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Selection for Loss of Tetracycline Resistance by *Escherichia coli*

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An improved medium for the direct, positive selection of tetracycline-sensitive clones from a population of tetracycline-resistant strains of *Escherichia coli* is described.

Various genetic techniques have been developed requiring the excision of the tetracycline-resistant transposon Tn*10* from an insertion site within a gene (5). Furthermore, several plasmid vector systems allow genetic cloning by insertional inactivation of tetracycline resistance (3). A direct, positive selection for tetracycline-sensitive bacteria would be extremely valuable for these studies. Bochner et al. (2) recently described a technique for the direct selection of tetracycline-sensitive (Tc^s) clones from a predominantly tetracycline-resistant (Tc^r) population of bacteria. They developed a selective medium based upon the inhibition of Tc^r bacteria by the lipophilic chelating agents fusaric acid or quinaldic acid. This medium was found to be effective for the selection of Tc^s revertants of strains of *Salmonella typhimurium* which were Tc^r due to the insertion of the transposon Tn*10* in their chromosomes (2). In addition, the authors reported success in selecting Tc^s revertants of the *Escherichia coli* strain JC1036 (*thr-1 leu-6 ara-14 proA2 lacY1 tsx-33 galK2 his-4 rpsL31 xyl-5 mtl-1 argE2 thi-1 srlC300*::Tn*10*). However, the medium described by Bochner et al. (2) is much less effective with most strains of *E. coli*. When most Tc^r strains of *E. coli* K-12 are plated on this medium, a high background of Tc^r colonies develops, necessitating extensive purification to isolate Tc^s colonies and eliminating the feasibility of direct replica plating. Thus, the usefulness of this medium in genetic studies with a variety of *E. coli* strains and in *E. coli* used as a host for genetic cloning is limited.

We sought to optimize the Tc^s medium of Bochner et al. (2) for application to *E. coli*. We initially attempted to modify their medium by varying the concentrations of the selective agents described by Bochner et al. (2). Varying the concentrations of NaCl, NaH_2PO_4, chlortetracycline hydrochloride, fusaric acid, and $ZnCl_2$ did not result in any significant improvement in differentiating between Tc^s and Tc^r strains (data not shown). Furthermore, neither the substitution of fusaric acid with quinaldic acid nor the use of both fusaric acid and quinaldic acid together enhanced the differentiation between Tc^s and Tc^r strains. However, we observed that although less than 10% of the colonies arising from fast-growing Tc^r strains of *E. coli* plated on this medium were Tc^s, somewhat better results were obtained with slow-growing strains. When the growth rate of strains was slowed, as in severely mutated strains or strains bearing a *recA* mutation, up to 50% of the colonies from the Tc^s plates showed the Tc^s phenotype. Thus, we reasoned that decreasing the nutrient concentration would slow the growth of the background Tc^r bacteria while allowing sufficient growth of Tc^s colonies. Improved differentiation of Tc^s and Tc^r strains was obtained by eliminating glucose and decreasing the concentration of tryptone to 0.5%. Similar results were obtained when nutrient broth (Difco) was substituted for the tryptone and yeast extract with less fastidious strains. The composition of this modified Tc^s medium is shown in Table 1. A comparison of the modified Tc^s medium with the medium described by Bochner et al. (2) was made with a variety of *fadR13*::Tn*10* strains of *E. coli* K-12 with different genetic backgrounds (Table 2). These results are shown in Table 3. For most strains tested, approximately 90% of the colonies obtained on the modified Tc^s medium were found to be Tc^s when directly replica plated as described in Table 3, whereas less than 10% of the colonies from the original Tc^s medium described by Bochner et al. (2) were found to be Tc^s (Table 3). The frequency of isolation of Tc^s clones on the modified Tc^s plates was ca. 10^{-4} per bacterium. Similar results were obtained for Tn*10* insertions at other chromosomal loci, including *fadL*::Tn*10*, *zji*::Tn*10*, *srlA*::Tn*10*, *zaf*::Tn*10*, and *zda*::Tn*10* (data not shown). As described above, the original Tc^s medium was somewhat more effective with slow-growing strains of *E. coli*. These results may explain the success of Bochner et al. (2) with strain JC1036. With slow-growing strains, the modified Tc^s plates also allowed 90% selection for Tc^s clones; however, 36 to 48 h of

incubation was required for growth. Thus, the modified Tc^s plates were found to be suitable for selection of Tc^s clones from Tc^r derivatives of all *E. coli* strains studied. Optimal selection resulted by plating 0.1 ml of a dilution containing 10^6 to 10^7 cells per ml and incubating for 24 to 48 h at 37°C.

