Characterization by Transformation of an Ampicillin-resistant Mutant of Pneumococcus

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SUMMARY

Mutagenesis of a transformable strain of pneumococcus by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine produced several mutants resistant to ampicillin. One of these was resistant to 0.1 µg. ampicillin/ml., but a purified solution of its DNA transformed the sensitive parent to three different levels of resistance at frequencies compatible with single, double and triple transformants. The higher levels of resistance were dependent on the presence of the genes conferring the lower resistances. Furthermore, transformants to the higher levels of resistance can be obtained at single or double frequency by using as recipient the strain already possessing the genes conferring the lower resistances. The expression times of the ampicillin genes are short (approximately 25 min.), but the actual times were difficult to determine since there was a delay of some 15 to 20 min. before the ampicillin exerted an effect on the sensitive strain under these conditions.

INTRODUCTION

In the course of experiments designed to map the relative positions of genes on the pneumococcus chromosome, it became necessary to make and characterize new markers to add to those already available. For this purpose, mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NNG) was performed and mutants having auxotrophic properties or antibiotic resistances were looked for. Amongst these were mutants resistant to ampicillin, and the characteristics relating to the transforming properties of one of these are described below.

MATERIALS AND METHODS

Organisms. *Diplococcus pneumoniae* strain cl 3, derived from strain R36A of Avery, MacLeod & McCarty (1944) by Ephrussi-Taylor (1951), sensitive to streptomycin and ampicillin. Stock cultures were kept as previously described (Butler, 1965).

Diplococcus pneumoniae strain r₂SQ, derived from strain r₂ of Ravin and Iyer (1961) by transformation, carrying the *ery-r2*, *str-41*, and *opt-r2* genes giving resistance to erythromycin, streptomycin and optochin respectively.

Media. The peptone media 'P' and 'NS' were prepared as previously described (Sicard, 1965; Butler, 1965), and the synthetic medium of Sicard (1965) was used in the mutagenic reaction with the following additions: glutamic acid 80 mg./l., aspartic acid 95 mg./l., tryptophan 100 mg./ml., serine 125 mg./l., proline 200 mg./l., phenylalanine 250 mg./l., adenine 18 mg./l. These compounds were added to allow the survival of auxotrophs.

Ampicillin. A fresh solution of ampicillin ('Penbritin', Beecham Research Laboratories, England) in 0.15 M-NaCl at the required dilution was prepared for each experiment.

Mutagenesis with NNG. Mutagenesis was obtained by allowing the NNG to act on an exponentially growing culture of strain cl 3. The organism was first grown in 'P' medium, washed and resuspended in supplemented Sicard medium. An aliquot of 1.0 ml. of this suspension was inoculated into 20 ml. supplemented Sicard medium and incubated at 37°. NNG at a final concentration of 20 µg./ml. was added at 50 min. and treatment was continued for 30 min. Under these conditions the NNG allowed 50 % survival of the culture. Samples were removed and plated in nutrient agar, incubated at 37° for 2 hr and a top layer of nutrient agar added containing ampicillin to give a final concentration of 0.025 µg./ml. The strain cl 3 is sensitive to ampicillin at a concentration of 0.0125 µg./ml. A check that the conditions gave good mutagenesis was provided by testing for the appearance of aminopterin-resistant mutants; if conditions are good, aminopterin-resistant mutants are obtained in high numbers (A. M. Sicard, private communication).

Preparation of deoxyribonucleic acid (DNA). The appropriate strain was inoculated into 750 ml. of medium containing 1 % (w/v) neopeptone (Difco), 0.85 % (w/v) yeast extract (Difco), 0.85 % (w/v) NaCl, 0.5 % (w/v) glucose and 31.5 % (w/v) glutamine (pH 7.1) and incubated at 37°. After 2½ hr phenol red solution was added, and as growth proceeded the lactic acid produced was neutralized by the addition of sodium hydroxide solution. When about 40 ml. of N-sodium hydroxide had been added, 55 g. hydrated sodium citrate was added and when dissolved, the culture was lysed by adding 4.5 g. sodium desoxycholate dissolved in a little water and incubated at 37° for 30 min. One volume of ethyl alcohol was stirred into the lysed culture, and the threads collected. Protein was removed by repeated shaking with chloroform and *sec*-octyl alcohol (Sevag, Lackman, & Smolens, 1938), the DNA again precipitated with one volume of ethyl alcohol and the fibres dissolved in sterile 0.15 M-NaCl.

