Mapping of the Pneumococcus Chromosome. Linkage between the Genes Conferring Resistances to Erythromycin and Tetracycline and Its Implication to the Replication of the Chromosome

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(Received 4 July 1972; revised 7 June 1973)

SUMMARY

In the process of constructing a map of the pneumococcus chromosome by transformation, a search for linkage groups involving the markers *ery-r2*, *str-r41*, *amiA-r1*, *opt-r2* and *tet-A* has been carried out. No linkage was found amongst the first four markers, but linkage was found between *ery-r2* and *tet-A*. The discrepancy between these results and the previously reported linkage between the loci conferring resistance to erythromycin and streptomycin is discussed. The anomalous position of the *ery-r2-tet-A* linkage in relation to the chromosome map obtained by the density-shift method, and the implications to the mode of replication of the chromosome, are discussed. Two models are proposed for the replication of the pneumococcal chromosome.

INTRODUCTION

A previous paper (Butler & Smiley, 1973) reported a beginning to the construction of a map of the pneumococcus chromosome by the density-shift method. To support and enlarge such a map, a search for linkage groups was carried out. Linkage between genes in bacterial transformation is taken to mean that the two genes concerned can be situated on the same piece of transforming deoxyribonucleic acid (DNA). Unlinked genes are therefore those which never occur on the same molecule of DNA, and double transformants obtained possessing such genes must be derived by the integration of two separate molecules of DNA resulting from independent events. In pneumococcus, the only definitive evidence for linkage groups concerns the gene conferring streptomycin resistance which is linked both to the mannose utilization gene and to loci conferring sulphanilamide resistance (Hotchkiss & Marmur, 1954). There are also reports by Ravin and his collaborators (Ravin, 1966; Ravin & Chen, 1967) that streptomycin and erythromycin are linked. The lack of linkage between the pairs streptomycin and optochin, aminopterin and optochin and streptomycin and bryamycin has been inferred by Rolfe & Ephrussi-Taylor (1961) in the course of density gradient experiments, and support for these conclusions has been given by Martin & Ephrussi-Taylor (1964) and by Louarn & Sicard (1969).

Except for the work of Ravin, no direct genetic evidence concerning the antibiotic resistance markers is available, and the work to be reported is designed to provide such evidence. The criteria used for linkage have been (i) the appearance of dilution curves; (ii) the frequencies of double transformants; and (iii) the co-transfer index of Nester & Lederberg (1961). In (i), double events giving rise to double transformants to unlinked genes will become rarer as the DNA concentration is reduced and hence the numbers of

Table 1. *Description of the pneumococcal strains used*

Strain	Genotype	Comments
Recipients		
cl3	Wild-type	Isolated by Ephrussi-Taylor (1951)
AmI	<i>amiA-rI</i>	Derived from strain cl3 (Sicard, 1964)
Donors		
cl3-s	$\left. \begin{array}{l} \textit{str-r4I} \\ \textit{ery-r2} \\ \textit{opt-r2} \\ \textit{tet-A} \end{array} \right\}$	Derived from cross cl3 $\times$ (r ₂ SQ)
cl3-r ₂		
cl3-Q		
cl3-TC3		
r ₂ SQ	<i>ery-r2 str-r4I opt-r2</i>	Derived from strain cl3 by transformation with DNA from strain TC3 derived from NNG mutagenesis of strain cl3 (Butler & Smiley, 1970, 1973)
AmIr ₂ SQ	<i>amiA-rI ery-r2 str-r4I opt-r2</i>	Derived from strain cl3 by transformation
TC3r ₂ Q	<i>tet-A ery-r2 opt-r2</i>	Derived from cross AmI $\times$ (r ₂ SQ)
TC3r ₂ S	<i>tet-A ery-r₂ str-r4I</i>	cl3-TC3 $\times$ (cl3-Q)
TC3r ₂ SQ	<i>tet-A ery-r2 str-r4I opt-r2</i>	TC3Q $\times$ (C3-r ₂)
TC3AmIr ₂ SQ	<i>tet-A amiA-rI ery-r2 str-r4I opt-r2</i>	cl3-TC3 $\times$ (cl3-s)
		TC3S $\times$ (cl3-r ₂)
		TC3r ₂ Q $\times$ (cl3-s)
		(AmIr ₂ SQ $\times$ (TC3r ₂ Q)

