Mapping of the pneumococcus chromosome: differences between recipient strains varying in *hex* property and the location of the *opt-r2* gene

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SUMMARY

Transformation studies in pneumococcus had shown that loci determining the resistance to erythromycin and streptomycin were unlinked when strain Cl3 was recipient but linked when strain SIII-I was recipient. This phenomenon also applies to other pairs of markers studied in these two recipients, no matter whether the transforming DNA was derived from strain Cl3 or strain SIII-I. Other differences between the two recipient strains were also revealed. Whereas competent cultures of strain Cl3 were composed of all competent cells, which was in agreement with previous reports of pneumococcal cultures, strain SIII-I normally gave a maximum average of 28 % competent cells. Strain SIII-I was unstable, since on repeated sub-culturing the competence peak profile changed and the value of 'fcq' increased. These properties were reflected in the two *hex⁻* strains 401 and R6x which were found to be similar to the 'altered SIII-I' strain. The results from the linkage studies have been applied to the chromosome map and have placed the *opt-r2* gene in the *str-r41*-containing chromosome arm.

1. INTRODUCTION

In a previous paper (Butler & Nicholas, 1973) a discrepancy was reported concerning the linkage between the genes conferring resistances to erythromycin and streptomycin in pneumococcus. Results of work in our laboratory showed no evidence of linkage between *ery-r2* and *str-r41* using strain Cl3 as recipient, although Ravin and his collaborators had shown linkage between *ery-r2* and *str-r53* using strain SIII-I as recipient (Ravin, 1966; Ravin & Chen, 1967). The *str-r53* marker is situated in the same locus as *str-r41* and may in fact cover the latter marker (Rotheim & Ravin, 1964), so that the discrepancy was not due to differences in the locations of the two streptomycin markers involved. It was suggested that the differences in linkage behaviour may be due to properties of the recipient organisms used, and a preliminary report of results supporting this view has been published (Butler, 1973). This paper will present further results indicating some of the differences between the properties of the two strains, and a comparison with two other *hex⁻* (Lacks, 1970) recipient strains. The results have also allowed the positioning of the *opt-r2* gene into its appropriate chromosome arm.

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2. MATERIALS AND METHODS

Organisms and sources of transforming DNA. Four strains were used as recipient organisms with DNA prepared from antibiotic resistance derivatives of each strain:

(*a*) Strain Clone 3 (Cl3), derived from the strain R36A of Avery, MacLeod & McCarty (1944) by Ephrussi-Taylor (1951). A description of the antibiotic re-sistant strains derived from strain Cl3 and used as the source of transforming DNA has been given previously (Butler & Nicholas, 1973).

(*b*) Strain SIII-I, kindly given by Dr A. W. Ravin, was originally derived by Ephrussi-Taylor (1951) by transformation of strain R36A by A66IIIN-DNA, which was followed by at least one mutational event during subsequent storage in the cold.

(*c*) Strain 401, derived from strain Cl3 by NNG mutagenesis by Tiraby, Claverys & Sicard (1973): it is *hex*⁻ and sensitive to all the antibiotics used.

(*d*) The strain R6x was kindly given by Dr Gérard Tiraby: it is sensitive to all the antibiotics used.

Stock cultures of all strains were maintained as previously described (Butler, 1965; Butler & Smiley, 1970).

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

Preparations of DNA. (*a*) The DNA from the antibiotic-resistant derivatives of strain Cl3 was prepared by Method II described previously (Butler & Nicholas, 1973).

(*b*) The DNA prepared from the derivatives of strain SIII-I carrying the markers *ery-r2* and *str-r53* was kindly given by Dr A. W. Ravin.

The *assays of transforming activity and of colony-forming units* were carried out as previously described (Butler & Nicholas, 1973). Chain lengths were not observed until later in the study and with strain SIII-I an average value of 2·68 was obtained from 21 observations. When the actual chain length was determined, then the colony-forming unit count was multiplied by the observed figure to give the viable count, otherwise the average values of 2·68 was used for strain SIII-I, 2·0 for strain Cl3, 6·0 for strain 401, and 2·38 for strain R6x.

