Genet. Res., Camb. (1972), **20**, *pp.* 231–237
Printed in Great Britain

The chromosomal integration of a β-lactamase gene derived from the P-type R-factor RP1 in *Escherichia coli*

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(*Received* 8 *May* 1972)

1. INTRODUCTION

The R-factor RP1, which carried the markers *amp neo tet*, confers resistance to penicillins by specifying the synthesis of Type IIIa β-lactamase. It was detected initially in a strain of *Pseudomonas aeruginosa* (Sykes & Richmond, 1970) and fragments spontaneously in *Ps. aeruginosa* strain Ps 18 to give clones which carry only the *amp* marker. Such clones are said to carry the element RP 1–1 since they are still capable of specifying the transfer of ampicillin and carbenicillin resistance to appropriate recipients in mating experiments. However, unlike lines carrying RP 1, they contain no detectable extrachromosomal DNA (Ingram *et al.* 1972), and the likely supposition is that the *amp* gene in these clones has become integrated into the bacterial chromosome together with the regions necessary to allow such strains to act as *amp* gene donors in mating experiments. It is probably cells of *Ps. aeruginosa* in which the *amp* gene has become integrated together with an RTF region that are responsible for the transfer of chromosomal markers reported by Holloway & Stanisich (1971).

When strains of *Pseudomonas aeruginosa* carrying RP 1–1 are mated with R⁻ strains of *Escherichia coli*, transfer of the *amp* gene takes place at a frequency of about 10^{-8}/recipient but the transferred gene is unstable, the surviving colonies are small and the transfer has all the characteristics of being abortive. However, if these small unstable colonies obtained on ampicillin or carbenicillin agar subcultured on similar medium for a number of cycles of plating and re-isolation, larger colonies emerge in which the *amp* gene is now stable (Ingram *et al.* 1972). Since there is no evidence that the RP 1–1 element has remained unchanged in the course of this process, the *E. coli* clones carrying a stable *amp* gene derived from RP 1–1 are said to carry RP 1–1*. This paper presents evidence that the *amp* gene in *Escherichia coli* (RP 1–1*) is part of the bacterial chromosome.

2. METHODS AND MATERIALS

Strains. Escherichia coli W 3110 (RP 1–1*) was obtained as described previously (Ingram *et al.* 1972). A *lac*⁻ derivative of this strain (*E. coli* UB1106) was isolated following treatment with *N*-methyl-*N'*-nitroso-*N*-nitroguanidine (Adelberg, Mandel & Chen, 1965) by picking a non-lactose fermenting colony from EMB

agar. The recipient strains JC5744 (*recA*$^+$ *lac*$^-$ *str*$^-$ *leu*$^-$) and JC2926 (*recA*$^-$ *lac*$^-$ *str*$^-$ *leu*$^-$), both free of R and F plasmids, were obtained from Dr E. M. Lederberg. The F prime factor used was a *lac015* derivative of F13 which carries *lac*$^+$, *proA*$^+$ and *proB*$^+$ (Scaife, 1967); it will be referred to simply as 'F*lac*$^+$' in the text. This particular F prime factor mobilizes the chromosome from the *lac* region and transfers it clockwise. The strain carrying this F prime factor, *E. coli* E7084 (∇ (*lac pro*)$_{\text{XIII}}$ *thi*$^-$ *str*$^-$) was obtained from Dr J. Scaife. This strain was chosen since its F factor gives rise to chromosomal transfer from a high proportion of the cells to which it is transferred by conjugation, provided they have no deletion in the *lac pro* region.

Escherichia coli UB1106 (RP 1–1*) containing *Flac* was obtained by mating *E. coli* E7084 with *E. coli* UB1106 (RP 1–1) and picking Lac$^-$ colonies growing on agar containing 200 μg carbenicillin/ml. (Transfer of RP 1–1* from *E. coli* UB1106 (RP 1–1*) to *E. coli* 7084 in this experiment is impossible because RP 1–1* has lost its ability to promote the transfer of carbenicillin resistance in the course of becoming stable in *E. coli*: Ingram *et al.* (1972).) The presence of the F factor in these strains was confirmed by their sensitivity to MS 2 phage. *E. coli* UB1107 (F$^-$ R$^-$ *leu*$^-$ *rif*^{r}) was an ampicillin-sensitive strain from the stock collection in this laboratory.

