Isolation of Amber Mutant of a P Group R Factor after Hydroxylamine Mutagenesis of a Transducing Lysate

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INTRODUCTION

The technique, used in bacterial mutagenesis, of exposing a generalized transducing phage lysate to hydroxylamine was first described for the *Salmonella typhimurium* phage P22 by Hong & Ames (1971), although it represents a variation on a principle long employed with transformation in *Bacillus subtilis* (e.g. Young, Smith & Reilly, 1969). Hong & Ames used the term 'localized mutagenesis' for the technique because of its usefulness in the isolation of mutations cotransducible with, and therefore close to, a given selected marker. This method of mutagenesis can be very efficient: for instance, when *Escherichia coli* K12 *ppc*⁺ transductants produced with a hydroxylamine-mutagenized P1 lysate were screened for mutations in the adjoining *argECBH* cluster, about 0.6 % were found to be arginine auxotrophs (Bretscher & Baumberg, 1976). Other attractions of this technique are: (i) hydroxylamine-induced mutations apparently occur randomly rather than in clusters as with nitrosoguanidine, and (ii) recessive mutations are expressed without need for growth and segregation after transduction (Hong & Ames, 1971). Its usefulness in isolating plasmid mutants was therefore tested.

METHODS

Bacterial, plasmid and phage strains. These are described in Table 1. All bacteria were derivatives of *E. coli* K12.

Media. Nutrient broth no. 2 and MacConkey agar were from Oxoid. Lennox broth (LB) was prepared according to Lennox (1955), with the addition of 2.5 mM-CaCl₂. LB agar plates contained 1.25 % (w/v) agar; LB soft agar contained 0.5 % (w/v) agar. Minimal salts medium and supplements were as described by Baumberg (1970). Ampicillin, kanamycin and tetracycline were used at 25, 25 and 10 µg ml⁻¹ respectively, unless otherwise noted.

Preparation and assay of P1_{kc} lysates. LB soft agar (2 ml), kept liquid in a 45 °C bath, was inoculated with 10⁶ to 10⁸ phage particles and 2 × 10⁷ to 2 × 10⁸ cells from either a mid-exponential to early stationary phase shaken LB culture or an overnight unshaken LB culture, and the mixture was poured on to LB agar plates pre-warmed to 37 °C. The plates were incubated at 37 °C for about 6 h, after which the semi-liquid soft agar layer was allowed to solidify at room temperature. LB (2 ml) was added, the soft agar macerated, and the whole was transferred to a test-tube for low-speed centrifuging. The supernatant lysate was stored at 4 °C over chloroform to maintain sterility. Lysates were assayed by the soft agar overlay method and those that contained 2 × 10⁹ or more plaque-forming units (p.f.u.)/ml were used in further experiments.

Mutagenesis of P1_{kc} lysates. This followed the method of Hong & Ames (1971). Lysate (5 to 10 ml; titre at least 10¹⁰ p.f.u./ml) was centrifuged at 38 000 g for 90 min at 15 °C, by

Table 1. *Bacterial, plasmid and phage strains*

Strain	Genotype	Origin
<i>Bacteria (Escherichia coli)</i>		
J62-1	F ⁻ <i>pro his trp lac nal-r</i>	N. Datta
CB-1	Hfr(p4X) <i>metB argCB-1</i>	N. Glansdorff
P4X	Hfr(p4X) <i>metB</i>	N. Glansdorff
P4XGN	Spontaneous <i>nal-r</i> mutant of P4XG, Hfr(p4X) <i>ppc</i>	A. E. Kadikiran
P4XB2	Hfr(p4X) <i>metB argR</i>	N. Glansdorff
E18M	Hfr(p4X) <i>argE18</i> }	Met ⁺ transductants of strains E18 and E34 (Cunin <i>et al.</i> , 1969)
E34M	Hfr(p4X) <i>argE34</i> }	
TLAO2	F ⁻ <i>argC6 trp lac</i>	N. Glansdorff
P4XB2E18	Hfr(p4X) <i>argR argE18</i> }	Isolated among Met ⁺ transductants of P4XB2 with P1kc grown on E18M or E34M
P4XB2E34	Hfr(p4X) <i>argR argE34</i> }	
P4XC6	Hfr(p4X) <i>argC6</i>	Isolated among Met ⁺ transductants of P4X with P1kc grown on TLAO2
C600	F ⁻ <i>thr leu thi lac str-r suII⁺</i>	NCIB10222
C600N	Spontaneous <i>nal-r</i> mutant of C600	
<i>Plasmid</i>		
R1822	Confers resistance to A, K, T;* incompatibility group P	R. H. Olsen
<i>Phages</i>		
P1kc		V. Bryson
N58	Amber mutant of phage T4	J. R. Guest
C427	Ochre mutant of phage T4	J. R. Guest
PRR1	RNA phage that infects cells carrying P group plasmids	R. H. Olsen