Assessment of linkage. Linkage has been assessed using three methods:

(*a*) The linkage index (l.i.) (Butler & Nicholas, 1973) defined as:

$$\text{calculated frequency/experimental frequency}$$

using the numbers of competent organisms in the competent culture to calculate the frequencies, determining the fraction competent by the Goodgal & Herriott formula (Goodgal & Herriott, 1961). For pneumococcus, the fraction competent ('fc') will also include the 'q' factor of Porter & Guild (1969) and hence equal 'fcq'. The 'q' factor may vary with the particular state of the transformable cell, so that it may well be more appropriate to regard the percentage figure obtained as the maximum possible for the competent cells, the actual figure depending on the value of 'q'.

If the ideal case of non-linkage occurred when the experimental frequency equalled the calculated frequency, the linkage index would equal 1·0; linked systems would have an l.i. of less than 1·0.

(b) The *cotransfer index* of Nester & Lederberg (1961) was defined as: $AB/A + B - AB$. The formula for this factor gives a value which in isolation gives no indication of the linkage state and must be referred to the calculation representing 'no linkage'. The authors suggested that this latter could be 'one-half the efficiency of transformation of either marker', i.e. $A/2N$ or $B/2N$, but since certain pneumococcal strains give quite different efficiency levels for different markers, this is unsatisfactory. In this paper, both $A/2N$ and $B/2N$ (where $N =$ cfu corrected for chain length and % component cells) have been calculated and their average taken as representing the 'unlinked' values. Linkage is indicated when the value of the cotransfer index is greater than that calculated for 'no linkage'.

(c) The *graphical method*, based on that used by Ravin (1966), which involved plotting DNA concentration against the ratio of double to single transformants. The curves attained give a measure of the degree of linkage between the markers concerned; as the dilution of DNA increases, then, if strongly linked, the values of the ratio approximate towards a constant, whilst, if weakly linked, the curves would be exponential, the curvatures being related to the degree of linkage, which, in turn could be regarded as representing the distance between the markers.

The majority of cases to be reported in this paper have been expressed both as a linkage index and a cotransfer index, and with very few exceptions, the two indices gave the same conclusions. Because of this agreement it seemed superfluous to continue to calculate both indices. The linkage index was favoured because of its convenience in calculation. However, values indicating weak linkages should be treated with caution since the index is dependent on a somewhat inaccurate determination of the number of competent cells. The order of linkage groups, from strong to weak, obtained by the graphical method compared well with that obtained from the linkage indices.

3. RESULTS

(i) *Percentage of competent cells in the competent cultures, or 'fcq'*

The percentage of competent cells, or more appropriately, 'fcq', in the competent culture was assessed by the application of the Goodgal & Herriott formula (1961) to the results obtained at saturating concentrations of donor DNA. It was necessary to confirm that, with strain Cl3 as recipient, 100 % of the cells in the competent culture were competent. For this, it was possible to use both multiply-marked DNA and mixtures of singly-marked DNA, since all pairs of markers, except the *tet-A ery-r2* pair, were unlinked. The results obtained with multiply-marked DNA gave percentages of competent cells ranging from 112 % to 282 % with an average of 185 %, whereas the singly-marked mixtures gave an average value of 301 %. The findings of values greater than 100 % is in agreement with the values obtained by Porter & Guild (1969) with strain Rx-1 which led to

their formulation of 'q'. Strain Cl3, therefore, would not exhibit spurious linkage, but have a value of 'q' greater than 1 and probably more often equal to 2.

With strain SIII-I as recipient to mixtures of singly-marked DNA at saturating concentrations, the results were quite different, as can be seen from the column headed 'normal' SIII-I in Table 1. In this case, the percentage value for 'fcq' representing the maximum percentage of competent cells in the competent culture fell in the range 22–34 with an average of 28%.

Table 1. *Maximum percentage of competent cells or values of 'fcq' in competent cultures of strain SIII-I, transformed by mixtures of singly-marked DNA, calculated by the Goodgal & Herriott formula*

	'Normal' SIII-I			'Altered' SIII-I	
Expt	Markers of double transformants*	Maximum % competent cells	Expt	Markers of double transformants	Maximum % competent cells
1	(ery-r2) (str-r41)	22	1	(amiA-r1) (opt-r2)	46
2	(str-r41) (opt-r2)	34		(tet-A) (amiA-r1)	45
3	(ery-r2) (opt-r2)	27			
	Average	28	2	(tet-A) (opt-r2)	63

* Markers in parentheses indicate that they were derived from different singly-marked preparations.

