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Positive Selection for Loss of Tetracycline Resistance

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A simple technique has been devised that allows direct plate selection of tetracycline-sensitive clones from a predominantly tetracycline-resistant population. The technique is especially useful in genetic methodologies based on the use of tetracycline resistance transposons, such as Tn*10*. Potential uses of the method include selection of deletion mutants, fine-structure mapping, generalized mapping, construction of multiply marked strains, elimination of tetracycline resistance transposons and plasmids, and cloning. The technique is based on our finding that tetracycline-resistant cells are hypersensitive to lipophilic chelating agents, such as fusaric acid. This finding supports the contention that certain metal ions critically facilitate tetracycline uptake and leads us to suggest possible molecular mechanisms for tetracycline resistance.

Expression of tetracycline resistance genes results in alterations of the host cell membrane. These membrane alterations interfere with tetracycline permeation, thereby rendering the cell tetracycline resistant. The precise mechanism of tetracycline exclusion is a subject of much speculation (10, 15).

One tetracycline resistance transposon, Tn*10*, is used widely as a tool in bacterial genetics. A repertoire of genetic methods has been developed based on the use of Tn*10* (26). The genetic utility of Tn*10* and other tetracycline resistance transposons and plasmids would be greatly enhanced by a simple method for selecting cells that have lost tetracycline resistance. We describe here a method we have devised for this purpose.

MATERIALS AND METHODS

Bacteria. The following strains of *Salmonella typhimurium* LT2 were used in this study: TA830 Δ*his*(*OGDCBHAF*)*644*, TA2789 Δ*his*(*OGDCBHAF*)*644 purF1741*::Tn*10*, TA3178 *dhuA1* Δ*hisF645 hisJ8908*::Tn*10*, TA2946 *dhuA1* Δ*hisF645 hisJ5645* (region I), TA2915 *dhuA1* Δ*hisF645 hisJ6773* (region III), TA2908 *dhuA1* Δ*hisF645 hisJ6766* (region V), TA1898 *dhuA1* Δ*hisF645 hisJ5624* (region VII), TA3000 *dhuA1* Δ*hisF645 hisQ6699* (region II), TA3029 *dhuA1* Δ*hisF645 hisQ6724* (region IV), TA3031 *dhuA1* Δ*hisF645 hisQ6726* (region VII), TA3034 *dhuA1* Δ*hisF645 hisP6729* (region X), and TA3056 *dhuA1* Δ*hisF645 hisP6751* (region XIII). A Tn*10*-containing strain of *Escherichia coli*, strain JC10236 (*thr-1 leu-6 ara-14 proA2 lacY1 tsx-33 galK2 his-4 rpsL31 xyl-5 mtl-1 argE3 thi-1 srlC300*::Tn*10*), also was used.

Disk sensitivity testing. Inhibitors were tested for toxicity on various strains by comparing the zones of inhibition around inhibitor-impregnated disks as previously described (2). Results were scored after overnight incubation (usually 16 h).

Chemicals. Tetracycline hydrochloride, chlortetracycline hydrochloride, fusaric acid, quinaldic acid, picolinic acid, 8-hydroxyquinoline, carbonylcyanide *m*-chlorophenylhydrazone, EDTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid, and quinolinic acid were from Sigma Chemical Co. 1,10-Phenanthroline, 2-picoline, 2-pyridylacetic acid hydrochloride, 2-pyrazinecarboxylic acid, pyrrole-2-carboxylic acid, picolinic acid *N*-oxide, and 2-mercaptopyridine *N*-oxide were from Aldrich Chemical Co. Pentachlorophenol, 3,3',4',5-tetrachlorosalicylanilide, 2,2'-bipyridine, thenoyltrifluoroacetone, nicotinic acid, and anthranilic acid were from Eastman Chemical Co. Ethylenediamine di(*o*-hydroxyphenyl acetic acid) was obtained from K & K Laboratories and was deferrated as previously described (31). Antibiotic-impregnated disks were from BBL Microbiology Systems. Tryptone and yeast extract were from Difco Laboratories.