Transfer experiments. Donor and recipient cultures, each containing about 5×10^8 bacteria/ml. in the late exponential phase, were mixed in equal proportions and incubated statically at 35° for 4 hr. Samples were then withdrawn, diluted as appropriate in nutrient broth, and plated on agar containing 200 μg carbenicillin/ml. to select Amp^R progeny. Streptomycin (50 μg/ml.) was commonly used as a counterselective agent. These Amp^R clones were then tested for their genetic constitution as described in the Results section.

Media. All growth and mating experiments were carried out in nutrient broth. The presence of the *leu*$^+$ marker was determined on minimal agar plates (Clowes & Hayes, 1968) supplemented with all the L-amino acids save L-leucine. The presence of *lac*$^+$ was detected on EMB-lactose agar (Clowes & Hayes, 1968).

Symbols. Resistance to neomycin and kanamycin in these strains is caused by the amino-glycoside phosphorylating enzyme (Davies, Brzezinska & Benveniste, 1972) and is specified by the *neo* gene. Resistance to ampicillin and to carbenicillin (*amp*) is due to Type IIIa β-lactamase (Sykes & Richmond, 1970; Richmond & Sykes, 1973), an enzyme distinct from the product of the *ampA* genes (Eriksson-Grennberg, 1968). The biochemical basis of tetracycline resistance is still somewhat obscure (Franklin, 1967). In this paper the genes responsible for this property are referred to collectively as *tet*.

Antibiotics. The carbenicillin and rifampicin used for selection purposes were the gifts of Dr R. Sutherland (Beecham Research Laboratories, Brockham Park, Surrey, England) and Drs F. Knüsel and F. Kradolfer (CIBA–Geigy, Basel, Switzerland), respectively.

3. RESULTS

Mobilization of the amp *gene in* E. coli (*RP 1–1*)*

The failure of *Escherichia coli* UB1106 (RP 1–1*) to act as a donor of the *amp* gene in conjugation experiments with a range of different recipients, the absence of extrachromosomal covalently closed circular DNA and the failure to 'cure' the strain of its *amp* marker with sodium dodecyl sulphate (Tomoeda, Inuzuka, Kubo & Makamura, 1968) all suggest that this gene may be chromosomally integrated in this strain of *Escherichia coli* (Ingram *et al.* 1972).

To confirm this location it is necessary to show linked transfer of *amp* and a chromosomal gene to an appropriate recipient. This has been achieved by introducing an F prime factor into *E. coli* UB1106 (RP 1–1*) and examining these clones for their transfer properties, using an ampicillin-sensitive, leucine-requiring strain as recipient. The F prime derivative of *E. coli* UB1106 (RP1–1*) is an 'intermediate male', so the pattern of transfer to a recipient is less simple than would be the case if the F factor had been carried solely in the integrated state. If *amp* is chromosomally located in the donor, linked transfer of *leu*$^+$ and *amp* should occur to a *recA*$^+$ recipient by chromosomal transfer, and the recipients receiving these genes would be F$^-$ and *lac*$^-$ unless infection with an *Flac*$^+$ factor also occurred as a second, independent event. Double infection of this type was commonly encountered in these experiments – see later. If a RecA$^-$ recipient were to be used, linked transfer of *leu*$^+$ and *amp* should be all but abolished since the only transfer of this type that could take place would be as an F′ *leu*$^+$ *amp* factor (Low, 1968) and this plasmid would be expected to arise only extremely rarely, if at all, with this *Flac*$^+$ factor.

Escherichia coli UB1106 (RP1-1*) containing the *Flac*$^+$ factor was mated with *E. coli* JC5744 and the Amp^R progeny selected. Whereas ampicillin resistance could not be transferred to an R$^-$ strain of *Escherichia coli* from *E. coli* UB1106 (RP 1–1*) (Ingram *et al.* 1972), resistant colonies were obtained at a frequency of about 10^{-5}/donor from the *Flac*$^+$ derivative of this strain (Table 1). These resistant colonies were then scored for the presence of *lac*$^+$ by plating on EMB agar, and for F by scoring for MS2 phage sensitivity. Two types of colony were found: about 60% were Amp^R Lac$^+$ and MS2^S and the remainder Amp^R Lac$^-$ and MS2^R. No MS2^S Lac$^-$ nor MS2^R Lac$^+$ colonies were obtained (Table 1). The isolation of Lac$^-$ MS 2^R colonies suggested that some chromosomal transfer of *amp* was taking place while the Lac$^+$ MS 2^S colonies might have arisen either by transfer of an F′ *lac*$^+$ *amp* or by double infection.

