Genet. Res., Camb. (1968), **11**, *pp.* 119–124
Printed in Great Britain

Study of the association of resistance to two drugs in a transferable determinant in *Salmonella typhimurium*

By E. S. ANDERSON, MARIANNE V. KELEMEN, CHRISTINE M. JONES and J.-S. PITTON*

Enteric Reference Laboratory, Public Health Laboratory Service Colindale Avenue, London, N.W. 9

(*Received* 8 *December* 1967)

1. INTRODUCTION

The nature of the association between the genes for drug resistance in transferable resistance factors (R-factors) has been a matter of some speculation. As the result of transduction experiments, Watanabe & Fukasawa (1961) suggested that an R-factor consisted of a linear linkage group which carried all the resistance determinants and the transfer factor responsible for the mobility of the complex. In spite of this postulated linearity, the strains of Shigella described by the Japanese workers, which were usually resistant to several drugs, transferred their resistances together, and interrupted mating experiments failed to separate individual resistances. This behaviour, which is representative of a class of multi-resistant R-factors found in Shigellae, Salmonellae and other Enterobacteria, does not support the concept of linear linkage between the determinants. In contrast, work in this laboratory exposed another class of R-factors, found at least in Salmonellae and *Escherichia coli*, in which not only could individual resistances be separated in transfer, but the transfer factor itself, apparently in the pure state, could be obtained in recipient lines (Anderson & Lewis, 1965*a*, *b*; Anderson, 1966, 1967). It was evident that a single linear linkage group did not accurately describe such R-factors. The behaviour of the multi-resistant R-factor studied was the result of the independent interaction of three separate resistance determinants with the transfer factor, of which probably only a single copy existed in the host cell (Anderson & Lewis 1965*b*; Anderson, 1966).

Most of our work has been pursued on a strain of phage type 29 of *Salmonella typhimurium* resistant to ampicillin (A), streptomycin (S), sulphonamides (Su), tetracycline (T) and furazolidone. Furazolidone resistance has not been transferred from this strain so far and it will be ignored. The transfer factor in this strain has been given the symbol Δ (Anderson & Lewis, 1965*b*). The strain can thus be designated 29ASSuTΔ. Unless it is specifically needed, the Δ symbol will be omitted.

It was pointed out previously that whereas A and T were separate determinants transferring independently of each other, S and Su, although independent of A and T, always transferred together to recipient lines (Anderson & Lewis, 1965*a*, *b*). Spontaneous loss of streptomycin resistance is always accompanied by loss of sulphonamide resistance, and vice versa. Unless the two resistances are different facets of activity of one gene, therefore, the SSu determinant carries separate but closely linked genes for resistance to streptomycin and sulphonamides. This article offers experimental support of the hypothesis that two resistance genes are concerned.

* Present address: University Institute of Medical Microbiology, University of Geneva, Geneva, Switzerland.

2. MATERIALS AND METHODS

Strain 29ASSuT was irradiated with ultraviolet light and grown overnight in nutrient broth. Master plates of the culture were then prepared on nutrient agar. Replica plating on to the same medium containing penicillin (100 μg/ml), streptomycin (40 μg/ml) or tetracycline (20 μg/ml) was then used to detect clones that had lost resistance to one or more antibiotics.

Bacterial crosses were performed by the methods described earlier (Anderson & Lewis, 1965*a*, *b*). Crosses in which transfer of sulphonamide resistance was being investigated were plated on Diagnostic Sensitivity Test Medium (Oxoid) containing 1·0 % of lactose and 100 μg/ml of sulphathiazole. Transfer of other resistances was detected on MacConkey agar containing suitable concentrations of the respective antibiotics.

Minimal inhibitory concentrations (MIC) of penicillin were determined by the method of Anderson & Datta (1965). Release of β-lactamase by ampicillin-resistant strains was compared on starch-nutrient agar plates developed with iodine-penicillin solution (Anderson & Lewis, 1965*a*).

3. RESULTS

The observations to be reported here were made on a line resulting from exposure of 29ASSuT to ultraviolet light for 20 sec, which killed 80% of the bacteria. The line concerned had lost its streptomycin resistance and is therefore designated 29ASuT. It still carries the Δ transfer factor. Table 1 shows the results of crossing this line with K12F$^-$ (=K12).

Table 1. *Cross of 29ASuT with K12*

Parents	Progeny	Frequency
Donor × Recipient		
29ASuT × K12	K12A	10^{-2}
	K12ASu	2×10^{-3}
	K12T	10^{-7}

Resistances: A = ampicillin, Su = sulphonamide, T = tetracycline.