RESULTS

Development of a selective medium. We compared the sensitivities of Tet^s and Tet^r bacteria to a wide variety of inhibitors in order to find one that would provide a selection against the tetracycline resistance gene(s) carried on transposon Tn*10*. Our rationale was that alterations in the bacterial cell membrane which decrease the permeability of tetracycline (14, 16, 21) might simultaneoulsy increase cell susceptibility to other toxic compounds, thereby conferring a selective disadvantage.

The inhibitors were examined on a rich medium containing ingredients 1 through 6 in Table 1. Since tetracycline resistance is an inducible trait (14, 21), chlortetracycline was included to generate the membrane modifications associated with tetracycline resistance. When chlortetracycline is autoclaved in broth it is denatured so as to lose its toxicity toward Tet^s cells while retaining its inducing ability for Tet^r cells (R. Rodriguez, personal communication).

TABLE 1. *Composition of selective plates*

Ingredient[a]	Amount per liter
1. Agar	15 g
2. Tryptone	10 g
3. Yeast extract	5 g
4. Sodium chloride	10 g
5. Glucose	2 g
6. Chlortetracycline hydrochloride	0.05 g[b]
7. $NaH_2PO_4 \cdot H_2O$	10 g
8. Fusaric acid (2 mg/ml)	6 ml
(or quinaldic acid [10 mg/ml])	(10 ml)
9. Zinc chloride (20 mM)	5 ml

[a] Ingredients 1 through 7 were autoclaved for 20 min and cooled before addition of ingredients 8 and 9.

[b] A 0.05-g amount was found to be optimal for inducing the Tet^r phenotype in Tet^r cells without causing toxicity to Tet^s cells.

The sensitivities of otherwise isogenic Tet^s and Tet^r cells were compared on these plates by the disk sensitivity method. No differences were detected in the sensitivities to the following inhibitors: methenamine mandelate, streptomycin, gentamicin, ethambutol, isoniazid, sulfathiazole, colistin, bacitracin, vancomycin, neomycin, oleandomycin, cephaloglycin, polymyxin B, ampicillin, carbenicillin, phenylethyl alcohol, trimethoprim, nalidixic acid, novobiocin, 2-nitroimidazole, 3-oxauracil, isoguanine, and 8-azaguanine. Of all the compounds initially tested, only one, picolinic acid, was found to be slightly more toxic to Tet^r strains.

We sought to optimize the hypersensitivity of Tet^r strains to picolinic acid by varying the temperature, ion content, and pH. Temperature variation had only a minor effect, 37°C (or 42°C) being slightly preferable to 30°C. The hypersensitivity was improved slightly by increasing the concentration of Na^+ while omitting K^+. However, a very significant improvement was achieved by lowering the pH to 5.5. This modification increased the toxicity of picolinic acid as well as its preferential inhibition of Tet^r cells. The low pH, high Na^+ modification was easily achieved by inclusion of NaH_2PO_4 (ingredient 7) in the formula. When chlortetracycline was omitted from the medium, Tet^r and Tet^s strains exhibited identical sensitivities to picolinic acid. This confirmed that the gratuitous inducer of tetracycline resistance was an essential component of the medium.

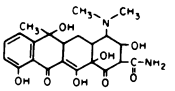
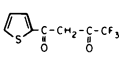
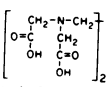
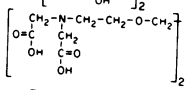
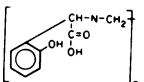
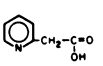
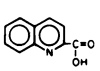
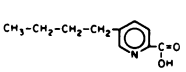
Mode of action of picolinic acid. The toxicity of picolinic acid (Table 2, compound 9) is at least partially attributable to chelation of metal ions (12, 13, 22). However, picolinic acid is also a membrane-permeating weak acid (pK_a = 5.5) and therefore is potentially toxic as a proton conductor. A closely related compound, pyrithione (Table 2, compound 19), though also a chelating agent, is thought to act primarily as a proton conductor (9). The increased toxicities of these compounds at low pH are compatible with either mode of action.