Preparation of lysates. The organism was grown for about 2 hr in 10 ml. 'P' medium supplemented with asparagine and glutamine, centrifuged and resuspended in 1.0 ml. of the same medium. To the homogeneous suspension was added 0.1 ml. of 5 % (w/v) sodium desoxycholate and incubated at 37° for 30 min. The lysates were then kept at 4° until required, and their activity remained stable for several weeks.

Assay of transforming activity. The method used was essentially as described previously (Butler, 1965); differences in the technique which eventually arose will be described below.

RESULTS

Isolation of ampicillin-resistant mutants. As a result of the treatment with NNG, several colonies were isolated resistant to ampicillin at the challenging concentration of 0.025 µg./ml. Sensitivity curves were constructed, and the organisms were found to fall into two groups showing resistance to either 0.1 µg./ml., or 0.05 µg./ml. Strain Amp 3, which fell into the first group, was selected for further study.

Transformation with Amp 3 as donor. Transformation was carried out by using purified DNA prepared from the mutant strain Amp 3 as donor and strain cl 3 as recipient. Since mutants of lower resistance had been isolated it was of interest to ascertain whether the DNA from Amp 3 could give transformants resistant only to

0.1 $\mu\text{g./ml.}$ or whether it was also able to transform to lower resistances. After exposure to the DNA, aliquots of the culture were plated in nutrient agar and allowed to express for 2 hr before challenging with several concentrations of ampicillin. The results, given in Fig. 1, showed that the DNA transformed to three levels of resistance, 0.03 $\mu\text{g./ml.}$, 0.06 $\mu\text{g./ml.}$ and 0.09 $\mu\text{g./ml.}$ Transformants at the 0.03 level were obtained at a frequency some 10^2 greater than those at the 0.06 level, which themselves occurred at a frequency some 10^2 greater than those at the 0.09 level, indicating that these three levels of resistance were the result of single, double and triple transforming events respectively.

Fig. 1. Transformation of strain cl 3 to ampicillin resistance by DNA from strain Amp 3.
●, Sensitivity curve of strain cl 3; ▲, ampicillin-resistant transformants.

At dilutions which allowed both 0.03 (i.e. Amp 03) and 0.06 (Amp 06) transformants to form colonies on the same plate, Amp 06 could be distinguished from Amp 03 by their much larger colony size. This explains the projection back, in Fig. 1, of the Amp 06 curve to concentrations below 0.03 $\mu\text{g./ml.}$

Expression time. In the first instance, the expression times at the 0.03 and 0.06 levels were investigated by crossing strain cl 3 with 0.09-DNA. The double layer technique always gave the full number of transformants even at zero time, and hence expression was allowed in the reaction tube after a 4 min. exposure to the DNA followed by treatment with DNase for 1 min. Samples were then plated at intervals directly into nutrient agar containing ampicillin at concentrations of 0.03 $\mu\text{g./ml.}$ and 0.05 $\mu\text{g./ml.}$ Full expression was complete at about 4 to 5 min. for the 0.03 transformants and 9 min. for the 0.06 transformants. The curves obtained are shown in Fig. 2.

These expression times are very short, and it was considered that they may be artifacts. It seemed possible that the ampicillin was not exerting an immediate effect

on the organism, either because (a) competent organisms or organisms being transformed were insensitive to ampicillin, or (b) the ampicillin required a period of time before the bacteriocidal action occurred with perhaps a period of bacteriostatic activity, allowing expression to occur.

Action of ampicillin on transforming organisms. To test the effect of ampicillin on a culture which was in the process of being transformed, strain Cl 3 was exposed to r_2 SQ-DNA for 4 min., treated with DNase for 1 min. and ampicillin at concentrations of

Fig. 2. Apparent expression curves of genes conveying ampicillin resistance to the 0.3 μg./ml. and 0.6 μg./ml. levels in the cross Cl 3 × (Amp 3). ●, Transformants to Amp 0.3 as singles; ▲, transformants to Amp 0.6 as doubles.