Replica plating of the Lac$^-$ MS 2^R colonies on to plates lacking L-leucine showed that all these colonies were prototrophic for leucine as well as carbenicillin resistant and this observation confirms the view that chromosome transfer had been responsible for the emergence of this class of recipient colonies (Table 1).

Examination of the Lac$^+$ MS 2^S colonies in the same way showed that all these had also received *leu*$^+$. However, in this case double infection had occurred, as could be shown by outcrossing these recipient colonies with an R$^-$F$^-$ leucine-

Table 1. *Progeny obtained when* Escherichia coli *UB1106 (RP1–1*) carrying* Flac *was mated with the RecA+ recipient* E. coli *JC5744*

Phenotype	No. of colonies	Freq. of transfer	% of total
A. Amp^R [Str^R] progeny			
Amp^R Str^R	817	$\sim 10^{-5}$	100
Amp^R Str^R $MS2^R$ Lac^- Leu^+	330	$\sim 4 \times 10^{-6}$	40·4
Amp^R Str^R $MS2^S$ Lac^+ Leu^+	487	$\sim 6 \times 10^{-6}$	59·6
B. Leu^+ [Str^R] progeny			
Leu^+ Str^R	450	$\sim 10^{-3}$	100
Leu^+ Str^R $MS2^R$ Lac^- Amp^R	434	$\sim 10^{-3}$	96·5
Leu^+ Str^R $MS2^S$ Lac^+ Amp^R	16	$\sim 3 \times 10^{-5}$	4.5

Amp^R [Str^R] colonies were selected on plates containing carbenicillin and streptomycin, and the Leu^+ [Str^R] progeny on plates containing streptomycin but lacking leucine.

requiring strain of *E. coli*. Twenty independent Lac^+ Leu^+ $MS2^S$ Amp^R colonies were mated with *E. coli* UB1107 and Leu^+ [Rif^R] progeny selected on plates containing 20 μg. rifampicin/ml. but lacking leucine. The ratio of donor to recipient in this cross was reduced to 0·1, and the duration of mating to 50 min. in order to reduce the incidence of double transfer as far as possible. The Leu^+ colonies obtained were then scored for the presence of *lac*$^+$, for their reaction to MS 2 phage and for their sensitivity to carbenicillin. More than 90% of all Leu^+ colonies obtained in this experiment were resistant to carbenicillin, but only about 5 % were Lac^+ and $MS2^R$. These results suggest strongly that the Lac^+ Leu^+ MS 2 Amp^R colonies obtained in the cross shown in Table 1 were the result of double infection, MS 2-sensitivity and *lac*$^+$ probably coming to the recipient *via* the *Flac*$^+$, and *amp leu*$^+$ by chromosomal transfer.

In a second series of experiments the cross between *E. coli* UB 1106 (RP1–1)* containing *Flac*, on the one hand, and *E. coli* JC5744 on the other, was repeated but Leu^+ [Str^R] progeny were selected. Transfer of *leu*$^+$ occurred at a frequency of about 10^{-3}/donor (Table 1), which confirms that the donor in this cross is capable of initiating a relatively high level of chromosome transfer. The leu^+ colonies obtained in this way were than scored for the presence of *lac*$^+$, for carbenicillin resistance and for sensitivity to MS2 phage as described earlier. All were found to be carbenicillin-resistant but only about 4% of these were also $MS2^S$ and Lac^+. Twenty colonies which had received *leu*$^+$, *lac*$^+$ and which were sensitive to MS2 phage were outcrossed with *E. coli* UB1107 to assess the linkage of the various markers. Transfer of *Flac*$^+$ occurred independently of *leu*$^+$ and *amp* suggesting that the colonies carried *Flac*$^+$ unlinked genetically to *amp* and *leu*$^+$, at least in the majority of the bacteria in the clones.

Table 2. *Amp*^{R} [*Str*^{R}] *progeny obtained when* Escherichia coli *UB1106* (*RP 1–1**) *carrying* F*lac was mated with the RecA recipient* Escherichia coli *JC2926*

Phenotype	No. of colonies	Freq. of transfer	% of total
Amp^R Str^R	568	$\sim 10^{-8}$	100
Amp^R Str^R MS2^S Lac$^-$ Leu$^-$	51	$\sim 10^{-9}$	8·9
Amp^R Str^R MS2^S Lac$^+$ Leu$^-$	517	$\sim 10^{-8}$	91·9

Transfer to a RecA$^-$ recipient

If transfer of *amp* is indeed occurring by the chromosomal route in these experiments, then recovery of carbenicillin-resistant progeny in a RecA$^-$ recipient should be much less frequent than in RecA$^+$ lines, and, moreover, there should be no incorporation of both *amp* and *leu*$^+$ into the recipient chromosome.