To help determine which property of picolinic acid is responsible for the hypersensitivity of Tet^r cells, we compared the sensitivities of Tet^r and Tet^s cells to compounds that are either (i) nonchelating proton conductors (carbonylcyanide *m*-chlorophenylhydrazone, pentachlorophenol, tetrachlorosalicylanilide); (ii) weakly acidic, lipophilic chelators (Table 2, compounds 2 through 5); or (iii) strongly acidic, hydrophilic chelators (Table 2, compounds 6 through 8). Compared with Tet^s cells, the Tet^r cells exhibited normal sensitivity to all three nonchelating proton conductors (data not shown), but hypersensitivity to six of seven chelators (Table 2). This result suggests that chelation, not proton conduction, is an essential factor in the hypersensitivity of Ter^r cells to picolinic acid.

To gain additional insight into the mode of action of picolinic acid, 11 structurally related compounds were tested for their abilities to inhibit Tet^r versus Tet^s cells. Since the ring nitrogen and carboxylic acid oxygen of picolinic acid form the divalent chelate, most of the compounds selected are altered in the pK_a or the geometrical arrangement of these functions. A few of the analogs chosen have altered lipophilicity. Results of the survey, summarized in Table 2 (compounds 9 through 20), yielded several interesting findings. First, chelation was again seen to be critical to the selective toxicity of picolinic acid. 2-Picoline and nicotinic acid (compounds 10 and 12), which are incapable of chelation, did not preferentially inhibit Tet^r cells. Second, increasing the lipophilicity of picolinic acid greatly improved the selective toxicity to Tet^r cells. Quinaldic acid and fusaric acid (compounds 16 and 17) have preserved the chelation structure of picolinic acid, but contain an additional fused aromatic ring or butyl group to increase lipophilicity. The improved selectivity against Tet^r cells engendered by these seemingly minor modifications was striking (see also Fig. 1). This may be due to preferential partitioning of these chelators in the cell membrane. Three compounds, (nicotinic acid, quinolinic acid, and pyrazine-2-carboxylic acid, [compounds 12 through 14]) were actually more toxic to Tet^s than to Tet^r cells. We can offer no simple explanation for this reversal.

Effect of cations on hypersensitivity of Tet^r cells to chelators. Ten biologically important cations were tested for their abilities to counteract the hypertoxicities of the four most selective chelators. This was done by using the

TABLE 2. *Sensitivities of Tet^s versus Tet^r cells to chelating agents and picolinic acid analogs*[a]

Compound (trivial name)	Structure	mg per disc	Zone of inhibition[b] tet^s	Zone of inhibition[b] tet^r	S or R[c]
1. tetracycline		0.3	24c	8c	R
2. 8-hydroxyquinoline		0.1	22t	21c/26t	S
3. 1,10-phenanthroline		0.02	14t	11c/14t	S
4. 2,2'-bipyridine		0.2	19c/25t	20c/27t	S
5. thenoyltrifluoroacetone (TTA)		0.04	14c	19c	S
6. ethylenediamine tetraacetic acid (EDTA)		1	14t	17t	S
7. ethyleneglycol-bis(β-aminoethyl ether)N,N' tetraacetic acid (EGTA)		1	8t	8t	-
8. ethylenediamine di(o-hydroxy-phenyl acetic acid) (EDDA)		0.4	11t	18c	S
9. pyridine-2-carboxylic acid (picolinic acid)		0.2	13c/17t	16c/24t	S
10. 2-methyl pyridine (2-picoline)		18.86	9c	9c	-
11. pyridine-2-acetic acid (2-pyridylacetic acid)		1	9t	8t	-
12. pyridine-3-carboxylic acid (nicotinic acid)		2	10t/19vt	10t	R
13. pyridine-2,3-dicarboxylic acid (quinolinic acid)		1	25c	22c/26t	R
14. pyrazine-2-carboxylic acid (2-pyrazine carboxylic acid)		2	7t/19vt	<6	R
15. pyrrole-2-carboxylic acid		1	10t/22vt	10c/21vt	S
16. quinoline-2-carboxylic acid (quinaldic acid)		0.2	10c	14c/30t	S
17. 5-butyl picolinic acid (fusaric acid)		0.02	8c/12t	19c/22t	S
18. picolinic acid N-oxide		1	12vt	15t	S
19. 2-mercaptopyridine N-oxide (pyrithione)		0.01	21c	26c	S
20. o-amino benzoic acid (anthranilic acid)		1	14c	13c	-

[a] The disk sensitivity method was used to compare a Tet^s strain, TA830, with an isogenic Tet^r strain, TA2789, on plates containing ingredients 1 through 7 from Table 1.