such transformants will fall off more steeply than those due to linked genes. Criterion (ii) is permissible in pneumococcal transformation since the numbers of competent organisms in a competent culture are high (Fox & Hotchkiss, 1957; Nester & Stocker, 1963; Porter & Guild, 1969), and it does not give rise to the spurious linkage observed by Anagnostopoulos & Crawford (1961) for transformation in *Bacillus subtilis* due to comparatively low numbers of competent cells in a competent culture of *B. subtilis* (Cahn & Fox, 1968; Nester & Stocker, 1963).

MATERIALS

Organisms. The wild-type strain used was the transformable strain of *Diplococcus pneumoniae* known as Clone 3 (cl3) derived from the strain R36A of Avery, MacLeod & McCarty (1944) by Ephrussi-Taylor (1951). All other strains used were derived from cl3 and are listed in Table 1. Stock cultures were maintained as previously described (Butler, 1965; Butler & Smiley, 1970).

Media. The peptone media 'P' and 'NS' were prepared as previously described (Sicard, 1964; Butler, 1965).

Preparation of DNA. Two methods of preparation of the donor DNA were used: *Method I.* This method has been described by Butler & Smiley (1970), and was based on Sicard (1965) and Martin & Ephrussi-Taylor (1964). Deproteinization was by the Sevag method (Sevag, Lackman & Smolens, 1938). *Method II.* A forced culture of the pneumococci was prepared in the same way as for method I. The extraction of this culture was then carried out by modification of the methods of Berns & Thomas (1965), Kirby (1965), and Bacon, Overend, Lloyd & Peacocke (1967). The culture (1500 ml) was centrifuged and resuspended in 150 ml of sodium citrate saline (SCS) consisting of 0.015 M-C₃H₅ (COONa)₃·2H₂O (trisodium citrate) and 0.15 M-NaCl. At this point, it was often stored overnight at 4 °C. Fifteen ml of 20 % (w/v) sodium dodecyl sulphate in SCS were added, giving a final concentration of 2 % sodium dodecyl sulphate, and incubated at 37 °C for 45 min. Pronase (Type VI, Sigma

Chemical Company, St Louis, U.S.A.) was then added to give a final concentration of 1 mg/ml, and incubated at 37 °C for 2 h. An equal volume of a mixture consisting of 250 ml 90 % phenol in water, 35 ml *m*-cresol, and 2.5 g 8-hydroxyquinoline, was added and mixed gently for 20 min at room temperature. After centrifuging at 2500 rev./min for 20 min, the DNA-containing layer was carefully taken off with a wide-bore pipette and NaCl added to a final concentration of 3 % (w/v). The phenol extraction was repeated with 0.5 vol. of the phenol mixture. The precipitate from the first phenol extraction was further extracted with SCS overnight and given the second phenol extraction. The phenol was removed from the DNA solution by washing twice with diethyl ether, and the ether removed by evaporation under reduced pressure. An equal volume of ethyl alcohol was added and the DNA spooled, redissolved in SCS and re-spoiled on the addition of a volume of ethyl alcohol. The DNA was then finally dissolved in sterile 0.15 M-NaCl.

Assay of transforming activity. The method used was essentially as described previously (Butler, 1965), except that 1.0 ml of the competent culture was added to 0.1 ml of suitable dilutions of the DNA solutions. Double transformants were assayed by the addition of a top layer of nutrient agar containing the two antibiotics after full expression had been allowed for the gene having the longest expression time. The same concentrations of antibiotics were used for doubles as for single transformants.