(ii) *Instability of strain SIII-I*

The regimen required for maintaining competence involved repeated sub-culturing in an attempt to keep the culture in comparable conditions. After protracted periods of sub-culturing, it was noticed that the competence peak profile of strain SIII-I changed. The 'normal' peak profile of strain SIII-I was a low plateau, giving relatively low numbers of transformants over a period of some 30 min. This is in contrast to the peak profile of strain Cl3 which gave a very high peak of transformable cells. The tip of the peak existed for only a few minutes although the whole peak may cover a period of some 30 min. These were the profiles of the cultures that gave rise to the average values of competent cells in the competent culture of 28% for strain SIII-I and 185% for strain Cl3. However, after prolonged subculturing the first part of the plateau of the strain SIII-I profile developed a small peak, before falling to give the more normal plateau. The values of 'fcq' in the competent cultures were then determined using saturating concentrations of mixtures of singly-marked DNA, and, as can be seen from the column headed 'altered SIII-I' in Table 1, the value had increased, indicating that the percentage of competent cells in such cultures had increased. The higher the peak, the larger was the value of 'fcq'.

(iii) *Linkage between the erythromycin and streptomycin resistance genes*

The homologous cross between strain SIII-I as recipient and DNA from the SIII-I derived strain carrying the markers *ery-r2* and *str-r53* was carried out, using varying concentrations of the DNA. The results, expressed as the linkage

index (l.i.), indicate that quite strong linkage was present between these two markers (Table 2, column 1). However, when the heterologous cross, Cl3x (SIII-I-*str-r53 ery-r2*) was made, no linkage was found (Table 2, column 3), although the DNA preparation was the same as that used in the first cross. Further, with the other heterologous cross, SIII-Ix (Cl3-*str-r41 ery-r2*), in which DNA derived from strain Cl3 was used with SIII-I as the recipient, linkage was indicated (Table 2, column 5). These interpretations of the linkage indices were supported by a consideration of the cotransfer indices (Table 2, columns 2, 4 and 6). Hence, the recipient appeared to be the all important factor influencing the realisation of the linkage between these two markers.

Linkage between other markers

Further dilution curves were carried out using strain SIII-I as recipient to multiply-marked DNA extracted from strains derived from strain Cl3 in order to investigate possible linkages between combinations of the markers *str-r41*, *ery-2*, *opt-r2*, *amiA-r1* and *tet-A*. Examples of the values of the linkage indices and the cotransfer indices obtained are given in Table 3, the two indices giving the same conclusions regarding the linkage state. Average values of the linkage indices for all the pairs studied, normalized to the *ery-r2 str-r41* pair, are given in Table 4 (column (i)) and can be compared with the values obtained with strain Cl3 (column (iv)) (Butler & Nicholas, 1973). Whereas with strain Cl3 as recipient only one pair, *tet-A ery-r2*, was linked, with nine other pairs unlinked, with strain SIII-I as recipient six pairs were linked and only four pairs were found to be unlinked, namely *tet-A opt-r2*, *tet-A amiA-r1*, *amiA-r1 ery-r2* and *amiA-r1 opt-r2*.

(v) *Linkage results by the graphical method*

The curves obtained by plotting DNA concentrations against the ratio of double to single transformants AB/A or AB/B (Ravin, 1966), are shown in Fig. 1, from which it can be seen that the pair *tet-A ery-r2* shows strong linkage (55 %), with *tet-A str-r41* and *ery-r2 str-r41* being very much more weakly linked, followed by *str-r41 opt-r2*, with *tet-A opt-r2* and *ery-r2 opt-r2* showing marginal linkage. The combinations with the *ami A-r1* gene show no linkage giving curves similar to those obtained with mixtures of singly-marked DNA. Comparison with the l.i. values showed only two differences which occurred in the border zone between linkage and non-linkage where *tet-A opt-r2* gave both a linkage index and cotransfer index indicating non-linkage, and *amiA-r1 str-r41* an l.i. and cotransfer index indicating weak linkage.