* A, ampicillin; K, kanamycin; T, tetracycline. In descriptions of the phenotype of strains carrying this R factor or its derivatives, superscripts r and s denote resistance and sensitivity respectively.

which time over 99 % of the phage had sedimented. The supernatant was removed, and mutagenesis mixture (3 ml) was added to the pellet. The mutagenesis mixture contained (per ml): 0.3 ml phage buffer (Clowes & Hayes, 1968); 0.6 ml 0.1 M-sodium phosphate buffer pH 6, containing 1 mM-EDTA; and 0.1 ml freshly prepared 1 M-hydroxylammonium chloride, adjusted to pH 6 with 10 M-NaOH and containing 1 mM-EDTA. The phage particles were gently resuspended in this mixture with a glass rod and incubated at 37 °C for 40 h. They were then centrifuged as above, washed twice with phage buffer, and finally resuspended in LB. The suspension was kept over chloroform till use. Sometimes the mutagenized phage became incapable of transduction after a few days' storage at 4 °C, and so it was used as quickly as possible. During mutagenesis, the plaque-forming titre fell rapidly though to a variable extent, initially by as much as 10-fold every 4 h. Transducing ability was, however, relatively little affected. This was to be expected, since phage genomes rapidly accumulate mutations that render them incapable of productive infection.

Transduction procedure. Unmutagenized P1kc at multiplicities of 0.01 to 0.5, or various quantities of mutagenized P1kc, were added to a late-exponential phase LB recipient culture. After incubating for 10 min at 37 °C, the mixtures, and controls without phage, were centrifuged and resuspended in minimal salts; both were then plated on selective media.

Testing of transductants. Transductants selected for resistance to one antibiotic were streaked on to two MacConkey agar plates each containing one of the other antibiotics to

which R1822 confers resistance. Alternatively, antibiotic-sensitive transductants were sought by replica plating the selection plate directly on to MacConkey agar plates containing one of the other antibiotics. Both methods worked. Resistance to phage PRR1 was checked either by spotting a high-titre suspension of PRR1 on to a lawn of the isolate in soft agar on a nutrient agar base, or by spotting the phage on to a streak of culture on nutrient agar plates (surface spot test).

Conjugation procedure. A mid-exponential phase nutrient broth donor culture was mixed with an overnight nutrient broth recipient culture in the proportion 1:10 by vol. The mixture was incubated at 37 °C for 2 h; it and its parental controls were then centrifuged, resuspended in minimal salts, diluted as appropriate and plated on selective media.

Isolation and testing of revertants. Overnight nutrient broth cultures of strains carrying a mutant R1822 plasmid and an *argE* or *argC* amber mutation were centrifuged and resuspended in 0.5 vol. of minimal salts. Minimal plates lacking arginine were spread with the suspension (0.05 to 0.25 ml) and incubated for 3 to 5 days at 37 °C. Revertant colonies were picked directly into nutrient broth (2 ml), grown overnight at 37 °C, and spot tested in soft agar with T4 amber mutant N58, T4 ochre mutant C427, and PRR1.

RESULTS AND DISCUSSION

Isolation of mutants of R factor R1822 after hydroxylamine mutagenesis of a transducing lysate

P_kc lysates of strain J62-1 carrying the R1822 plasmid were prepared, treated with hydroxylamine, and used to transduce strain CB-1. Transductants were selected on MacConkey agar containing either ampicillin, kanamycin or tetracycline, and also on minimal media supplemented with either methionine or arginine, so that frequencies of transduction of plasmid markers could be compared with those of chromosomal markers. For unknown reasons, no transductants ever appeared on kanamycin at 25 µg ml⁻¹, although a few appeared when the concentration was lowered to 10 µg ml⁻¹. Relative numbers of transductants inheriting the various selected markers A^r:K^r:T^r:Arg⁺:Met⁺ were approximately 10:1:10:200:100 for untreated lysates; after treatment of the lysate with hydroxylamine, the ratio of Arg⁺ and Met⁺ to A^r and T^r increased by a factor of 5 to 10. The untreated lysate gave one Arg⁺ transductant per 1.4 × 10⁵ p.f.u. The equivalent figure cannot be given for the hydroxylamine-treated lysate, since little or no plaque-forming ability remained. However, assuming that the centrifugations before and after mutagenesis pelleted 100 % of the particles and that the final resuspension separated them completely, one would expect a frequency of one Arg⁺ transductant per 6.7 × 10⁵ p.f.u. in the untreated lysate.