0.03 μg./ml., 0.06 μg./ml. and 0.09 μg./ml. were added. Samples were then plated in agar at intervals, given full expression for streptomycin resistance (2 hr) before overlaying with streptomycin agar. Maximum numbers of streptomycin transformants were obtained until 15 to 20 min. exposure to the ampicillin, when the number of transformants dropped, indicating that the organisms were insensitive to the bacteriocidal action of the ampicillin up to that time. Furthermore, there was no effect by the ampicillin during the first 5 min. after the end of the DNase treatment so that the rise seen at the early part of the curve in Fig. 2 was a true increase in the number of transformants with increase in the time of expression. It was possible that a bacteriostatic action of the ampicillin occurred which was negated on plating in the ampicillin-free medium. However, a culture of strain Cl 3 prepared in exactly the same way (i.e. to produce

competence) but not treated with DNA gave a growth curve showing a doubling of the number of colony-forming units for the first 15 to 20 min. followed by a fairly rapid drop. This showed that there was no bacteriostatic action at the earlier exposure times, and that the bacteriocidal action occurred only after 20 min. exposure. This culture was competent at the time of exposure to the ampicillin but a non-competent culture tested in the same way gave essentially similar results.

From these results it can be concluded that the expression times of 4 to 5 min. for the 03 transformants and 9 min. for the 06 transformants are apparent times and must be increased by the 15 to 20 min. necessary for the ampicillin to exert its effect under these conditions. The procedure adopted for scoring for ampicillin transformants is to allow exposure of the recipient organism to the DNA for 15 to 30 min. and then to plate aliquots directly into nutrient agar containing ampicillin.

Table 1. Resistance levels in $\mu\text{g./ml.}$ of ampicillin transformants appearing at frequencies corresponding to singles, doubles and triples

Recip.	Donor DNA	Resistance level ($\mu\text{g./ml.}$) of ampicillin transformants		
		Singles	Doubles	Triples
cl 3	Amp 09	0.03	0.06	0.09
cl 3	Amp 06	0.03	0.06	—
cl 3	Amp 03	0.03	—	—
Amp 03	Amp 06	0.06	—	—
Amp 03	Amp 09	0.06	0.09	—
Amp 03	Amp 03	—	—	—
Amp 06	Amp 09	0.09	—	—
Amp 06	Amp 03	—	—	—

Table 2. Apparent expression times of the 06 and 09 genes as single or double transformants

Cross	Apparent expression time (min.)	
	Singles	Doubles
cl 3 $\times$ (Amp 09)	4.5 (03)*	9 (06)
Amp 03 $\times$ (Amp 09)	4.5 (06)	10 (09)
Amp 06 $\times$ (Amp 09)	5 (09)	—

* Figures in parentheses give the resistance level of the transformants.

Crosses between the ampicillin transformants. The single, double and triple transformants obtained by the cross cl 3 $\times$ (Amp 09) were isolated and lysates of the strains prepared. Crosses were then made using these lysates as donors and strain cl 3 or the resistant transformants as receptors. The results obtained are summarized in Table 1. The higher levels of resistance could not be obtained as single transformants from cl 3, but could be obtained by using as recipient the transformant having a resistance one step lower than that of the donor DNA. Similarly, resistances at the 09 level could be obtained at doubles frequency in the cross Amp 03 $\times$ (Amp 09). Hence, the higher resistances are the result of the organism possessing two or more of the genes conferring ampicillin resistance. The 06 level requires two genes, and the 09 level requires three genes.

Expression of the 06 and 09 levels as singles and doubles. The apparent expression times for transformation to the 06 and 09 levels as single or double transformants were

determined in the same manner as described above. The results are summarized in Table 2. Both the o6 and o9 genes when transformed as singles have the same apparent expression time as the o3 gene transformed as a single into cl 3, whilst the o9 gene transformed as a double had the same time as the o6 gene with cl 3, as recipient.

DISCUSSION

The results show that Amp 3 contained three loci conveying resistance to ampicillin which, although transmitted independently (i.e. are unlinked), are dependent on each other in a sequential fashion for the manifestation of the higher resistance levels. The lowest level of resistance, o3, was obtained at the frequency of single transformants in the cross cl 3 $\times$ (Amp o9), whereas o6 was obtained at doubles frequency and o9 at trebles frequency. Furthermore, in the cross Amp o3 $\times$ (Amp o9), o6 was obtained at singles frequency and o9 at doubles frequency. Hence resistance at the o6 level could not be obtained in the absence of the o3 gene, and the o9 level could not be obtained in the absence of both o3 and o6 genes.

