effect of tetracycline-chelocardin combinations studied by a paper strip method on plates inoculated with E. coli K-12 R IP111 (tet A) and E. coli K-12 R IP69 (tet B).*

induction process to be studied in liquid media.

Kinetics of induction and deinduction. Kinetics of induction and deinduction were studied by the technique already used with *S. aureus* and erythromycin by Weisblum et al. (17) in flasks containing 5×10^{-6} M tetracycline inoculated with *E. coli* K-12 R IP111 (*tet* A) and *E. coli* K-12 R IP69 (*tet* B). Almost all of the bacterial population was able to grow on plates containing 2.5×10^{-4} M and 5×10^{-4} M tetracycline, respectively, after 10 min of incubation. Tetracycline resistance of both *tet* A and *tet* B appeared to be inducible by a subinhibitory concentration of tetracycline. Conversely, bacteria able to grow on plates containing tetracycline and 10^{-5} M chelocardin did not appear with *E. coli* K-12 R IP111 (*tet* A). In flasks containing *E. coli* K-12 R IP69 (*tet* B), bacteria able to grow on tetracycline and chelocardin appeared after 10 min of incubation, and the whole population was phenotypically resistant after 30 min (Fig. 3). If tetracycline was omitted from the test plate, induced cells failed to form colonies, After 2 h of incubation, flasks containing 5×10^{-6} M tetracycline were diluted 10^{-4}-fold. The induced culture was cultivated in

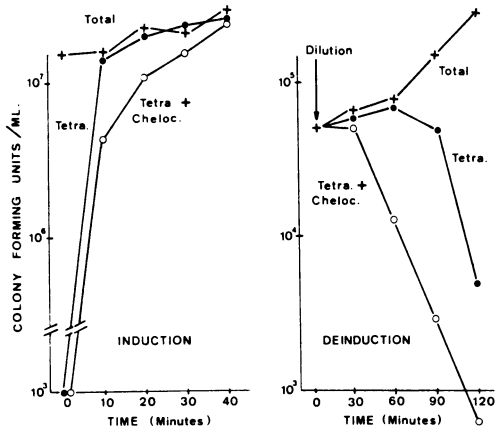

FIG. 3. *Kinetics of induction and deinduction. At the indicated time the number of bacteria able to grow on antibiotic-free agar and on agar containing 10^{-5} M chelocardin and/or 5×10^{-4} M tetracycline was calculated.*

antibiotic-free liquid medium and plated on agar containing inhibitory concentrations of tetracycline and chelocardin. The percentage of bacteria able to grow dropped suddenly after 60 min. This fact shows that such bacteria appear susceptible to chelocardin again after deinduction.

Mutants resistant to chelocardin. Selection of mutants resistant to chelocardin was comparatively studied with *tet* A and *tet* B strains on gradient plates containing 0 to 100 μg of chelocardin per ml and inoculated with 10^9 bacteria of *tet* A and *tet* B strains. No colonies or very few small colonies of the three *tet* A strains appeared in the inhibition area. Conversely, 300 to 400 colonies of normal size appeared in this zone with the three *tet* B strains. After isolation on antibiotic-free medium, these bacteria were stably resistant to chelocardin. By the membrane technique, the calculated mutation rate to chelocardin was 3×10^{-7} to 5×10^{-7} for *E. coli* K-12 R IP69 (*tet* B). The IC_{50} values of *E. coli* K-12 R IP69 (*tet* B) uninduced and mutant are shown in Fig. 4. The IC_{50} values of the mutants were 30 μg/ml. This level of resistance to chelocardin was stable after 50 generations. By conjugal transfer from a mutant of *E. coli* K-12 to a susceptible *E. coli* K-12, this level of resistance to chelocardin was easily transmitted.

DISCUSSION

The relative bacteriostatic activity of antibiotics belonging to the same group is hardly established by conventional determination of the minimal inhibitory concentrations because the standard deviation of this determination is rather high. According to the results of the International Collaborative Study report on antibiotic susceptibility testing (6), the standard deviation of the reference agar dilution method, in the case of tetracycline, for three strains studied by 12 laboratories was, respectively, 1.12, 1.21, and 1.00 $\log_2$. Consequently only two- or fourfold differences are significant. By determination of IC_{50} values, the standard deviation for tetracycline determination is 10-fold less. The relative activity of all the members of the tetracycline group of antibiotics appears to be clear-cut on *E. coli* K-12 bearing R plasmids coding for tetracycline resistance. The data presented here show a constant increase in activity of each new tetracycline derivative in the order they have been introduced in clinical practice. If the tetracycline group of antibiotics is ranked in the order oxytetracycline, tetracycline, demethylchlortetracycline, doxycycline, minocycline, and chelocardin, each drug appears more active on tetracycline-resistant strains than the preceding one. It has therefore been claimed that a new derivative is active against "tetracycline-resistant" strains. This assertion is strictly dependent on estimation of the critical level of discrimination between susceptibility and resistance. Despite