[b] The zone of inhibition is characterized both quantitatively and qualitatively. Numbers denote the diameter (in millimeters) of the zone around a 6-mm paper disk. Letters denote the extent of growth of cells within the zone as clear (c), slightly turbid (st), turbid (t), or very turbid (vt). Slash (/) indicates double zone (see, for example, the zone around the quinaldic acid disk in Fig. 1B).

[c] S, the Tet^r strain is more sensitive than the Tet^s strain; R, Tet^r strain is more resistant than the Tet^s strain; –, identical sensitivities of the Tet^r and Tet^s strains.

disk sensitivity method on plates formulated with ingredients 1 through 7 (Table 1), with or without the supplement of an additional cation. Results of this study are summarized in Table 3.

In general, the monovalent cations (Na^+, K^+, and Li^+) and two divalent cations (Ca^{2+} and Mg^{2+}) had no significant effect on the toxicities of the chelators to Tet^s cells or on their hypertoxicities to Tet^r cells. Fusaric acid toxicity was decreased by Fe^{3+}. Quinaldic acid toxicity was increased by Cu^{2+}. 8-Hydroxyquinoline toxicity was increased by Zn^{2+} or Cu^{2+} and decreased by Fe^{2+} or Fe^{3+}. 1,10-Phenanthroline toxicity was decreased by Zn^{2+}, Cu^{2+}, Fe^{2+}, or Fe^{3+}.

Of the 10 cations tested, only Mn^{2+}, Fe^{2+}, and Fe^{3+} were broadly effective at antagonizing the hypersensitivity of Tet^r cells. Manganese was active at equalizing the zones of inhibition and eliminating the selection of Tet^s clones by all chelators except 8-hydroxyquinoline. Iron, on the other hand, decreased the hypersensitivity of Tet^r cells to all four chelators, but was not as active as manganese at eliminating the selection of Tet^s clones by fusaric acid and quinaldic acid. Fe^{2+} was significantly more effective than Fe^{3+} against 8-hydroxyquinoline. These results point to manganese or iron or both as the principal targets of the lipophilic chelators.

Chelation of zinc is known to be a factor in the antimicrobial activity of chelating agents (1,

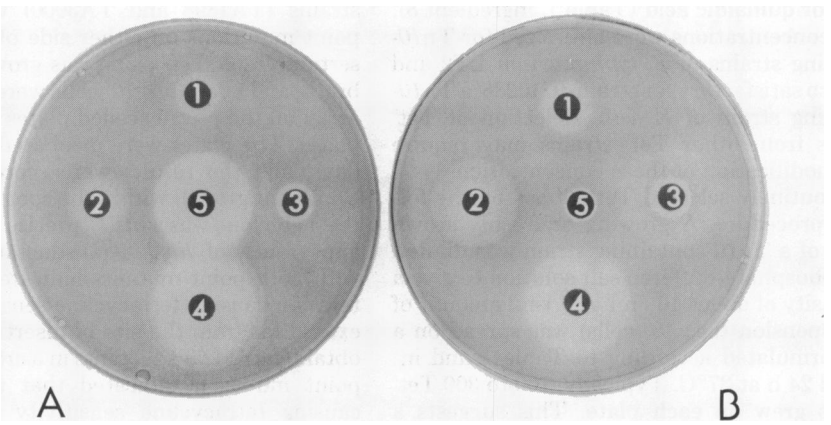


FIG. 1. *Disk sensitivity method used to compare a Tet^s strain, TA830 (A), with an isogenic Tet^r strain, TA2789 (B), on plates containing ingredients 1 through 7 (Table 1). Paper disks (6-mm diameter) contained the following: (1) 0.2 mg of picolinic acid, (2) 0.02 mg of fusaric acid, (3) 0.2 mg of quinaldic acid (4) 0.3 mg of chloramphenicol, and (5) 0.3 mg of tetracycline.*

TABLE 3. *Effects of cations on the selective toxicities of chelating agents*[a]