(vi) *Comparison of strain SIII-I with two other recipient strains*

Strain SIII-I is said to be *hex⁻* (see Table 5) whilst strain Cl3 is *hex⁺* and hence it was of interest to ascertain whether other *hex⁻* strains behaved similarly to strain SIII-I with respect to linkages between pairs of markers. Two strains were selected as recipients, strain 401, known to be *hex⁻*, and a strain first thought to be R6. The efficiencies of integration of the *ery-r2*, *tet-A* and *opt-r2* genes compared

Table 2. *Linkage of genes conferring resistance to streptomycin and erythromycin: values of the linkage index (l.i.) and contransfer index (CoI) of double transformants using multiply-marked DNA*

Expt	Relative DNA conc.	SIII-I × (SIII-I str-r53 ery-r2) Linkage index (1)	Cotransfer index ×10⁻² (2) Exptl	Calc.	C13 × (SIII-I str-r53 ery-r2) Linkage index (3)	Cotransfer index ×10⁻² (4) Exptl	Calc.	SIII-I × (C13 str-r41 ery-r2) Linkage index (5)	Cotransfer index ×10⁻² (6) Exptl	Calc.
1	0·3 sat.	0·58	1·14	0·657						
2	Sat.	0·69	1·16	1·09						
	Sat.	0·58	1·38	0·786						
	0·3 sat.	0·69	0·881	0·526						
	0·15 sat.	0·61	0·675	0·362						
	0·075 sat.	0·54	0·354	0·196						
	0·035 sat.	0·25	0·489	0·122						
	Av.	0·56	—	—						
3	0·3 sat.	—	—	—	2·20	0·212	1·01			
	0·15 sat.	—	—	—	1·41	0·064	0·23			
	Av.				1·81	—	—			
4	Sat.	—	—	—	—	—	—	0·64	1·46	0·937
5	Sat.	—	—	—	—	—	—	0·78	1·14	0·887

Table 3. *Double transformants involving other markers with strain SIII-1 as recipient: values of the linkage index and cotransfer index using multiply-marked DNA derived from strain Cl3*

Relative DNA concn.	*ery-r2 str-r41*			*tet-A str-r41*			*tet-A ery-r2*			*amiA-r1 ery-r2*			*amiA-r1 str-r41*		
	Linkage index	Cotransfer index $\times 10^{-2}$		Linkage index	Cotransfer index $\times 10^{-2}$		Linkage index	Cotransfer index $\times 10^{-2}$		Linkage index	Cotransfer index $\times 10^{-2}$		Linkage index	Cotransfer index $\times 10^{-2}$	
		Exptl	Calc.		Exptl	Calc.		Exptl	Calc.		Exptl	Calc.		Exptl	Calc.
Sat.	0·68	1·43	0·942	0·57	1·28	0·730	0·019	57·6	0·0701	1·61	0·597	0·964	0·79	1·21	0·942
Sat.	0·64	0·763	0·490	0·63	0·738	0·458	0·013	52·0	0·0441	2·51	0·231	0·592	0·80	0·726	0·575
0·25 sat.	0·64	0·439	0·333	0·78	0·332	0·262	0·009	43·3	0·0259	3·01	0·103	0·330	0·82	0·385	0·319
0·10 sat.	0·42	0·400	0·172	0·43	0·291	0·126	0·005	46·6	0·0126	2·16	0·0919	0·200	1·26	0·169	0·215
0·05 sat.	0·23	0·367	0·0920	0·44	0·162	0·0717	0·003	44·9	0·0654	1·88	0·0695	0·130	0·98	0·127	0·124
0·025 sat.	0·20	0·101	0·0433	0·32	0·248	0·0469	0·002	40·9	0·0439						
0·01 sat.				0·20	0·271	0·0214	0·0005	43·5	0·0191						
Av.	0·47			0·48			0·007			2·33			0·93		

Table 4. *Values of linkage indices of double transformants using multiply-marked DNA derived from strain Cl3 with strains SIII-I, 401 and R6x as recipients compared with those obtained with strain Cl3 as recipient*

