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SHORT PAPERS

A method for selecting streptomycin-sensitive mutants of R factors which adenylate streptomycin*

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1. INTRODUCTION

This laboratory has been studying R factor-mediated resistance to streptomycin (Sm)§ and Spc and has found that certain R factors inactivate both drugs by adenylation (Harwood & Smith, 1969*a*, *b*; Smith, 1969; Smith *et al.*, in preparation). Whether these resistances are mediated by the same or different enzymes remains one of the important questions raised by these observations. An analysis of R factors with mutations affecting these resistances would help answer this question, and techniques for enriching for such mutants would facilitate such studies. The penicillin technique (Davis, 1948) can be used to select Spc^S mutants but cannot be used to select Sm^S mutants because of the bactericidal action of Sm. We have presented evidence in an accompanying paper that Sm-adenylate does not cause phenotypic suppression *in vivo* and that R factor-infected CSD (Gorini & Kataja, 1964) mutants of *E. coli* do not form clones when plated on media containing Sm at concentrations which do not exceed the capacity of the adenylating enzyme (Harwood & Smith, 1969*b*). These observations suggested that this technique might be used to enrich for the desired Sm^S R factor mutants. Our findings presented in this paper confirm this proposal.

2. MATERIALS AND METHODS

(i) Media

Nutrient Broth: Trypticase Soy Broth (Baltimore Biological Laboratory).

Minimal medium: as described by Davis & Mingioli (1950) but without citrate.

Nutrient and minimal agar consisted of the corresponding broth solidified with 1·5% agar.

Levine's EMB agar (BBL) was used in some experiments.

Antibacterial drugs were added to autoclaved agar at concentrations indicated or described earlier (Marsh & Smith, *J. Bact.*, in press).

Buffer: minimal medium but without $(NH_4)_2SO_4$.

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§ Abbreviations used see p. 261.

(ii) Bacterial strains

The following strains of *E. coli* were used: Rc (B, arg⁻, CSD, Sm^R) (Gorini & Kataja, 1964); AB 1932-1 is a spontaneous mutant resistant to 100 μg/ml of Nal (Walton & Smith, 1969) derived from AB 1932 (K_{12}, F^-, arg⁻, met⁻, xyl⁻, gal⁻, lac⁻, T_6^R) obtained from Dr E. Adelberg.

RE 130 is an R factor originally carried by a natural isolate of *E. coli* which mediates Trx, Hg^R, Su^R, Sm^R, Spc^R, Tc^R, Cm^R (Harwood & Smith, 1969*a*).

(iii) Technical methods

U.v. source: GE germicidal lamp.

Cells to be exposed to u.v. were grown overnight in nutrient broth, centrifuged, washed twice and resuspended in buffer, and exposed to 900 ergs/mm² u.v. while constantly stirred. This dose of u.v. decreased the viable cell count by $2{\cdot}5 \times 10^{-4}$. The cells were diluted 1/3 in broth, reincubated overnight at 37 °C, diluted in buffer, and plated on EMB agar for viable counts and on minimal drug media.

Clones picked for study were purified thrice on the medium on which they were originally plated. Single colonies were then picked to broth, incubated overnight, and tested for patterns of drug resistance and arginine auxotrophy.

Viable cell counts and tests for drug resistance were performed as described (Marsh & Smith, *J. Bact.*, in press). Arginine auxotrophy was tested on minimal medium by either spreading a clone to isolated colonies or inoculating a suspension of 10^5 bacteria on to the test plate with a replicate inoculator. Conjugations were performed as described (Watanabe, 1964). Recombinant clones were selected on EMB agar containing Nal, 100 μg/ml and either Tc 20 μg/ml or Cm 20 μg/ml (AB 1932-1 recipient) or Sm 1000 μg/ml and either Tc or Cm as above (Rc-recipient).