The transfer of T will be ignored from now on. The transfer frequency of A from 29ASuT to K12 is identical with that of A from 29ASSuT to the same recipient (Anderson & Lewis 1965*a*). As would be expected from the resistance spectrum of the donor strain, the SSu determinant of 29ASSuT has disappeared. It has been replaced in 29ASuT by a new determinant, ASu, the transfer frequency of which to K12 is lower than that of A by a factor of about 0·2. This transfer frequency is similar to that of SSu from 29ASSuT to K12 (Anderson & Lewis, 1965*a*, *b*). Moreover, it was demonstrated that all recipients that had received Su had also become resistant to ampicillin. It was therefore assumed that irradiation had resulted in the elimination of S from the SSu determinant, and that a copy of A had been inserted into the hiatus so created. Alternatively, disturbance of the base sequence of the S gene might have resulted in its inactivation, and the A gene might have become attached elsewhere on the linkage group. The possibility of conversion of the S gene into the A gene was not seriously considered. For the sake of simplicity the first hypothesis is treated as correct for the time being, though no suggestion will be advanced concerning the mechanism of integration of A at the S site.

Anderson & Lewis (unpublished observations) demonstrated that the A determinant produced a characteristic pattern of phage restriction in phage type 36 of *S. typhimurium*. The SSu determinant does not produce such restriction. Nor does the ASu determinant, and in this respect it is indistinguishable from SSu.

If ASu has really replaced SSu in 29ASuT, the following predictions can be made:

(1) The transfer frequency of ASu should be the same as that of SSu.

(2) With the exception of the difference in drug resistance, ASu should be operationally homologous with SSu.

(3) Despite its A gene, the ASu determinant should be operationally different from the A determinant.

(4) If the A gene of ASu is fully functional, a strain carrying both A and ASu should show a higher level of ampicillin resistance than a strain carrying either determinant alone.

The strains used in testing these hypotheses are shown in Table 2.

Table 2

Recipient strains	Determinant carried	Designation
K12F⁻	—	K12
K12	ASu	K12ASu*
S. typhi	—	*S. typhi*
S. typhi	ASu	*S. typhi*ASu
S. typhimurium phage-type 36	A	36A
S. typhimurium phage-type 36	ASu	36ASu

* The Δ transfer factor was initially absent from all lines used as recipients.

The results obtained can be summarized as follows:

It has already been shown (see Table 1) that the transfer frequency of ASu to a sensitive recipient strain is similar to that of SSu, and this has since been confirmed in a number of experiments.

Superinfection with SSu of a strain already carrying ASu

The frequency of transfer of SSu fell to between 10^{-1} and 10^{-2} of its normal level. For example, *S. typhi* accepted SSu from K12 at a frequency of about 10^{-4}. *S. typhi*ASu accepted SSu from K12 at a frequency of about 10^{-5}. The lines so formed, which can be designated *S. typhi*ASu/SSu, were all unstable and segregated pure SSu lines with high frequency. Replica-plating experiments with ASu/SSu lines showed that within a few hours about 70% of colonies were segregants pure for SSu. Less than 1% were pure ASu segregants. Tests of the residual colonies, which were ASu/SSu hybrids, revealed that they too were undergoing a segregation pattern similar to that of the parent line.

The pattern of segregation was the same when the hybrids carried the Δ transfer factor as when they did not.

It was thus evident that all *S. typhi*ASu/SSu hybrids formed in these crosses were unstable; that their instability resulted from competition between ASu and SSu, probably for a single site in the bacterial cell; that their competition for a site in the host cell was independent of their possible association with the Δ transfer factor; and that the results of their competition were weighted in favour of SSu, perhaps because it constituted the 'wild' form of the determinant. Similar results were obtained when K12ASu and 36ASu were superinfected with SSu.

Superinfection with ASu of a strain already carrying A

If ASu is operationally homologous with SSu but not with A, it should enter a line carrying A with the same frequency as SSu, and the lines so formed should be stable.

As an example we shall give give the results of superinfecting 36A with ASu from K12ASu. Selection on medium containing sulphathiazole showed that ASu entered 36A at a frequency of 3×10^{-4}, the same as that of SSu. The hybrid lines 36A/ASu formed in this way were stable. When 36A/ASu was crossed with K12, the A determinant transferred at its normal frequency, and the ASu determinant transferred at the frequency characteristic of SSu.

Activity of the A gene of ASu

The MIC of penicillin for 36A is 3000 μg/ml; 36ASu has the same MIC of penicillin as 36A. There was no distinguishable difference in the diameter of zones of β-lactamase diffusion from colonies of 36ASu and 36A grown on starch-nutrient agar plates developed with iodine-penicillin solution. It can thus be concluded that the A gene in ASu is as fully functional as that in the pure A determinant. It would therefore be expected that both ampicillin resistance genes would be active in hybrids such as 36A/ASu. The penicillin MIC of 36A/ASu lines proved to be 6000 μg/ml, that is, precisely twice that of 36A and 36ASu lines. This establishes that each A gene is fully active and is unaffected by the presence of the other. As the ampicillin (penicillin) resistance activity of the A gene results from the production of a β-lactamase, it is inferred that A/ASu hybrids produce twice as much of this enzyme as do lines that carry only A or ASu. This assumption has not yet been tested experimentally.

It will be recalled that the original variant line 29ASuT, resulting from irradiation, transferred both A and ASu to K12. This suggested that it carried both the A and the new ASu determinants. Because 29ASuT carries two functional copies of the A gene, therefore, while the parent strain 29ASSuT from which it was descended carries only one, the penicillin MIC of 29ASuT should be double that of the parent strain. This was confirmed by the finding that the penicillin MIC of 29ASuT was 6000 μg/ml, while that of 29ASSuT was 3000 μg/ml.