Double transformant	Linkage index with recipient strains			
	SIII-I* (i)	401* (ii)	R6x* (iii)	C13† (iv)
tet-A ery-r2	0·012	0·006	0·031	0·012
tet-A opt-r2	1·63	0·50	0·81	1·28
tet-A str-r41	0·78	0·48	1·30	1·39
str-r41 opt-r2	0·98	0·95	0·78	2·10
ery-r2 opt-r2	0·85	0·61	0·85	2·10
ery-r2 str-r41	0·47	0·61	1·28	3·20
amiA-r1 str-r41	0·88	—	—	2·20
amiA-r1 ery-r2	2·14	—	—	1·50
tet-A amiA-r1	14·2	—	—	5·50
amiA-r1 opt-r2	1·37	—	—	—

* Values normalized to *ery-r2 str-r41*.   † From Butler & Nicholas (1973).

Table 5. *Marker efficiency, when str-r41 = 1·0, with strains 401, R6x and SIII-I as recipients using multiply-marked donor DNA derived from strain Cl3*

Strain	Marker				
	str-r41	ery-r2	tet-A	opt-r2	amiA-r1
401	1·0	0·54	—	0·57	—
		0·51	0·73	0·62	—
		0·78	0·42	0·54	—
Average		0·61	0·68	0·58	—
R6x	1·0	0·81	—	0·63	—
		0·87	0·99	0·64	—
		0·88	1·01	—	—
Average		0·85	1·00	0·64	—
SIII-I	1·0	0·76	—	0·60	0·74
		0·98	—	0·68	0·81
Average		0·87	—	0·64	0·78

to *str-r41* were determined, using multiply-marked DNA derived from strain Cl3, and are given in Table 5. The *ery-r2* figure for strain 401 is comparable to those given by Tiraby and his colleagues (Tiraby & Sicard, 1973; Tiraby *et al.* 1973), whilst the value for *opt-r2* is slightly higher, thus confirming the *hex⁻* nature of strain 401. 'Strain R6' appears also to be *hex⁻*, and hence was identified as strain R6x (Tiraby, Fox & Bernheimer, 1975). It will be noted that these *hex⁻* strains gave efficiencies which, although similar for each marker, were mostly below 1·0, and it may be that each, in fact, possesses some low *hex* activity.
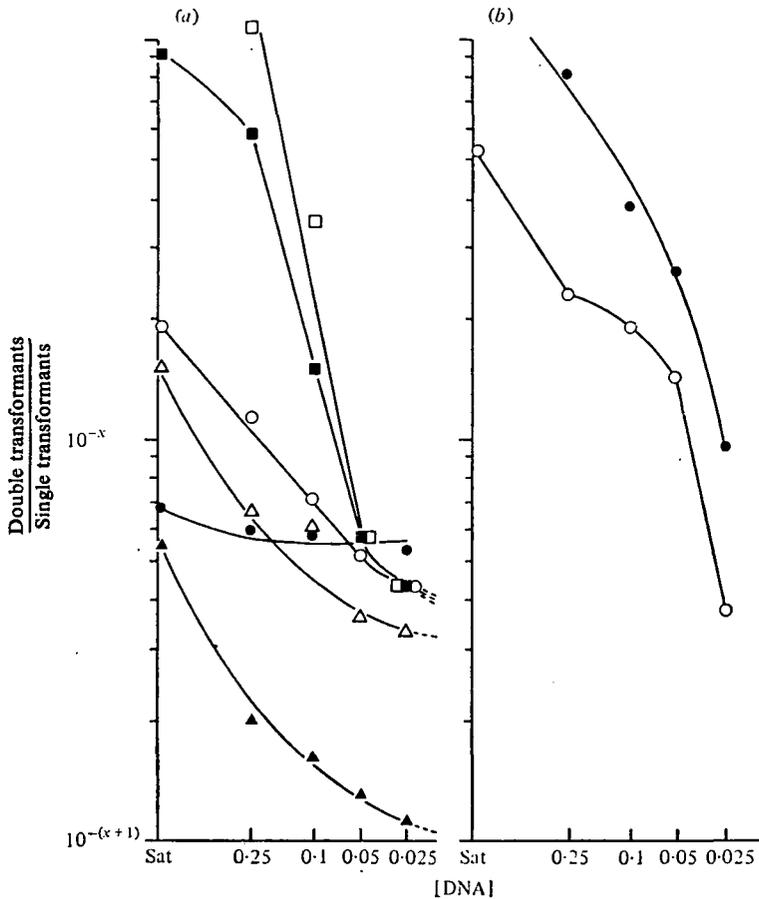
Fig. 1. Plots of DNA concentration against the ratio of double transformants to single transformants obtained from dilution curves using strain SIII-I as recipient to strain Cl3-derived donor DNA. (a) ●, *tet-A ery-r2*, $x = 1$; ○, *ery-r2 str-r41*, $x = 2$; ▲, *str-r41 opt-r2*, $x = 2$; △, *tet-A str-r41*, $x = 2$; ■, *tet-A opt-r2*, $x = 3$; □, *ery-r2 opt-r2*, $x = 3$. (b) ●, *amiA-r1 str-r41*, $x = 3$; ○, *amiA-r1 ery-r2*, $x = 3$.