3. RESULTS

E. coli Rc will form colonies when plated on Min supplemented with either arginine or Sm. Although *E. coli* Rc/RE 130 will also grow on Min with arginine, the presence of an R factor which mediates Sm^R by adenylation appears to be incompatible with the growth of arg⁻ cells on Min–Sm (Harwood & Smith, 1969*b*). Therefore, growth on such medium ought to occur only when the cells have become either arg^+ or have lost the Sm^R locus of the R factor.

In order to increase the frequency of cells bearing R factors which have mutations of the Sm^R locus, a culture of *E. coli* Rc/RE 130 was treated with ultraviolet light and aliquots were plated on Min–Sm, Min–Sm–Su or Min–Sm–Cm. These latter drugs were included in the medium of certain plates after preliminary studies revealed that the clones appearing on Min–Sm were either arg^+ revertants or had R factors mediating only Tc^R. It was hoped, therefore, that the presence of Su or Cm would select R factor mutants with other (and varying) genotypes.

Colonies with two distinct growth rates were obtained on each medium. Those forming macrocolonies at 24 h were studied independently from those forming smaller colonies after 4 days. Large colonies were found on each of the three media at a frequency of $1{\cdot}6 \times 10^{-6}$ of total viable cells. Small colonies were found at frequencies of $4{\cdot}8 \times 10^{-3}$ on Min–Sm, $2{\cdot}5 \times 10^{-3}$ on Min–Sm–Cm, and $1{\cdot}8 \times 10^{-5}$ on Min–Sm–Su (Table 1).

The 16 large colonies studied from each of the three media retained their R-factor drug resistances but were arg^+. Of the 142 small colonies studied, 107 were arg⁻ and had Sm^S R factors; 26 were slow-growing arg^+ cells, possibly due to suppressor mutations, and had unaltered R factors; 8 had both an arg^+ phenotype and a mutant R factor. None of the

190 colonies examined had both its original arginine auxotrophy as well as an R factor mediating Sm^R.

Of the 46 small colonies picked from Min–Sm, 45 had R factors mediating resistance only to Tc, and one had lost its R factor. Of the 48 small clones from Min–Sm–Cm, 5 had R factors mediating resistance to Tc Cm; 32 had resistance to Hg Su Tc Cm; and 1 had resistance to Hg Su Sm Spc Cm. Thirty of the 48 small clones from Min–Sm–Su had R factors which mediated resistance to Hg Su Tc Cm, and 1 had resistance to Hg Su Sm Spc Cm. All R factors retained their ability to transfer by conjuagtion.

Table 1. *Genotype of clones arising from selection*

			Genotype of clones studied		
Selective media	Colony size	Total clones studied	Arginine requirement	R factor patterns of resistance	No. of clones
Min–Sm	Large	16	+	Hg Su Sm Spc Tc Cm	16
	Small	46	−	Tc	45
Min–Sm–Cm	Large	16	+	Hg Su Sm Spc Tc Cm	16
	Small	48	+	Hg Su Sm Spc Tc Cm	9
			+	Hg Su Tc Cm	5
			+	Hg Su Sm Spc Cm	2
			−	Hg Su Tc Cm	27
			−	Tc Cm	5
Min–Sm–Su	Large	16	+	Hg Su Sm Spc Tc Cm	16
	Small	48	+	Hg Su Sm Spc Tc Cm	17
			+	Hg Su Sm Spc Cm	1
			−	Hg Su Tc Cm	30

As noted above, 34 of the slow-growing clones were arg^+; 8 of these clones also had mutant R factors. In order to determine if a genetic alteration of the R factor could suppress the arg^- mutation of the host chromosome, R factors from 13 such clones, including 4 of the mutant R factors, were transferred by conjugation to *E. coli* AB 1932-1 and, in turn, back into *E. coli* Rc; the arginine phenotype of these clones was then re-examined. None of the R factors studied suppressed the arg^- mutation; the arg^+ prototrophy must have resulted therefore from a chromosomal mutation(s).

