THE SELECTION OF GENETIC RECOMBINATIONS WITH BACTERIAL GROWTH INHIBITORS

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Genetic exchanges between cells of *Escherichia coli*, strain K-12, were first demonstrated with a nutritional selective method (Tatum and Lederberg, 1947; Lederberg, 1947). A variety of mutants of K-12 were obtained with growth factor deficiencies that prevented growth in a synthetic agar medium. Recombinations between different mutant strains in mixed culture were selected by inoculating washed suspensions into synthetic agar plates. The colonies that appeared 24 to 48 hours later represented cells that had exchanged genetic factors so that they possessed the full complement needed to synthesize all of their own growth substances. To detect such recombinants, selection is essential, as they make up only about one one-millionth of the mixed bacterial culture.

In principle, other selective agents besides nutritionally deficient medium could be used to detect recombinants. Bacterial growth inhibitors should lend themselves to this application, particularly as inhibitor-resistant mutants can be obtained more readily than nutritional mutants in many organisms. The method would consist first of isolating two mutants, each resistant to one inhibitor, but sensitive to the other. Then, recombinants resistant to both inhibitors would be selected from mixed cultures by platings into medium containing both substances. As with nutritional selections, it is essential to distinguish between spontaneous mutations and recombinations. This is best accomplished by studying the behavior of independent genetic factors, not influenced by the selective method, which should show reassortments among the selected dually resistant recombinants.

In this paper an experiment with *Escherichia coli* K-12, using streptomycin and azide as selective agents, will be shown to support the feasibility of this approach.

MATERIAL AND METHODS

For genetic studies two stocks of K-12 have been developed that carry a variety of genetic differences. Each of the characteristics mentioned has been obtained in a separate mutational step by methods previously described (see Lederberg, 1949). W-677 requires threonine (T-), leucine (L-), and thiamine (B_1-), fails to ferment lactose, maltose, xylose, or mannitol, and is resistant to phage T5 (T5-R). W-478 requires methionine, ferments each of the foregoing sugars, and is

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sensitive to T5 (T5-S). Long experience with these stocks has shown that each of the characters is quite stable, especially when the stocks are maintained on ordinary complete media, which do not select for reversions.

After preliminary trials with a number of agents, streptomycin and sodium azide were selected as satisfactory inhibitors for experiments on *E. coli*. A streptomycin-resistant mutant of W-677, 677-*sr*, was obtained by plating about 10^{10} cells altogether into several plates of nutrient agar containing 100 μg per ml of streptomycin base. One colony grew out and was purified by serial restreakings of single colonies on ordinary nutrient agar. Strain 677-*sr*, as noted in table 1, proved to carry the same genetic markers enumerated above for W-677. Similarly, an azide-resistant mutant of W478, 478-*azr*, was isolated from plates of nutrient agar containing M/500 sodium azide. Strain 478-*azr* likewise was unaltered in other properties as compared with W-478.

Streptomycin is an unusually suitable selective agent for two reasons: the inhibition of sensitive cells is complete, so that the plates remain clear, and the spontaneous mutation conferring resistance to streptomycin is very infrequent, about once per 10^{10} divisions (Newcombe and Hawirko, 1949). Azide is not quite so suitable in either respect. Azide-containing plates, heavily inoculated with sensitive cells, eventually become diffusely turbid, and the mutations for azide resistance occur rather frequently, about once per 10^8 divisions. However, other growth inhibitors tested, including penicillin, chloromycetin, heavy metal salts, and brilliant green, were, for *E. coli*, even less satisfactory.

In conducting crosses, it was found necessary to grow the parents together in broth for several hours before plating them into azide-streptomycin agar, as very poor yields of recombinants were obtained from cultures mixed just before plating. This difference from the nutritional selective technique may be attributed to the prompt and irreversible inhibition of sensitive cells by the compounds used here.

The combination streptomycin and iodoacetate resistance gave results very similar to those about to be described, except that the inhibition of the parental cells was less complete and recombinant colonies had to be picked from a rather dense background of parental growth.

EXPERIMENTAL RESULTS

A detailed experiment is summarized in table 1. The parents (677-*sr* and 478-*azr*) were grown, individually, overnight in 10 ml Difco "pennassay broth" at 37 C. One-tenth-ml samples of each were inoculated into the same fresh tube of this medium, and individually into separate tubes for controls. After 8 hours' incubation, the cultures had reached a density of about 10^9 cells per ml. Four-tenth-ml samples were mixed with 20 ml nutrient agar that had been supplemented with streptomycin and azide, 100 μg per ml and M/500, respectively. After 48 hours' incubation, the plates were examined for colonies. Such dually resistant colonies might be *azr-sr* recombinants, *azr* mutants from 677-*sr*, or *sr* mutants from 478-*azr*. In order to characterize them, colonies at the surface were picked for further tests.

In the control series one streptomycin-resistant mutant was obtained from 7×10^9 cells of 478-*azr*. This mutant proved to be streptomycin-dependent and could not be cultivated on plain agar. Platings of the 677-*sr* control yielded 7 *azr* mutants per 10^9 cells. The mixed culture gave an average of 100 colonies per plate or 250 dually resistant cells per 10^9.