Assay of colony-forming units. A portion of the competent culture was taken and suitable dilutions were plated in triplicate in nutrient agar containing horse blood. After overnight incubation the colonies were counted. No correction was made for chain lengths. The object was to relate the number of transformant colonies to the cell number, and since a transformant colony is derived from either a single transformant or from several transformants present in a chain, it seemed appropriate to relate these to the total colony-forming units (c.f.u.) rather than to a viable count which has been corrected for chain lengths. This reasoning holds when assessing the relative frequencies of single transformants but, on reflection, would give spurious results when applied to the assessment of frequencies of double transformants. Either cell of a chain of, for example, two cells would give rise to a singly marked transformant colony, but either one or the other of the cells must receive both the two hits necessary to produce the transformant colony doubly marked to two unlinked markers. Hence, although in the former case the second cell in the chain could have been disregarded, it should not be ignored when calculating frequencies in the latter case. From many observations of chain lengths carried out on similar strains under the same cultural conditions, the average chain length would not be expected to be greater than two (and might even be lower); hence all frequency calculations have been made using the c.f.u. count multiplied by two.

RESULTS

Experiments were carried out using strains Cl3 and Am1 as recipients, and DNA prepared by method I from the strains as listed in Table 1. Either full dilution curves were made, or else a saturating and non-saturating concentration of the donor DNA were used for the production of transformants. Platings were made in triplicate for all single transformants and also for all the possible combinations of double transformants. At the same time, a count of the c.f.u. in the recipient culture was carried out. The dilution curves were plotted, and the frequencies of transformation for each transformant class were determined. The frequencies for the double transformants were compared to the calculated frequencies expected as the maxima for unlinked systems, derived by multiplying together the frequencies of the two

single events. It is convenient to make this comparison by calculating the linkage index (l.i.):

$$\text{Linkage index} = \frac{\text{Calculated frequency}}{\text{Experimental frequency}}$$

The linkage index provides a useful assessment of the degree of linkage. If the ideal case of non-linkage occurred when the experimental frequency equalled the calculated frequency, the linkage index would equal 1.0; however, it is more usual for unknown factors to appear, giving rise to non-ideal cases (Porter & Guild, 1969), when an l.i. greater than 1.0 would occur. Linked systems would have an l.i. of less than 1.0.

In some cases, the co-transfer indices (CoI) of Nester & Lederberg were calculated. This index was defined by Nester & Lederberg (1961) as

$$\text{Co-transfer index} = \frac{A_1 B_1}{A_1 + B_1 - A_1 B_1}$$

where A_1 and B_1 are the number of single transformants to genes A and B respectively, and $A_1 B_1$ is the number of double transformants. Complete linkage would give an index equal to 1.0.

It is convenient to describe the results in two groups, those not involving and those involving the *tet-A* marker.

Experiments not involving the tet-A marker. The linkage indices and the co-transfer indices of double transformants of combinations of the *str-r41*, *ery-r2*, *opt-r2* and *amiA-r1* markers, at saturating and non-saturating concentrations of DNA, are given in Table 2. Both strains Cl3 and AM1 were used as recipients, with a preparation of donor DNA from strain r₂SQ and two preparations of DNA from strain AM1r₂SQ. In each case, the frequency of transformation obtained experimentally was always lower than the calculated frequency, indicated by the linkage index having values greater than 1 and by the co-transfer index values being very low, so that there was no evidence of linkage (or co-transfer) between any of the markers. Fig. 1 is representative of the dilution curves obtained, showing that the numbers of double transformants fell off with dilution of the DNA at a faster rate than the number of single transformants.

Dilution curves were also made with mixtures of DNA preparations where one marker was on one DNA preparation and the other marker on the other DNA preparation, so that co-transfer would be impossible. The values of the linkage index and the co-transfer index for the double transformants *str-r41 ery-r2*, *str-r41 opt-r2*, and *amiA-r1 str-r41* are given in Table 3. These values are of the same order as those given in Table 2, particularly for the *str-r41 ery-r2* double, confirming that they behave as unlinked markers.