4. DISCUSSION

These findings confirm and extend our initial proposal that Sm-adenylate does not direct phenotypic suppression *in vivo* and, therefore, that CSD arg^- mutants of *E. coli* infected with R factors mediating Sm-adenylation can form colonies on Min–Sm only following a genetic alteration resulting in either arginine prototrophy or a Sm^S R factor (Harwood & Smith, 1969*b*).

Although not all clones arising from this selective method have R factors with the desired Sm^S mutation, the present results recommend its use for enriching for such mutants. Thus, the frequency with which clones with R factor mutation(s) were found was 3 times that of cells which had no R factor mutation but which grew slowly on the selective media because of an alteration to arg^+, and 10–3000 times that of cells which were fast-growing arg^+ revertants, from which they could be distinguished by colony

size. This technique has also been found to be useful (D. H. Smith, N. Prescott & J. A. Janjigian, in preparation) in enriching for Sm^S mutants of R factors mediating Sm^R by phosphorylation (J. Davies, personal communication) and for Km^S R factors mediating Km^R by phosphorylation (Kondo *et al.* 1968).

The observation that all bacteria found on Min–Sm following u.v. mutagenesis had R factors mediating only conjugation and Tc^R remains to be explained. These findings might suggest a preferred breakage point on the R factor genome, which if true would be of interest in light of the early observation that fi^+ R factors frequently lose all properties except Tc^R when introduced into *Salmonella typhimurium* (Watanabe & Lyang, 1962). In any event, the inclusion of other drugs in Min–Sm medium guarantees the selection of mutant R factors with varying patterns of resistance. In these studies, mutant R factors which mediate four different phenotypes were found: Trx–Hg–Su–Tc–Cm; Trx–Tc–Cm; Trx–Tc; Trx–Spc–Sm–Hg–Su–Cm. We have not yet determined if these genotypes resulted from the mutagenic effect of the u.v. or its ability to enhance genetic recombination (Jacob & Wollman, 1955). The linkage groups found are consistent, however, with previous maps of the genome of certain R factors (Watanabe & Fukasawa, 1961). The possibility of exploiting this technique to determine a genetic map of R factor loci should be examined.

Although not all linkage groups on this R factor were interrupted by the u.v. treatment, we consider it significant that of the 112 Sm^S R factor mutants studied, all were Spc^S, and that all Sm^R R factors were also Spc^R. The results of further genetic analysis of the relation between the R factor-mediated adenylation (inactivation) of Sm and Spc will be presented elsewhere (D. H. Smith, J. A. Janjigian, N. Prescott & P. W. Anderson, in preparation).

SUMMARY

An Arg^- conditionally streptomycin (Sm)-dependent strain of *E. coli* B that carries an R factor mediating Sm adenylation cannot grow on minimal agar containing 20 μg Sm/ml unless the strain either becomes Arg^+ or loses the ability to adenylate Sm (Harwood & Smith, 1969*b*). We have therefore studied the efficacy of this selection with respect to enriching for Sm^S mutants of such R factors. Following u.v. mutagenesis, cells were plated on the selective medium; of the clones which were examined, Arg^- R-Sm^S colonies were found up to 3000 times more frequently than Arg^+ R-Sm^R colonies.

ABBREVIATIONS USED

Sm, streptomycin; Spc, spectinomycin; Hg, mercuric chloride; Su, sulphadiazine; Tc, tetracycline; Cm, chloramphenicol; Km, kanamycin; Nal, nalidixic acid; Trx, intercellular transfer by conjugation; *arg*, arginine; *met*, methionine; *xyl*, xylose; *lac*, lactose; *gal*, galactose; CSD, conditionally streptomycin-dependent; Min, minimal medium; Min–Sm, minimal medium with Sm 20 μg/ml; Min–Sm–Su, minimal medium with Sm 20 μg/ml and Su 800 μg/ml; Min–Sm–Cm, minimal medium with Sm 20 μg/ml and Cm 5 μg/ml.

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