Amp o6 transformed cl 3 to give both Amp o3 and Amp o6 as transformants, and Amp o9 transformed Amp o3 to give both Amp o6 and Amp o9. Hence, the pneumococci show the presence of three unlinked genes conferring resistance to ampicillin, namely (a) *amp-A* conferring resistance to 0.03 $\mu\text{g./ml.}$, (b) *amp-B*, which in the presence of *amp-A* confers resistance to 0.06 $\mu\text{g./ml.}$ and (c) *amp-C* conferring resistance to 0.09 $\mu\text{g./ml.}$ provided the organism also possesses *amp-A* and *amp-B*. Strains possessing the latter two systems are similar to strain r_4 of Schaeffer (1956), a pneumococcus which, although resistant to 50 $\mu\text{g. streptomycin/ml.}$, gave a transformant in the first instance resistant to 10 $\mu\text{g./ml.}$ which was then transformed by the same DNA to the full resistance of 50 $\mu\text{g./ml.}$ The sulphonamide system described in pneumococcus by Hotchkiss & Evans (1958) however is very different; in this case the three individual markers *a*, *b*, and *d* potentiated each other to give higher resistances but each marker itself could be transformed as single units. The penicillin-resistant mutants of pneumococcus described by Hotchkiss (1951) were similar to our ampicillin-resistant mutants in the sense that the DNA from the penicillin-resistant mutants transformed to resistance levels lower than that of the donor DNA. However, they were very different in that no transformations to the highest resistance level possessed by the donor DNA were described even after further transformation on a resistant transformant. Also, the expression time of the penicillin mutants was more than 90 min.

It is worth noting that the three resistance loci were produced in the same organism by a single mutagenic treatment by NNG.

As suspected, the very short expression times of 4 to 5 min. were found to be artifacts due to the delay in the manifestation of the ampicillin action. Even so, an expression time of 20 to 25 min. is still short when compared to the other resistance markers known in pneumococcus, such as 70 min. for *opt-r2*, 90 min. for *ery-r2* and 120 min. for *str-41*. It is interesting to note that the apparent expression times become the same for all three loci when being transformed as singles or as doubles, the time for the singles being about half that of the doubles. Hence it would appear that the second marker in the double transformants could not be expressed until after the first marker had completed an essential step in its expression.

Nester (1964) showed that competent cultures of *Bacillus subtilis* were insensitive to penicillin, from which it was deduced that such cultures were not dividing, and it is believed that they are in a latent state. The results concerning the effect of ampicillin on the pneumococcal competent culture show that these cultures are not similar to the *B. subtilis* cultures in this respect since a doubling in the colony-forming units of the competent culture occurred first, followed by a rapid drop to 1 % survival after 50 min. exposure to the ampicillin.

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REFERENCES

- AVERY, O. T., MACLEOD, C. M. & MCCARTY, M. (1944). Studies on the chemical nature of the substance inducing transformation of pneumococcal types. Induction of transformation by a desoxyribonucleic acid fraction isolated from *Pneumococcus* type III. *Journal of Experimental Medicine* **89**, 137.
- BUTLER, L. O. (1965). A co-precipitation method for the preparation of transforming DNA from small samples of low density bacterial cultures. *Journal of General Microbiology* **39**, 247.
- EPHRUSSI-TAYLOR, H. (1951). Transformations allogènes du pneumocoque. *Experimental Cell Research* **2**, 589.
- HOTCHKISS, R. D. (1951). Transfer of penicillin resistance in pneumococci by the desoxyribonucleate derived from resistant cultures. *Cold Spring Harbor Symposia on Quantitative Biology* **16**, 457.
- HOTCHKISS, R. D. & EVANS, A. H. (1958). Analysis of the complex sulfonamide resistance locus of pneumococcus. *Cold Spring Harbor Symposia on Quantitative Biology* **23**, 85.
- NESTER, E. W. (1964). Penicillin resistance of competent cells in deoxyribonucleic acid transformation of *Bacillus subtilis*. *Journal of Bacteriology* **87**, 867.
- RAVIN, A. W. & IYER, V. N. (1961). The genetic relationship and phenotypic expression of mutations endowing pneumococcus with resistance to erythromycin. *Journal of General Microbiology* **26**, 277.
- SEVAG, M. G., LACKMAN, D. B. & SMOLENS, J. (1938). The isolation of the components of streptococcal nucleoproteins in serologically active form. *Journal of Biological Chemistry* **124**, 625.
- SCHAEFFER, P. (1956). Analyse génétique de la résistance à la streptomycine chez le pneumocoque. *Annales de l'Institut Pasteur, Paris* **91**, 323.
- SICARD, A. M. (1965). A new synthetic medium for *Diplococcus pneumoniae*, and its use for the study of reciprocal transformation at the Ami-A locus. *Genetics, Princeton* **50**, 31.