Although the medium of Bochner et al. (2) was effective with certain strains of *E. coli* useful for genetic cloning, it was ineffective with several strains extensively used for this purpose, including strain C600. We have found that the modified Tc^s plates work well with strain C600 also (Table 3), further extending the usefulness of this technique. Strains containing the intact pBR322 plasmid were unable to grow on the modified Tc^s plates, whereas recombinant plasmids with insertions in the *Bam*HI or *Hin*dIII sites of the Tc^r gene of pBR322 grew readily on these plates (data not shown). In contrast, the medium of Bochner et al. (2) allowed growth of a heavy background of Tc^r cells containing the intact pBR322. Inclusion of ampicillin to prevent plasmid segregation did not affect the efficiency of the modified Tc^s plates in these studies. In addition, H. Moyed and K. Bertrand (personal communication) have used these media during studies involving cloning of Tc^r fragments of Tn*10* into the plasmid pACYC177 in *E. coli*. They found the modified Tc^s plates to be much more effective for selection of Tc^s and fusaric acid-resistant derivatives of such clones.

The modification of the Tc^s medium described in this paper should increase the usefulness of this technique for workers studying *E. coli* ge-

TABLE 1. *Composition of modified Tc^s plates*

Ingredient[a]	g/liter
Agar	15
Tryptone broth[b]	5
Yeast extract	5
Chlortetracycline hydrochloride (12.5 mg/ml)[c]	4[d]
NaCl	10
$NaH_2PO_4 \cdot H_2O$	10
Fusaric acid (2 mg/ml)	6[d]
$ZnCl_2$ (20 mM)	5[d]

[a] Agar, tryptone broth, yeast extract, chlortetracycline hydrochloride, and NaCl were dissolved in 1 liter of distilled water, autoclaved for 20 min at 121°C, and cooled to ca. 45°C before addition of $NaH_2PO_4 \cdot H_2O$ and fusaric acid as separately sterilized solutions.

[b] Alternatively, the tryptone and yeast extract may be substituted with nutrient broth for less fastidious strains.

[c] Chlortetracycline hydrochloride was prepared as a 12.5-mg/ml solution and stored in the dark at 4°C before use.

[d] Milliliters per liter.

TABLE 3. *Efficiency of Tc^s selection with original and modified Tc^s media with E. coli strains*

Strain	No. of colonies in following medium[a]					
	A			B		
	Tc^s	Tc^r	% Tc^s	Tc^s	Tc^r	% Tc^s
RS3040	7	171	3.93	171	7	96.07
LS5700	11	60	15.49	80	9	89.89
LS5701	3	86	3.49	82	7	92.13
LS5702	10	56	17.86	76	13	85.39
LS5703	0	89	0	79	10	88.76

[a] Medium A was the original Tc^s medium described by Bochner et al. (2). Composition of this medium was as follows: 15 g of agar, 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, 2 g of glucose, 0.05 g of chlortetracycline hydrochloride, 10 g of $NaH_2PO_4 \cdot H_2O$, 6 ml of fusaric acid (2 mg/ml), and 5 ml of 20 mM $ZnCl_2$ per liter. Medium B was the modified Tc^s medium described in Table 1. Bacteria were grown to ca. 6×10^8 cells per ml in rich broth medium (6) and diluted to an appropriate density in M9 minimal medium (6), and 0.1-ml portions were plated on the above media and on rich broth plates for viable cell counts. Cultures were incubated for 24 to 48 h at 37°C. Colonies from the Tc^s plates were replica plated by stippling onto Tc^s plates, rich broth plates containing 20 μg of tetracycline per ml, and rich broth plates.

TABLE 2. *Bacterial strains used*

Strain	Genotype[a]	Source
RS3040	*fadR13*::Tn*10*	R. Simons et al. (7)
JC1552	*argG6 metB1 his-1 leu-6 trp-31 mtl-2 xyl-7 malA1 gal-6 lacY1 str-104 tonA2 tsx-1 supE44*	A. Clark strain via CGSC[b]
LS5700	JC1552 *fadR13*::Tn*10*	This work
JK268	*dadR1 trpE61 trpA62 tna-5 purB58*	J. Kuhn strain via CGSC[b]
LS5701	JK268 *fadR13*::Tn*10*	This work
H680	*purB51 trp-45 his-68 tyrA2 thi-1 lacY1 gal-6 malA1 xyl-7 mtl-Z strA125 tonA2 tsx-70 supE44*	P. de Haan strain via CGSC[b]
LS5702	H680 *fadR13*::Tn*10*	This work
C600	*thi-1 thr-1 leu-6 lacY1 tonAz1 supE44*	R. Appleyard strain via CGSC[b]
LS5703	C600 *fadR13*::Tn*10*	This work

[a] Tn*10* insertions were as previously described (4, 5). When an insertion is not within a known gene, it is given a three-letter symbol starting with *z*, and the second and third letters indicate the numerical position on the *E. coli* K-12 linkage map (1) (e.g., *zaf* corresponds to 5 min).
and the third letter (*e*) indicates that the insertion is in the *E. coli* chromosome.

[b] Obtained from B. Bachmann, Coli Genetic Stock Center (CGSC), Yale University, New Haven, Conn.

netics and using *E. coli* as a host for genetic cloning.

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