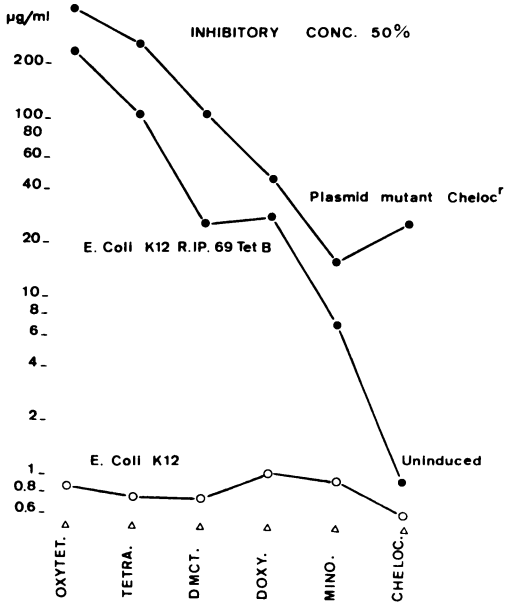

FIG. 4. *IC_{50} values to oxytetracycline, tetracycline, demethylchlortetracycline, doxycycline, minocycline, and chelocardin of E. coli K-12 R IP69 tet B uninduced and a mutant obtained on a gradient plate containing 0 to 100 μg of chelocardin.*

continuous increase in activity against any given R plasmid, the range of the phenotypic expression of resistance to minocycline of *E. coli* (*tet* B) is slightly higher than the range of *E. coli* R (*tet* A) to doxycycline.

Only *E. coli* K-12 harboring R IP7 (*tet* A) was as susceptible to minocycline as *E. coli* K-12 without an R plasmid. Chelocardin appeared to be the most active against all the six substrains of *E. coli* K-12 studied (all determinations were below 1.5 μg/ml). In addition, *E. coli* K-12 coding for the *tet* A character appeared to be as susceptible to chelocardin as *E. coli* K-12 J5. Conversely, the IC_{50} values of *E. coli* K-12 harboring a plasmid coding for *tet* B characters were slightly but significantly higher. The differences between the *tet* A and *tet* B characters were broadly confirmed by studies on inducibility of tetracycline resistance.

When tetracycline and chelocardin combinations were studied by diffusion techniques, using antibiotic paper strips containing antibiotics, a so-called "antagonism" appeared only for the three *E. coli* K-12 strains harboring plasmids coding for the *tet* B character. This kind of phenomenon has already been observed with a quite different group of antibiotics. In clinical isolates of *S. aureus* resistant to erythromycin but susceptible to spiramycin, Chabbert (2), using different kinds of diffusion techniques, noted that low concentrations of erythromycin apparently antagonize spiramycin activity. This observation has been subsequently extended to oleandomycin (8), lincomycin, and pristinamycin (component I) (1). In such strains, termed by Garrod (8) "dissociated" type, erythromycin resistance is inducible by a low concentration of erythromycin. Spiramycin and others macrolides, if inducers at all, are poor. In a combination of erythromycin and spiramycin obtained by various geometric arrangements of impregnated papers in agar diffusion or by checkerboard techniques in liquid media, erythromycin has been shown to induce resistance to macrolides. Constitutive mutants resistant to the macrolides are easily obtained (17).

Weisblum et al. (17) characterized in detail the process of induction and deinduction by erythromycin: the binding of [^{14}C]erythromycin 50*S* ribosome subunits from induced, uninduced, and constitutive resistant strains. Lai and Weisblum (11) were able to relate resistance to enzymatic methylation of 23*S* RNA.

The results presented here show that oxytetracycline, tetracycline, demethylchlortetracycline, doxycycline, and minocycline are able to "antagonize" chelocardin in *E. coli* K-12 bearing plasmids coding for *tet* B character. The same kind of phenomenon is observed in kinetics of induction and deinduction by tetracycline of chelocardin resistance performed by the method used by Weisblum for erythromycin. In addition, constitutive mutants resistant to chelocardin have only been obtained from *E. coli* bearing the *tet* B character.

Those facts lead us to the following conclusions: (i) the existence of two types of tetracycline resistance characters is confirmed; (ii) any study on resistance mechanisms to tetracyclines has to take into account the *tet* A and *tet* B character; (iii) the "antagonism" phenomenon described here is a useful tool for this discrimination; and (iv) in enteric bacteria coding for *tet* B character, resistance to tetracycline might be related to an inducible enzyme different from the enzyme responsible for tetracycline resistance in *E. coli* bearing the *tet* A character.

The inducible enzyme responsible for tetracycline resistance has been considered as affecting permeability to the drug. The striking similarity of the phenomena reported here with those observed with *S. aureus* and macrolides suggest that it would be interesting to investigate whether, in the case of the *tet* B character, the enzyme might be acting on the 30*S* ribosomal subunit.

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