To test this hypothesis, the cross examined in the experiments shown in Table 1 was repeated but with *E. coli* JC2926 in place of JC5744 as recipient (Table 2). Only about one-thousandth as many Amp^R [Str^R] colonies were obtained in the RecA$^-$ recipient. Moreover, examination of these colonies for the presence of *lac*$^+$ and *leu*$^+$ and for their sensitivity to MS2 phage revealed two phenotypes only: over 90% were Lac$^+$ Leu$^-$ and Amp^R, while the remaining 8% were Lac$^-$ MS2^S Leu$^-$ and Amp^R. Indeed, no Leu$^+$ colonies were ever found when RecA$^-$ recipient was used.

Whereas Amp^R Str^R MS2^S Lac$^+$ colonies derived from the RecA$^+$ recipient proved to have two unlinked gene groups, this was not the case for colonies of this phenotype derived from the RecA$^-$ host. Outcrossing experiments to strain UB1107 showed that all the markers behaved as a single linkage group and that transfer of *amp* to the ampicillin-sensitive recipient was always accompanied by the transfer of *lac*$^+$ and F (i.e. MS2 sensitivity). It seems likely therefore that the *amp* gene was transferred to *E. coli* JC2926 as an F′ *amp lac*$^+$, and that the incorporation of *amp leu*$^+$ into the recipient chromosome which was the commonest event when a RecA$^+$ recipient was used, cannot take place in a RecA$^-$ strain.

Similarly, outcrossing of the Amp^R Str^R MS2^S Lac$^-$ clones to UB1107 showed linked transfer of carbenicillin resistance and sensitivity to MS2 phage in all cases; this pattern suggests the transfer of an F′ *amp* plasmid. The properties of the F′ *amp* and F′ *amp lac*$^+$ factors will be described in detail elsewhere. Transfer of both these plasmids may occur to a RecA$^+$ recipient but would not be detected because of the high level of *leu*$^+$ *amp* transfer by the chromosomal route (cf. Tables 1 and 2).

4. DISCUSSION

The highly linked transfer of *leu*$^+$ and *amp*, independent of F and *lac*$^+$, to a RecA$^+$ recipient but not to a RecA$^-$ strain otherwise isogenic, is good evidence that *amp* is integrated in the donor strain *E. coli* UB1106 (RP 1–1*). Such a conclusion certainly accords well with the other information available for this strain: it contains no plasmid DNA (at least in the covalently closed circular form) and the *amp*

gene cannot be 'cured' by treatment with sodium dodecyl sulphate (Ingram *et al.* 1972).

These experiments only give preliminary information on the map position of this *amp* gene in *E. coli*. All *leu*$^+$ colonies obtained by chromosomal transfer are also carbenicillin resistant which argues that the site of integration of *amp* is very close to *leu* (map position: 1 minute; Taylor, 1970). In this connexion it must be stressed that the *amp* gene examined here was derived from RP1 and is distinct from the *ampA* gene mapped at 80 minutes in the *E. coli* chromosome by Eriksson–Grennberg (1968).

There has been speculation from time to time as to where R-factor mediated genes arose initially (see, for example, Watanabe, 1971). Were they derived from a bacterial chromosome in a manner similar to the way in which F-prime factors are generated? Or did the genes evolve on extrachromosomal replicons in the first place? The experiments reported here do little to resolve this point. What they do show, however, is that it is perfectly possible for a gene hitherto encountered only on plasmids, but nevertheless extremely common among enteric bacteria in the environment (Jack & Richmond, 1969; Richmond, Jack & Sykes, 1971), to exist as a stable entity in the chromosome of *Escherichia coli*. Moreover the gene may be picked up from this location by interaction with a superinfecting plasmid such as F.

This investigation was supported by a CAPS award to R.B.S. jointly from the Science Research Council and Glaxo Research Limited, and by a Programme Grant from the Medical Research Council to M.H.R. for the investigation of epidemiological and molecular behaviour of R factors and other plasmids. We are grateful to Beecham Research Limited for generous gifts of carbenicillin and to CIBA-GEIGY for similar gifts of rifampicin.

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