Cation[b] (mM)	Zone of inhibition[c]							
	Fusaric acid		Quinaldic acid		8-Hydroxyquinoline		1,10-Phenanthroline	
	Tet^s	Tet^r	Tet^s	Tet^r	Tet^s	Tet^r	Tet^s	Tet^r
None	12t	16c/23t*[d]	8st	13c/29t*	19t	25c/30t*	12t	9c/17t*
Na^+ (100)	13t	19c/23t*	9st	13c/27t*	20t	25c/29t*	12t	9c/14t*
K^+ (10)	13t	15c/23t*	9st	11c/27t*	27t	22c/30t*	14t	9c/15t*
Li^+ (10)	12t	16c/23t*	10st	12c/28t*	24t	24c/31t*	12t	9c/17t*
Ca^{2+} (1)	12t	15c/22t*	10st	11c/27t*	21t	22c/30t*	13t	8c/17t*
Mg^{2+} (1)	11t	14c/21t*	8st	12c/29t*	19t	24c/30t*	11t	9c/17t*
Zn^{2+} (0.1)	11t	15c/20t*	10st	12c/27t*	15c/21t	28c/30t*	9t	9t
Cu^{2+} (0.1)	10t	16c/19t*	10c	12c/30t*	16c/26t	24c/29t*	9t	9t
Mn^{2+} (0.1)	13t	14t	8st	11st	20t	21c*	12t	14t
Fe^{2+} (0.1)	12t	9c/15t*	9st	11c/13t	8t	13t	8t	9t
Fe^{3+} (0.1)	8t	9c/15t*	10st	11c/13t*	8t	18st*	8t	9t

[a] The same procedures and symbols described in the footnotes of Table 2 were used.

[b] Cation supplements were added as chloride salts by allowing 0.3 ml of a 100-fold-concentrated solution (except 0.6 ml of a 50-fold-concentrated NaCl solution) to soak into 30 ml of medium overnight.

[c] The amounts of the compounds added to the disks are given in Table 2.

[d] An asterisk indicates the presence of Tet^s satellite colonies growing around the disk as described in the text.

12, 13, 18, 37), and we noticed that zinc improved the growth of Tet^s colonies on plates containing fusaric acid (and, to a lesser extent, quinaldic acid). Apparently, these chelators exhibit some residual toxicity to Tet^s cells due to chelation of zinc. Thus, we recommend the addition of 0.1 mM zinc chloride (Table 1, ingredient 9) when fusaric or quinaldic acid is used to select Tet^s clones.

Selection of Tet^s clones by using fusaric or quinaldic acid. Fusaric and quinaldic acids gave the highest degrees of selectivity against Tet^r cells in disk sensitivity tests, and we therefore designed selection conditions with these substances. The optimal concentration on plates was found to be 12 μg/ml for fusaric acid or 100 μg/ml for quinaldic acid (Table 1, ingredient 8). These concentrations were optimized for Tn*10*-containing strains of *S. typhimurium* LT2 and were also satisfactory for strain JC10236, a Tn*10*-containing strain of *E. coli*. Selection of Tet^s variants from other Tet^r strains may require slight modification of these concentrations.

We routinely selected Tet^s clones by the following procedure. A growing or freshly grown culture of a Tn*10*-containing strain was diluted into a phosphate-buffered salt solution to give a cell density of about 10^7/ml. A 0.1-ml amount of this suspension (i.e., 10^6 cells) was spread on a plate formulated according to Table 1 and incubated 24 h at 37°C. Typically, 100 to 300 Tet^s colonies grew on each plate. This suggests a frequency of loss of tetracycline resistance of from 1 in 10^4 to 3 in 10^4, in agreement with previous measurements (24).

Virtually all fusaric acid- or quinaldic acid-resistant clones out of several hundred that were characterized in various experiments proved to be Tet^s. One exceptional case is described in Discussion. Thus, selection of cells resistant to fusaric or quinaldic acid on plates formulated by the recipe in Table 1 constitutes a reliable selection for Tet^s cells. It is conceivable, however, that some classes of Tet^s variants are not selected for by this method.

Certain applications of the method may require the use of a minimal medium instead of a complex medium. To obtain a selective minimal medium, the formula was modified by omitting the tryptone, yeast extract, and sodium chloride and replacing them with 2 g of NH_4Cl, 0.1 g of $MgSO_4 \cdot 7H_2O$, and 2 g of K_2HPO_4 per liter. Also, the amount of fusaric acid added was reduced to 1 ml/liter.