Experiments involving the tet-A marker. The values of the linkage index and co-transfer index at the varying concentrations of DNA throughout a dilution curve for transformants involving the pairs of markers *tet-A str-r41*, *tet-A amiA-r1*, *tet-A ery-r2* and *tet-A opt-r2* are given in Table 4. In the two former cases the experimental frequencies were all lower than the calculated frequencies whilst the *tet-A ery-r2* double clearly showed linkage. Preparations of DNA from two different (but allied) strains gave evidence of co-transfer. The values of the linkage index for the *tet-A opt-r2* double are interesting, since they show a change from weak linkage at the higher DNA concentration to non-linkage at the lower concentrations except for the very lowest concentration, although the values of the co-transfer index all indicated non-linkage. The meaning of this behaviour is not clear. Fig. 2 gives typical dilution curves and it can be seen that the curve for *tet-A ery-r2* double transformants follows the slope of the curves for the *tet-A*, the *ery-r2* and the *opt-r2* single transformants, whilst the *tet-A opt-r2* double transformant fell away rapidly as the DNA was diluted, the

Table 2. *Double transformants not involving the tet-A marker: values of the linkage index (l.i.) and the co-transfer index (Col) of the double transformants using multiply marked DNA*

Experiment*	Relative [DNA]	<i>str-r4I ery-r2</i>		<i>str-r4I opt-r2</i>		<i>ery-r2 opt-r2</i>		<i>amiA-rI str-r4I</i>		<i>amiA-rI ery-r2</i>	
		l.i.	Col	l.i.	Col	l.i.	Col	l.i.	Col	l.i.	Col
1	Saturating	1.3	0.0092	—	0.0092	—	0.006	—	—	—	—
2	Saturating	4.1	0.0032	1.4	0.0022	1.6	0.0018	—	—	—	—
3	Saturating	4.2	0.009	3.5	0.0067	3.0	0.0067	—	—	—	—
	0.15 Sat.	5.4	0.0026	2.6	0.002	2.7	0.0018	—	—	—	—
4	Saturating	1.7	0.0077	1.1	0.0096	—	0.012	—	—	—	—
	0.15 Sat.	1.1	0.0052	—	0.0044	—	0.0049	—	—	—	—
5	0.15 Sat.	4.3	0.0022	2.1	0.0025	1.2	0.0024	2.6	0.0012	—	—
6	Saturating	2.8	0.0071	1.4	0.0064	1.9	0.043	1.9	0.014	1.5	0.015
Average	Saturating	2.8	0.0072	1.9	0.0068	2.2	0.0062	1.9	0.014	1.5	0.015
	0.15 Sat.	3.6	0.0033	2.4	0.0030	2.0	0.0030	2.6	0.0012	—	—

* Experiment 1 had strain am1 as recipient with strain r₃sq-DNA. All other experiments had strain cl3 as recipient with strain am1r₃sq-DNA. Experiment 2 used a different preparation of DNA from experiments 3, 4, 5 and 6.

Fig. 1. Dilution curves for transformants to the markers *str-r4I* ($\circ$, $x = 5$), *ery-r2* (Δ , $x = 4$), *opt-r2* ($\square$, $x = 4$), *str-r4I ery-r2* ($\bullet$, $x = 3$), *str-r4I opt-r2* ($\blacktriangle$, $x = 3$) and *ery-r2 opt-r2* ($\blacksquare$, $x = 2$) resulting from the cross $\text{cl3} \times (\text{r}_{25}\text{Q})$.