(vii) *Peak profiles and values of 'fcq'*

The finding that strain SIII-I was normally giving competent cultures in which only a maximum of 28 % of the cells were competent made it necessary to check the percentage of such cells in competent cultures of strains 401 and R6x. Transformations were therefore carried out using saturating concentrations of mixtures of singly-marked donor DNA using both strains 401 and R6x as recipients, and the results obtained were applied to the Goodgal & Herriott formula for the calculation of 'fcq' for the competent culture. The results given in Table 6 show that the average values obtained were 48 % for strain 401 and 60 % for strain R6x. With strain SIII-I there appeared to be a correlation between the value of 'fcq' and the competence peak profile with an increase in the value as the competence

Table 6. *Maximum percentage of competent cells or 'fcq' in competent cultures of strains 401 and R6x, using saturating concentrations of mixtures of singly-marked donor DNA derived from strain Cl3*

	Expt	Markers of double transformants*	% Competent cells
Strain 401			
	1	(*ery-r2*) (*opt-r2*)	49
	2	(*str-r41*) (*opt-r2*)	40
		(*ery-r2*) (*str-r41*)	48
	3	(*tet-A*) (*str-r41*)	56
		Average	48
Strain R6x			
	1	(*str-r41*) (*opt-r2*)	68
	2	(*tet-A*) (*str-r41*)	53
		Average	60

* Markers in parentheses indicate that they were derived from different singly-marked preparations.

plateau changed to a peak. The peak profile for each strain were reminiscent of those of the 'altered' strain SIII-I in which the percentage value of 'fcq' increased to above 60 %.

(viii) *Linkage*

Dilution curves were carried out using multiply-marked donor DNA derived from strain Cl3 with both strains 401 and R6x as recipients in order to investigate possible linkages between combinations of the markers *str-r41*, *ery-r2*, *tet-A* and *amiA-r1*. Extreme difficulty was found when scoring for double transformants involving the *amiA-r1* gene, and no results have been obtained for combinations including this marker. The average values of the linkage indices for all pairs studied, normalized to the *ery-r2 str-r41* pair, are given in Table 4 (columns (ii) and (iii)). The *tet-A ery-r2* pair again shows strong linkage with either recipient. With strain 401 all other pairs tested appeared to show linkage, although the *str-r41 opt-r2* index is very close to 1·0, and the curves obtained by plotting the ratio of double to single transformants, shown in Fig. 2, indicate that the *tet-A opt-r2* and *ery-r2 opt-r2* pairs are unlinked. With strain R6x the pairs *tet-A str-r41* and *ery-r2 str-r41* gave indices indicating non-linkage, although all the other pairs gave values in the doubtful zone.