4. DISCUSSION

It is assumed in this discussion that the resistance determinants are extrachromosomal in location.

The properties of the ASu determinant suggest that it carries separate genes for ampicillin resistance and sulphonamide resistance, and therefore that the S gene of the SSu determinant is also distinct from Su. In the irradiated clone 29ASuT in which the ASu determinant was formed, a copy of the A gene has been substituted for the S gene of SSu, with the same close linkage to Su as S. The determinant would of course be expected to carry other genes controlling its structure, operation and replication, and its actual size is at present unknown. In terms of drug resistance, however, it is probably a linkage group of two genes as previously suggested (Anderson & Lewis, 1965*b*).

The competition between ASu and SSu which results in loss of one or the other (mostly in loss of ASu), suggests that there is only one site in the host cell that can accommodate either ASu or SSu. This leads to the hypothesis that under normal conditions there is only one copy of any resistance determinant in the cell. The precise doubling of the penicillin MIC in hybrids carrying both A and ASu supports this hypothesis.

The high segregation rate of ASu/SSu hybrids suggests that, unless a determinant can occupy its specific integration site, perhaps on the cell membrane, it cannot multiply, so that it will ultimately be diluted out of the hybrid population. As the Su region of SSu is

apparently unaltered in the ASu determinant, it might be inferred that the attachment region of each determinant is in the neighbourhood of the Su gene. However, as the only regions of these determinants yet defined are A, S and Su, and as the actual size of the determinant in terms of DNA is unknown, the region of attachment may be one of which the identity is as yet unknown. Nevertheless, it is reasonable to assume that competition between ASu and SSu probably results from affinity of an identical region of each determinant for the unique site in the cell into which it can integrate.

The fact that the segregation pattern of ASu/SSu hybrids is the same whether or not the Δ transfer factor is present, indicates that the cellular integration site for which ASu and SSu are competing is distinct from the integration site of Δ into the cell, and that the competition is independent of the possible association of the determinants with Δ. From this it may be assumed that, in most cells carrying determinants such as A and SSu and transfer factors such as Δ, each element is an independent replicon occupying its specific integration site, and therefore that the determinants are dissociated from the transfer factor which mediates their introduction into fresh hosts. This supports the hypothesis previously advanced that, in R-factors such as SSuΔ, the SSu determinant is usually dissociated from the Δ transfer factor, so that most cells will transfer only Δ. Only the few cells in which the two components are in the associated state will transfer the SSuΔ resistance factor (Anderson & Lewis, 1965*b*). Once transferred, an R-factor such as SSuΔ will again dissociate, so that the determinant and the transfer factor will integrate into their respective sites in the new host cell.

Work to be published later suggests that in R-factors such as TΔ, where the tetracycline resistance determinant T is closely linked to the transfer factor, only the transfer factor occupies an integration site in the cell. In such cases the determinant is maintained by the fact that its replicon is operationally linked with that of the transfer factor.

This work is being continued and further observations will be reported in a later article.

SUMMARY

A strain of *S. typhimurium* carrying transferable determinants, one for resistance to ampicillin (A), another for resistance to streptomycin and sulphonamides (SSu), was irradiated with ultraviolet light. A clone resulting from this treatment had lost streptomycin resistance and now carried the A determinant and a new determinant, ASu. Except for coding for ampicillin resistance, the ASu determinant was homologous with SSu. The A moiety of ASu produced ampicillin (penicillin) resistance of the same degree as the original A determinant. It was therefore concluded that irradiation had resulted in the elimination of the S gene in the SSu determinant and its replacement by an A gene to form ASu. Experiments with the ASu and SSu determinants suggest that there is normally only one copy of a resistance determinant in the host cell and only one cell site into which it can integrate.

One of us (J-S. Pitton) is grateful to the Fonds National Suisse pour la Recherche Scientifique for a grant.

REFERENCES

ANDERSON, E. S. (1966). Influence of the Δ transfer factor on the phage sensitivity of Salmonellae. *Nature, Lond.* **212**, 795–799.

ANDERSON, E. S. (1967). Facteurs de transfert et résistance aux antibiotiques chez les Entérobactéries. *Ann. Inst. Pasteur* **112**, 547–563.

ANDERSON, E. S. & DATTA, N. (1965). Resistance to penicillins and its transfer in Enterobacteriaceae. *Lancet* i, 407–409.

ANDERSON, E. S. & LEWIS, M. J. (1965*a*). Drug resistance and its transfer in *Salmonella typhimurium*. *Nature, Lond.* **206**, 579–583.

ANDERSON, E. S. & LEWIS, M. J. (1965*b*). Characterization of a transfer factor associated with drug resistance in *Salmonella typhimurium*. *Nature, Lond.* **208**, 843–849.

WATANABE, T. & FUKASAWA, T. (1961). Episome-mediated transfer of drug resistance in Enterobacterieaceae. III. Transduction of resistance factors. *J. Bact.* **82**, 202–209.