Table 3. Double transformants not involving the *tet-A* marker: values of the linkage index (l.i.) and co-transfer index (CoI) of double transformants resulting from mixtures of singly marked DNA

Relative [DNA]	$\text{cl3} \times (\text{cl3-s})(\text{cl3-r}_2)$ <i>str-r4I ery-r2</i>		$\text{cl3} \times (\text{cl3-s})(\text{cl3-Q})$ <i>str-r4I opt-r2</i>		$\text{cl3} \times (\text{Aml})(\text{r}_{25}\text{Q})$ <i>amiA -rI str-r4I</i>	
	l.i.	CoI	l.i.	CoI	l.i.	CoI
Saturating	1.9	0.0066	4.0	0.0015	—	—
0.5 Sat.	1.8	0.0058	3.8	0.0009	—	—
0.25 Sat.	—	—	—	—	4.5	0.0020
0.15 Sat.	2.5	0.0039	5.9	0.0003	—	—
0.075 Sat.	2.3	0.0032	4.5	0.0002	—	—
0.038 Sat.	3.2	0.0015	3.3	0.00004	—	—
0.015 Sat.	2.7	0.0010	—	—	—	—
0.0075 Sat.	3.2	0.0005	—	—	—	—
Average	2.5	0.0032	4.3	0.0007	4.5	0.0020

Table 4. Double transformants involving the *tet-A* marker: values of the linkage index (l.i.) and co-transfer index (CoI) of the double transformants using multiply marked DNA

Relative [DNA]	<i>tet-A str-r4I</i> cl3 × (Tc3Amlr ₂ SQ)		<i>tet-A amiA-rI</i> cl3 × (Tc3Amlr ₂ SQ)		<i>tet-A ery-r2</i> cl3 × (Tc3Amlr ₂ SQ)		<i>tet-A opt-r2</i> cl3 × (Tc3r ₂ SQ)	
	l.i.	CoI	l.i.	CoI	l.i.	CoI	l.i.	CoI
1.0 Sat.	1.2	0.022	2.4	0.0020	0.024	0.437	0.90	0.012
0.5 Sat.	1.2	0.016	5.9	0.0007	0.031	0.388	0.90	0.010
0.14 Sat.	1.2	0.013	2.3	0.0020	0.019	0.212	1.8	0.004
0.07 Sat.	1.7	0.007	5.4	0.0007	0.016	0.162	1.7	0.003
0.035 Sat.	1.6	0.005	11.7	0.0003	0.0095	0.161	1.7	0.002
0.014 Sat.	—	—	—	—	0.011	0.088	1.2	0.002
0.007 Sat.	—	—	—	—	0.0065	0.105	0.8	0.002
cl3 × (Tc3r ₂ Q)								
1.0 Sat.	—	—	—	—	0.016	0.170	—	—
0.29 Sat.	—	—	—	—	0.012	0.142	—	—
0.15 Sat.	—	—	—	—	0.010	0.114	—	—
0.075 Sat.	—	—	—	—	0.0058	0.126	—	—
0.029 Sat.	—	—	—	—	0.0037	0.132	—	—
0.015 Sat.	—	—	—	—	0.0026	0.099	—	—
0.0075 Sat.	—	—	—	—	0.0016	0.090	—	—

points for the lower dilution being well below the abscissa. A control dilution curve was carried out using a mixture of two DNA preparations, one containing the *tet-A* marker and the other the *ery-r2* marker. In this case, co-transfer was impossible and double transformants must be resultants of double events. The curves obtained are shown in Fig. 3 and the numbers of *tet-A ery-r2* transformants fell off rapidly with decreasing DNA concentration.

DNA prepared by method II. DNA was prepared from strain Tc3r₂S by method II and dilution curves carried out for single transformants and for the three possible pairs of double transformants. The curves obtained are shown in Fig. 4 and the values of the linkage index of the double transformants are recorded in Table 5. Again, evidence of linkage was obtained for the *tet-A* and *ery-r2* markers, but no linkage was found for the *tet-A* and *str-r4I* markers, nor for the *ery-r2* and *str-r4I* markers. It is of interest to note that the *tet-A str-r4I* system gave higher linkage indices than the *ery-r2 str-r4I* system although *tet-A* and *ery-r2* are themselves closely linked. The reason for this is unknown, but perhaps it is due to an interference mechanism.