Deletions and inversions generated by loss of tetracycline resistance. Chromosomal deletions and inversions are known to occur at high frequency among Tet^s variants of Tn*10*-containing strains (24, 25). Therefore, we confirmed that deletion and inversion mutants constituted a substantial fraction of the Tet^s clones selected as fusaric acid resistant. Tet^s clones of strain TA3178 (a mutant defective in histidine transport due to a Tn*10* insertion in the *hisJ* gene at site B [30]) were selected by the standard procedure on fusaric acid-containing plates. After checking that these clones were indeed Tet^s, crosses with known point mutants in the region near site B were performed to determine the nature of the tetracycline-sensitive alteration. The crosses were performed on glucose minimal plates containing 0.25 mM D-histidine (as a histidine source) by seeding about 2.5×10^9 phage (P22 HT *int*201) that had been grown on strains (TA1898 and TA3000) which contain point mutations on either side of the *Tn*10 insertion. Each Tet^s clone was grown in nutrient broth, and about 5×10^7 cells were spotted in an array on the phage-seeded plates (12 clones per plate). The plates were incubated at 37°C for 3 days, and the results were scored as follows. Confluent growth within the spot indicated that the Tet^s clone was a *hisJ*$^+$ precise eductant. The appearance of *hisJ*$^+$ transductants in crosses with both point mutants indicated that the alteration causing tetracycline sensitivity did not extend far from the site of insertion. Failure to obtain *hisJ*$^+$ transductants in a cross with either point mutation indicated that the alteration causing tetracycline sensitivity extended out from the site of insertion, past the point mutation.

Of 50 clones tested, none was a precise eductant (i.e., *hisJ*$^+$), 23 (46%) were alterations within or very near the insertion site, and 27 (54%) were alterations extending outward from the insertion site into the chromosome (i.e., chromosomal deletions or inversions). Phage were grown on the latter 27 strains and backcrossed by the same method on nine *hisJ*, *hisQ*, or *hisP* point mutant strains (listed in Materials and Methods) to differentiate deletions from inversions, according to the recommendations of Kleckner et al. (24). Six of these (i.e., 12% of the original 50) Tet^s clones ultimately proved to be deletions. Kleckner et al. (24) measured a frequency of Tn*10*-induced deletions of 17% in a different strain of *S. typhimurium*.

DISCUSSION

Applications of the method. (i) Fine-structure mapping of a region of interest is greatly facilitated by the ease of generating a set of deletions around a Tn*10* insertion. (ii) Generalized mapping is facilitated by the high frequency

of localized chromosomal rearrangements (deletions and inversions) found among fusaric acid-resistant clones. First, Tn*10* is inserted in or near an unmapped gene of interest. Tet^s clones are then selected on rich medium and screened for acquisition of auxotrophy or loss of the ability to grow on various carbon sources (6). Identification of the biosynthetic or catabolic function(s) lost aids in map localization. (iii) Multiply marked strains are easily constructed, because the generation of a Tet^s clone leaves a genetic lesion at the original site of the insertion (24, 25). For example, we have constructed a strain with auxotrophic requirements for guanine, adenine, uracil, arginine, and nicotinic acid by repeating a two-step procedure. Tn*10* is inserted into a particular gene by transducing a strain to tetracycline resistance, and then a lesion is left behind when a fusaric acid-resistant (Tet^s) clone is obtained. (iv) Tn*10* insertions can be precisely eliminated from the chromosome by transduction. When 10^6 cells of Tet^r *S. typhimurium* (TA2789) were plated along with 10^8 P22 HT *int*201 phage, approximately 10% of the fusaric acid-resistant (Tet^s) cells that arose were transductants containing a restored *purF* gene. One transduction of this kind yielded a small percentage of clones that were fusaric acid resistant, but quinaldic acid sensitive and tetracycline resistant. At present we can offer no explanation for this unusual phenotype. (v) Clones can be selected which contain DNA inserted into tetracycline-resistant vehicles, such as pBR313 (4) and pBR322 (5). These plasmids have unique restriction sites in the tetracycline resistance genes so that when DNA is successfully cloned into these sites, tetracycline resistance is lost. Our method should simplify cloning by permitting direct selection of Tet^s presumptive clones as compared with the present negative selection methods (4). Reconstruction experiments have demonstrated the feasibility of this approach. In one *E. coli* background (strain CGSC 4517) the presence of pBR322 rendered the cells fusaric acid hypersensitive, as expected. When plated on fusaric acid-containing plates, fusaric acid-resistant clones arose at a frequency of about 10^{-2}. Most of these clones were Tet^s Amp^s and presumably cured of the plasmid. A derivative plasmid containing an insert of foreign DNA in the tetracycline resistance genes of pBR322 did not render this strain fusaric acid hypersensitive. Another strain of *E. coli*, 294, whose genetic character (EndoI$^-$ Thi$^-$ HsdR$^-$ HsdM$^+$ makes it more useful for cloning, is also rendered hypersensitive to fusaric acid by the presence of pBR322. Selection of Tet^s cells in this back- this background is improved greatly by increasing the level of sodium chloride in the plates to 20 g/liter. However, pBR322 did not increase the fusaric acid sensitivity of two other commonly used *E. coli* strains, C600 and HB101. Thus, the selection of a good starting strain is essential.