A total of 273 A^r and 403 T^r transductants isolated from lysates exposed to hydroxylamine, together with 198 A^r, 16 K^r and 147 T^r transductants obtained with an untreated control lysate, were tested for cotransduction of other plasmid markers. Among the former were found one A^s, one K^s and two T^s mutants, and 17 that failed to give a positive surface spot test with phage PRR1. No variants were found among the transductants obtained with the untreated lysate. The frequency of mutants among the transductants isolated using hydroxylamine-treated transducing phage was therefore 21/676 or 3 %, for the limited number of markers examined.

Strains carrying the four mutant plasmids that failed to confer resistance to one of the three antibiotics all retained resistance to the other pair of antibiotics and the plasmids were transmissible to strain P4XGN. However, although fertility was normal for the A^s and K^s mutants, the two T^s mutant plasmids showed diminished transmissibility and, unlike the A^s

and K^s mutants, gave weak or negative PRR1 spot tests in soft agar. The reason for this is not known. The plasmid phenotypes were the same in P4XGN as in CB-1, confirming that mutations were carried on the plasmid. The phenotype of the A^s mutant was interesting in that sensitivity to ampicillin was not completely lost; in this respect the mutant resembles that described by Curtis, Richmond & Stanisich (1973). The A^s isolate, when plated on MacConkey agar containing ampicillin at 10, 25, and 50 µg ml⁻¹, gave approximately 100, 1 and 0.01 % survival respectively at the three concentrations.

The 17 mutants that failed to give a positive surface spot test with PRR1 also failed to do so in soft agar; all carried the complete set of wild-type antibiotic resistances and were non-transmissible. As CB-1, the recipient strain for the transduction with mutagenized phage, is Hfr, it would appear that the mutant R1822 plasmids cannot be mobilized by F. In view of the specificity of mobilization of non-transmissible plasmids sometimes observed (see, for example, Williams Smith & Heller, 1973), this is not perhaps especially surprising. I shall denote the mutations in these mutant plasmids as *tra*⁻.

Characterization of amber mutants of R1822

In order to detect whether any of the plasmid mutations were of the amber or ochre chain-terminating types, *argE* or *argC* amber mutations were introduced into the isolates and co-reversion (indicating suppression) looked for. Each of the 21 mutants was used as recipient in crosses with strains P4XB2E18, P4XB2E34, or P4XC6 as donor, and selection was made for Met⁺ transconjugants (arginine being present in the selective medium) that retain the antibiotic resistances conferred by the plasmid concerned. Transconjugants were tested for the presence of the known amber mutations *argE18*, *argE34* or *argC6* by plating them on medium containing ornithine. Recombinants with any of these amber *arg* mutations grew well on ornithine, whereas those retaining *argCB-1*, which is a polar deletion, show only poor growth (Cunin *et al.*, 1969). Since the *metB* marker in CB-1 is closely linked to the *argECBH* cluster, the great majority of Met⁺ transconjugants were of the type sought.

Tra⁻ strains carrying a mutant plasmid and an *arg* amber mutation were grown in nutrient broth and spontaneous Arg⁺ revertants selected. Five to ten revertants were then tested for spot lysis by N58, C427 and PRR1 in soft agar. Of five *tra*⁻ mutant plasmids examined in this way with the three *arg* amber mutations, one, *tra-3*, was found to yield a positive PRR1 spot test when combined with the *argC6* and Arg⁺ revertants tested; furthermore, revertants that gave a positive spot test with PRR1 also gave positive spot tests with one or other or both of the T4 nonsense mutants N58 and C427. The R factor was transmissible from these revertants to P4XGN. However, P4XGN carrying that R factor failed to give a positive spot test in soft agar with PRR1 and the R factor was not transmissible. Both observations suggest that the Arg⁺ Tra⁺ phenotype in the revertants was due to amber or ochre suppressors. From one of these revertants, *tra-3* was transferred to C600, which carries the amber suppressor *suII*. In this host it conferred a weak sensitivity to phage PRR1 in soft agar, and was transmissible, though at a lower frequency than the parental R1822. The frequency of transfer to strain C600N was 8×10^{-7} to 3.5×10^{-6} per donor cell, compared with 1.4×10^{-4} for R1822. It appears that *suII* suppresses *tra-3* rather inefficiently: this may be due to a low frequency of suppression or to the glutamine inserted by *suII* not allowing optimal function of the polypeptide whose gene has suffered an amber mutation in *tra-3*. The latter would seem more probable since *suII* is known to be a relatively efficient suppressor (Kaplan, Stretton & Brenner, 1965).

Similar attempts were made to discover whether any of the four antibiotic-sensitivity mutations were amber or ochre. As yet there is no evidence for this.

In view of the remarkably wide host range of R1822 (Olsen & Shipley, 1973), the amber mutant described here may be useful in identifying amber or ochre mutations in these diverse bacterial species.

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