Clonal analysis. Strain cl3 was treated with DNA prepared from strain Tc3r₂SQ and scored for single transformants for the *ery-r2*, *tet-A* and *str-r4I* markers, and also for the double transformants *tet-A ery-r2*, *tet-A str-r4I* and *ery-r2 str-r4I*. At the same time a c.f.u. count was done on the recipient culture. The frequencies of each transformant type were calculated and those for the doubles compared with the calculated maximum frequencies for double events. Then 110 colonies of each transformant type were analysed for the presence of all these markers, and the results are summarized in Fig. 5. The total number of transformants and the frequency of each transformant type are given, with the calculated frequencies for the double transformants in square brackets. The analysis of the transformant types is given as the number of colonies having the indicated genotype. Of the single transformants to *ery-r2*, a high proportion was also resistant to tetracycline; similarly, the *tet-A* transformants exhibited a very strong tendency to be also erythromycin-resistant. These results must be interpreted as showing linkage between *ery-r2* and *tet-A*. Both the *tet-A*

Fig. 2. (a) Dilution curve for transformants to markers *tet-A* (○), *ery-r2* (△), and *tet-A ery-r2* (■), resulting from the cross $cl3 \times (rc3r_2Q)$. (b) Dilution curve for the transformants to the markers *tet-A* (○), *ery-r2* (△), *opt-r2* (□), *tet-A ery-r2* (●) and *tet-A opt-r2* (■), resulting from the cross $cl3 \times (rc3Am1r_2SQ)$.

str-r4I and the *ery-r2 str-r4I* double transformants had relatively high proportions of *tet-A ery-r2 str-r4I* triple transformants, the third marker being brought in by its linkage to the *tet-A* gene in the former and the *ery-r2* gene in the latter case. There was little evidence to support linkage between *ery-r2* and *str-r4I* and, taken with the previous results, it must be concluded that there is no such linkage.

Although the DNA concentration used in the transformation was just below saturating, it was still high enough to give *str* transformants at a frequency of 1 in 27 (i.e. 1 in 13.5 with reference to the uncorrected c.f.u. count). This frequency of transformation is still high enough to be able to give a number of double transformants to unlinked markers resulting from two events. This would account for the three *ery str* double transformants out of the 110 selected *ery* colonies; it may also help to explain the 12 colonies containing *str* as a third marker together with the *tet ery* amongst the 110 selected *tet-ery* double transformants, although we feel that some other factor inherent in the selection of the colonies analysed may contribute to this figure. The values of the linkage index and co-transfer index strongly indicate that this factor is not linkage.

Fig. 3. Dilution curves for the transformants to the markers *tet-A* (○), *ery-r2* (△), and *tet-A ery-r2* (●), resulting from the cross $cl_3 \times (cl_3-rc_3)(cl_3-r_2)$, i.e. using a mixture of two preparations of DNA, one containing the *tet-A* marker and the other the *ery-r2* marker.

Table 5. Values of the linkage index (l.i.) and co-transfer index (CoI) for double transformants involving the *tet-A* marker, using multiply marked donor DNA prepared by method II

Relative [DNA]	$cl_3 \times (rc_3r_2s) II$					
	<i>tet-A ery-r2</i>		<i>tet-A str-r4I</i>		<i>ery-r2 str-r4I</i>	
	l.i.	CoI	l.i.	CoI	l.i.	CoI
Saturating	0.089	0.208	6.8	0.0039	1.9	0.011
0.5 Sat.	0.068	0.154	8.9	0.0024	2.2	0.0071
0.25 Sat.	0.086	0.169	15.0	0.0011	2.8	0.0046
0.10 Sat.	0.051	0.154	18.5	0.0006	2.5	0.0029
0.05 Sat.	0.025	0.236	7.2	0.0010	3.4	0.0017
0.025 Sat.	0.014	0.175	23.0	0.0001	1.9	0.0012
0.01 Sat.	0.0033	0.195	—	—	1.2	0.0007
Average	0.048	0.184	13.2	0.0015	2.3	0.0042

