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

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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 sub-cultured 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 lacO15 derivative of F13 which carries lac+, proA+ and proB+ (Scaife, 1967); it will be referred to simply as ‘Flac+’ 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 (V (lac pro)K111 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*) was an ampicillin-sensitive strain from the stock collection in this laboratory.

Transfer experiments. Donor and recipient cultures, each containing about $5 \times 10^8$ 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 AmpR progeny. Streptomycin (50 µg/ml.) was commonly used as a counterselective agent. These AmpR 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 (RP1-1*)

The failure of Escherichia coli UB1106 (RP1-1*) 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 (RP1-1*) 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 (RP1-1*) (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− MS2 R colonies suggested that some chromosomal transfer of amp was taking place while the Lac+ MS2 S colonies might have arisen either by transfer of an F' lac+ amp or by double infection.

Replica plating of the Lac− MS2 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+ MS2 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<sup>R</sup> [Str<sup>R</sup>] progeny** | | | |
| Amp<sup>R</sup> Str<sup>R</sup> | 817 | ~ 10<sup>-5</sup> | 100 |
| Amp<sup>R</sup> Str<sup>R</sup> MS<sup>2</sup> Lac<sup>-</sup> Leu<sup>+</sup> | 330 | ~ 4 x 10<sup>-6</sup> | 40·4 |
| Amp<sup>R</sup> Str<sup>R</sup> MS<sup>2</sup> Lac<sup>+</sup> Leu<sup>+</sup> | 487 | ~ 6 x 10<sup>-6</sup> | 59·6 |
| **B. Leu<sup>+</sup> [Str<sup>R</sup>] progeny** | | | |
| Leu<sup>+</sup> Str<sup>R</sup> | 450 | ~ 10<sup>-3</sup> | 100 |
| Leu<sup>+</sup> Str<sup>R</sup> MS<sup>2</sup> Lac<sup>-</sup> Amp<sup>R</sup> | 434 | ~ 10<sup>-3</sup> | 96·5 |
| Leu<sup>+</sup> Str<sup>R</sup> MS<sup>2</sup> Lac<sup>+</sup> Amp<sup>R</sup> | 16 | ~ 3 x 10<sup>-5</sup> | 4·5 |

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

requiring strain of *E. coli*. Twenty independent Lac<sup>+</sup> Leu<sup>+</sup> MS<sup>2</sup>S Amp<sup>R</sup> colonies were mated with *E. coli* UB1107 and Leu<sup>+</sup> [Rif<sup>R</sup>] 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<sup>+</sup> colonies obtained were then scored for the presence of lac<sup>+</sup>, for their reaction to MS2 phage and for their sensitivity to carbenicillin. More than 90% of all Leu<sup>+</sup> colonies obtained in this experiment were resistant to carbenicillin, but only about 5% were Lac<sup>+</sup> and MS<sup>2</sup>R. These results suggest strongly that the Lac<sup>+</sup> Leu<sup>+</sup> MS2 Amp<sup>R</sup> colonies obtained in the cross shown in Table 1 were the result of double infection, MS2-sensitivity and lac<sup>+</sup> probably coming to the recipient via the Flac<sup>+</sup>, and amp leu<sup>+</sup> by chromosomal transfer.

In a second series of experiments the cross between *E. coli* UB1106 (RP1-1)* containing Flac, on the one hand, and *E. coli* JC5744 on the other, was repeated but Leu<sup>+</sup> [Str<sup>R</sup>] progeny were selected. Transfer of leu<sup>+</sup> occurred at a frequency of about 10<sup>-3</sup>/donor (Table 1), which confirms that the donor in this cross is capable of initiating a relatively high level of chromosome transfer. The leu<sup>+</sup> colonies obtained in this way were then scored for the presence of lac<sup>+</sup>, 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 MS<sup>2</sup>S and Lac<sup>+</sup>. Twenty colonies which had received leu<sup>+</sup>, lac<sup>+</sup> and which were sensitive to MS2 phage were outcrossed with *E. coli* UB1107 to assess the linkage of the various markers. Transfer of Flac<sup>+</sup> occurred independently of leu<sup>+</sup> and amp suggesting that the colonies carried Flac<sup>+</sup> unlinked genetically to amp and leu<sup>+</sup>, at least in the majority of the bacteria in the clones.
Table 2. AmpR [StrR] progeny obtained when Escherichia coli UB1106 (RP1-1*) carrying Flac was mated with the RecA recipient Escherichia coli JC2926

| Phenotype                        | No. of colonies | Freq. of transfer | % of total |
|----------------------------------|-----------------|-------------------|------------|
| AmpR StrR                        | 568             | ~ 10^{-8}         | 100        |
| AmpR StrR MS2S Lac- Leu-         | 51              | ~ 10^{-8}         | 8.9        |
| AmpR StrR MS2S Lac+ Leu-         | 517             | ~ 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 AmpR [StrR] 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 AmpR, while the remaining 8% were Lac- MS2S Leu- and AmpR. Indeed, no Leu+ colonies were ever found when RecA− recipient was used.

Whereas AmpR StrR MS2S 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 AmpR StrR MS2S 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 (RP1-1*). 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.

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Integration of a β-lactamase gene in E. coli

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