Design parameters of the selective medium. The medium given in Table 1 has several critical features. First, it contains heat-detoxified chlortetracycline to induce cell membrane changes responsible for tetracycline resistance without inhibiting growth of Tet^s cells. Acid-detoxified oxytetracycline (14) or other tetracycline analogs (3, 20, 32) are also capable of inducing tetracycline resistance adaptations without toxicity and presumably could be substituted for heat-detoxified chlortetracycline. Chlortetracycline hydrochloride may be omitted in selections against the tetracycline resistance of pBR322, since the resistance is expressed constitutively. Monobasic sodium phosphate and sodium chloride are included because they create environmental conditions of low pH (5.5) and high Na^+ (0.25 M). With some strains, such as the *E. coli* strain 294, it is important to add even more sodium chloride. Low K^+ concentration is not essential, but improves the selectivity slightly, most noticeably with 8-hydroxyquinoline (Table 3). The glucose, yeast extract, and tryptone constitute a rich growth medium that will support the growth of most viable enterobacteria. We have also been able to devise a minimal medium that achieves the same selectivity. Finally, a lipophilic chelator (fusaric acid or quinaldic acid) is the agent which, in a medium formulated with the ingredients described above, is responsible for selective inhibition of Tet^r cells.

Tetracycline uptake and the mechanism of tetracycline resistance. A sizable body of evidence (10, 15, 17, 33, 35) suggests that divalent cations are integrally involved in tetracycline permeation. Tetracyclines exist as dipolar ions at neutral pH and bind readily to cations in solution (11) and on membrane surfaces (7, 8). Presumably, the neutralization of negative charge upon cation binding facilitates movement across the cytoplasmic membrane. The primary change seen in induced Tet^r bacteria is a marked slowing of tetracycline permeation, especially at the stage of rapid initial uptake (28, 29). The principal mediator of this change appears to be a 34,000- or 36,000-molecular-weight protein (called the TET protein) that is synthesized and inserted into the cytoplasmic membrane (19, 23, 27, 34, 36).

A possible mechanism for retardation of tetracycline entry by the so-called TET protein is

suggested by our observation that induced Tet^r bacteria are hypersensitive to lipophilic chelators. We propose that the TET protein lowers the effective concentration of one or more species of metal ions in the cytoplasmic membrane. By lowering the effective concentration of tetracycline-binding metal ions, the TET protein could greatly slow tetracycline permeation. However, if these metal ions in the membrane serve an essential function (such as helping to maintain membrane integrity), then an induced Tet^r cell, having a lower effective concentration, will be more vulnerable to chelating agents. Chelators that tend to lodge in the membrane (e.g., fusaric acid and quinaldic acid) would predictably be more effective than other types of chelators (e.g. picolinic acid and pyrithione). As the integrity of the membrane becomes weakened, the high Na^+ concentration may create an additional electrolyte stress on the cell and promote lysis. Our results point to manganese or iron or both as the principal mediators of tetracycline permeation in *S. typhimurium*.

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