DISCUSSION

Comparisons of results obtained for crosses with multiply marked DNA with those obtained with mixtures of singly marked DNA showed that both the linkage index and the co-transfer index were of the same order for doubles involving the *str r4I*, *opt-r2*, *amiA-r1* and *ery-r2* markers. Since these markers could not be linked in the experiment involving mixtures of DNA when the markers were on different preparations, it follows that they

Fig. 4. Dilution curves for transformants to the markers *str-r41* (○), *tet-A* (□), *ery-r2* (△), *tet-A ery-r2* (■), *str-r41 ery-r2* (▲, count $\times 10$), and *tet-A str-r41* (●, count $\times 10$), resulting from the cross $cl3 \times (tc3r_2s)_{II}$.

were not linked with crosses involving multiply marked DNA. Hence the results confirm the lack of linkage between the *str-r41*, *opt-r2*, *amiA-r1* and *ery-r2* markers inferred by Rolfe & Ephrussi-Taylor (1961), Martin & Ephrussi-Taylor (1964) and Louarn & Sicard (1969). The similarities in the values of the indices were most marked with the *ery-r2 str-r41* double. However, Ravin (1966) and Ravin & Chen (1967) reported a weak linkage between *ery-r2* and *str-r53*. The *str-r53* marker is situated in the same locus as *str-r41* and may cover the latter marker. The discrepancy between the results presented here and those of Ravin may be due either to different methods of DNA preparation or to the use of different recipient strains. Ravin used a method of preparation similar to method I involving the Sevag technique of deproteinization. This may, in fact, produce conditions leading to breakage of the DNA. Method II, however, was physically much more gentle and produced a product having long strands on spooling. The results obtained with this preparation still gave co-transfer of *ery-r2* and *tet-A*, but there was again no evidence of linkage of *ery-r2* with *str-r41*. It would seem more probable that the differences are due to the two recipient strains. The strain used by the Ravin laboratory was strain sIII-I derived by Ephrussi-Taylor (1951), by transformation of R36A with $\lambda 66III$ -DNA which was followed by at least one mutational

Selected marker (s)			Genotype							
			N.G.	r_2	TCr_2	Tc	Tcs	S	r_2S	TCr_2S
r_2 : 1.1×10^6 ml $\frac{1}{74}$	110	tested	0	74	32	0	0	0	3	1
TCr_2 : 1.2×10^5 ml $\frac{1}{674}$ $\left[\frac{1}{3996}\right]$	110	tested	0	0	98	0	0	0	0	12
Tc: 1.6×10^6 ml $\frac{1}{54}$	110	tested	0	0	69	41	0	0	0	0
TCs : 2.3×10^3 ml $\frac{1}{36400}$ $\left[\frac{1}{1460}\right]$	110	tested	1	0	0	0	80	0	0	29
S: 3.0×10^6 ml $\frac{1}{27.0}$	110	tested	0	0	0	0	0	108	2	0
r_2S : 1.4×10^4 ml $\frac{1}{5638}$ $\left[\frac{1}{2000}\right]$	110	tested	0	0	0	0	0	0	97	13

Fig. 5. Clonal analysis of the cross $Cl_3 \times (TCr_2S)$. Experimental frequencies of the selected transformants are given, with the calculated frequencies for the double transformants in brackets; 110 colonies of each of a single and double transformant type were analysed and the number having the indicated genotype recorded. r_2 = *ery-r₂*; Tc = *tet-A*; S = *str-r4I*; N.G. = no growth.