The marked excess of dual resistants in the mixed culture immediately suggests that recombination of *azr* and *sr* factors has occurred. In order to confirm this conclusion 100 colonies from these plates were picked and purified by streaking on eosin methylene blue lactose agar, and reisolated colonies were tested for the various characters that distinguish W-478 from W-677. In addition, 14 dual-

TABLE 1

Segregation of genetic factors among 100 dually resistant organisms selected from a mixed population of azide- and streptomycin-resistant cells

NO. COLONIES	LACTOSE	MALTOSE	XYLOSE	MANNITOL	T5	NUTRITIONAL REQUIREMENTS
677-*sr* parent	−	−	−	−	R	T L B_1
478-*azr* parent	+	+	+	+	S	M
43	−	−	−	−	S	+ (4); M (1); TB_1 (3); B_1 (1)
28	+	−	−	−	S	TB_1 (9); T (1)
10	−	−	−	−	R	B_1 (4); T (2); TB_1 (4)
6*	−	−	−	−	R	TLB_1 (6)
4	+	−	+	−	S	+ (2); B_1 (1); L (1)
2	−	−	+	+	S, R	M (2)
2	−	+	+	+	S, R	M (1); B_1 (1)
1	−	−	+	−	R	B_1
1	−	+	−	−	S	M
1*	+	+	+	+	S	M

Fermentative tests were made on eosin methylene blue agar media; T5 responses by cross-streaking bacteria with phage. Nutritional tests were not made exhaustively except when needed to differentiate recombinants from mutants. The numbers in parentheses refer to the number of isolates with the indicated requirements (see text for symbols); "+" refers to a prototroph, i.e., no requirements.

* These isolates cannot be distinguished from a parent and are probably mutants.

resistant colonies derived from the 677-*sr* parent by itself were tested, and all agreed with 677-*sr* in all respects. It has not been feasible to collect appreciable numbers of streptomycin-resistant mutants, owing to the very low mutation rate, but no alteration of the genetic characters used here has been found in association with streptomycin resistance.

The results of the tests on the putative recombinants are given in table 1. Only 7 of the 100 colonies picked were indistinguishable from one or the other parent. In view of the observed frequency of spontaneous resistance mutations, it is likely that most or all of these 7 are mutants rather than recombinants.

The remaining 93 colonies were of a variety of recombination types. (The relatively high frequency of new combinations can be ascribed to the fact that

only those sexual progeny that have recombined *azr* and *sr* can be detected by current methods.) No attempt will be made here to interpret, in detail, the relative frequencies of the various recombination types in terms of the linkage relationships (see Lederberg, 1947). However, the linkage of xylose with mannitol fermentation, for example, which has been observed previously among nutritionally selected recombinants, is also very apparent in table 1.

DISCUSSION

This experiment realizes the expectation that genetic recombination in bacteria can be demonstrated with growth inhibitors, just as with nutritional selection. In view of previous results, this conclusion is far from surprising.

By itself the occurrence of more dually resistant cells in mixed cultures of single-resistant organisms is not entirely convincing as support for recombination. However, the selection of dual resistants can be used to accumulate possible recombinant cells among which the reassortment of independent, unselected markers can be studied. When, as shown in table 1, a variety of new combinations of such unselected markers can be identified, more nearly critical evidence for genetic recombination is at hand.

Many organisms that are not readily amenable to the isolation of nutritional mutants, e.g., the pathogenic cocci, should prove especially susceptible to genetic investigation by this procedure. The desiderata for suitable selective agents are fairly obvious. They should give a clear separation of susceptible and resistant cells in large populations. The number of resistant mutants should be reasonably small. Preferably, resistance should be discrete rather than quantitative. It is also important, if this method is to be given a fair trial, that the two agents used not interact (such as the synergistic effects of penicillin with iodoacetate), that there be no cross resistance to the two agents (e.g., streptomycin and streptothricin in *E. coli*), and that the dual resistant be viable. Some of these criteria can be verified, if necessary, by recovering dually resistant mutants from single resistants inoculated into a dually supplemented medium. Finally, there should be some assurance that the independent markers are unaltered by the selection procedure itself.

It is, of course, immediately evident that recombination might possibly play some role in the establishment of multiply resistant bacteria, under the influence of chemotherapy. However, we know, as yet, too little of the conditions and scope of genetic recombination among bacteria to entertain an informed opinion of the significance of this possibility.

SUMMARY

On the basis of previous experimental results, it should be possible to select for genetic recombinations in mixed bacterial cultures with the help of growth inhibitors. Azide- and streptomycin-resistant mutants were obtained from marked strains derived from *Escherichia coli* K-12. This strain had been shown previously to participate in genetic recombination by nutritional selective methods. Recombinant bacteria resistant to both streptomycin and azide were selected by

plating mixed cultures into media containing both compounds. It was shown that the majority of the dual resistants were recombinants, since they showed re-assortments of independent unselected characters, including nutritional requirements, sugar fermentations, and phage resistance. The general applicability of this technique to the detection and study of recombination in other bacteria is suggested.

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