Fig. 6. Map of the pneumococcal chromosome derived from density-shift experiments (Butler & Smiley, 1973). The distance between the *amp-ArI* marker and the *str-r4I* marker was taken as equal to 10.00 units, and the relative positions of the other markers calculated.

event. It therefore has a different history than the strain Cl_3 used as recipient in this laboratory. Further work has shown that there is indeed a difference in the linkage behaviour when strain SIII-1 is used as recipient compared to strain Cl_3 , and a preliminary report of these findings has been published (Butler, 1973).

The results described show that there is co-transfer of *ery-r₂* and *tet-A*. A previous paper (Butler & Smiley, 1973) described the relative positions of the markers obtained by the density-shift method and the map obtained is reproduced in Fig. 6. It will be seen that the replication order of the markers in this region of the chromosome is *-ery-r₂-str-r4I-tet-A-*, so that *str-r4I* is situated between *ery-r₂* and *tet-A*. Since *ery-r₂* and *tet-A* show linkage, the map would indicate that *str-r4I* should also be linked to both *ery-r₂* and to *tet-A*. This, however, is not the case, and hence there is a discrepancy between the linkage data and the

Fig. 7. Models proposed for the replication of the pneumococcal chromosome. (a) A model having one initiation point but bi-directional replication giving two replicating units; (b) a model having several initiation points with uni-directional replication in several replicating units.

map obtained by density shift. The density-shift method gives the order of replication of the markers, and this would be identical to the linear order of the markers on the chromosome only if the chromosome was composed of one replicon having one initiation point from which the replicating point travelled along the chromosome in one direction. It is possible, however, that more than one replicating unit may exist in the pneumococcal chromosome, and two models, depicted in Fig. 7, can be proposed, which would explain the discrepancies. In Fig. 7(a) a circular chromosome has been divided into two such units by having one initiation point from which replication proceeds in both directions around the chromosome. In this way, *ery-r2* and *tet-A* would be situated towards the end of one unit and *str-r41* would be situated towards the end of the other unit at such a distance from the initiation point that it would be replicated between *ery-r2* and *tet-A*. Evidence suggesting the presence of two replicating units has been put forward by Caro & Berg (1968, 1969) for the F^+ and F^- strains of *Escherichia coli*, by Masters & Broda (1971) also for *E. coli* and by Nishioka & Eisenstark (1970) for *Salmonella typhimurium*, all using transduction techniques, and by Yahara (1971) and Bird, Louarn, Martuscelli & Caro (1972) using DNA-DNA hybridization techniques. Evidence has also been presented in favour of bi-directional replication in *Bacillus subtilis* (Wake, 1972).

In Fig. 7(b) the chromosome, depicted for simplicity as a straight line, has been divided into several replicating units, each having its own initiation point with uni-directional replication. It could be postulated that initiation of each unit occurs simultaneously, that the units are of similar sizes and that the DNA synthesis in each unit occurred at similar rates. In this way, *ery-r2* and *tet-A* would occur towards the end of one unit whilst *str-r41* would occur towards the end of another unit. An extension of this model would allow for bi-directional replication from each of the initiation sites. Evidence of such a mechanism has been put forward in the study of the replication of mammalian chromosomes by Huberman & Riggs (1968) and by Callan (1972).

It seems that the model in *Pneumococcus* shown in Fig. 7(a) is more likely, in view of the evidence quoted above obtained by various independent groups of workers using different techniques. However, little is known concerning the replication of the pneumococcal chromosome, and the results reported above could be taken to support a multi-replicating

unit system. More light will be thrown on the problem as more mapping is achieved and more linkage groups are found.

Thanks are due for the technical assistance given by Mlle C. Genin, Fraulein M. Kobel and Fraulein A. M. Günther, and to the Medical Research Council for financial assistance for a part of this work. My thanks are also due to Professor A. M. Sicard for many useful and stimulating discussions during the course of the work, and for reading the manuscript.

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