4. DISCUSSION

The differing results concerning the linkage between the loci determining erythromycin and streptomycin resistance found in our laboratory and reported by the Ravin laboratory have been shown to be due to differences intrinsic in the recipient organisms used in each case. Furthermore, other loci were also linked when DNA derived from strain Cl3 was donor to strain SIII-I although they were unlinked when strain Cl3 was recipient. The reason for this can only be speculative
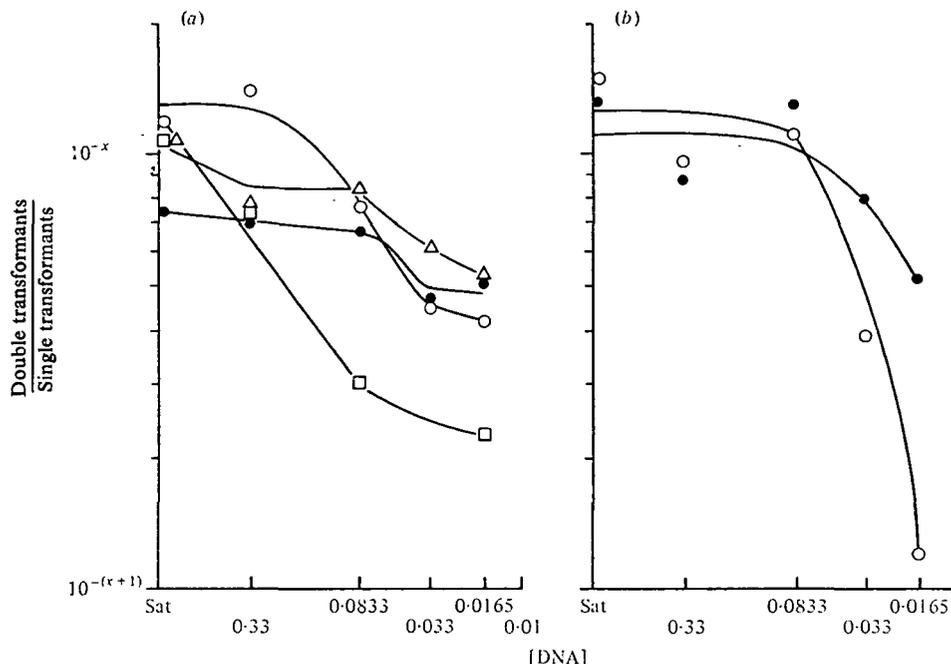
Fig. 2. Plots of DN Aconcentration against the ratio of double transformants to single transformants obtained from dilution curves using strain 401 as recipient to strain Cl3-derived donor DNA. (a) ●, tet-A-ery-r2, x = 1; △, str-r41 opt-r2, x = 2; ○, ery-r2 str-r41, x = 2; □, tet-A str-r41, x = 2. (b) ●, ery-r2 opt-r2, x = 2; ○, tet-A opt-r2; x = 3.

at this stage, but it may be that certain loci, e.g. the erythromycin and strepto-mycin loci, may lie closer together in strain SIII-I than in strain Cl3, so that a loop of DNA may be introduced which is not possible when strain Cl3 is recipient.

The curves obtained by plotting the ratios AB/A or AB/B against the DNA concentration give a measure of the degree of linkage between the markers con-cerned; as the dilution of DNA increases, then, if strongly linked the values of the ratio approximate towards a constant figure, whilst, if more weakly linked, the curves will be exponential, the curvature being related to the degree of linkage. By plotting the log log of the ratio against the log of the DNA concentration (Fazekas de St Groth, 1961), straight line relationships can be obtained, the slope of the line representing the degree of linkage, and hence the distance between the markers. The lines obtained for the six linked systems are shown in Fig. 3 and their slopes tabulated in Table 7. These slopes, regarded as representing distances between the genes, have enabled the positioning of the *opt-r2* gene. The replication map gave the order
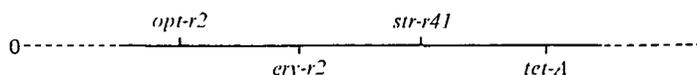
and there is also evidence that the pneumococcal chromosome replicates bi-direc-

tionally, with *ery-r2* and *tet-A*, in that order, near one terminus, and *str-r41* towards the other (Butler & Smiley, 1973; Butler & Nicholas, 1975). Hence, with these three genes used as reference markers, the application of the data places the *opt-r2* gene in the *str-r41* chromosome arm, as shown in Table 7.
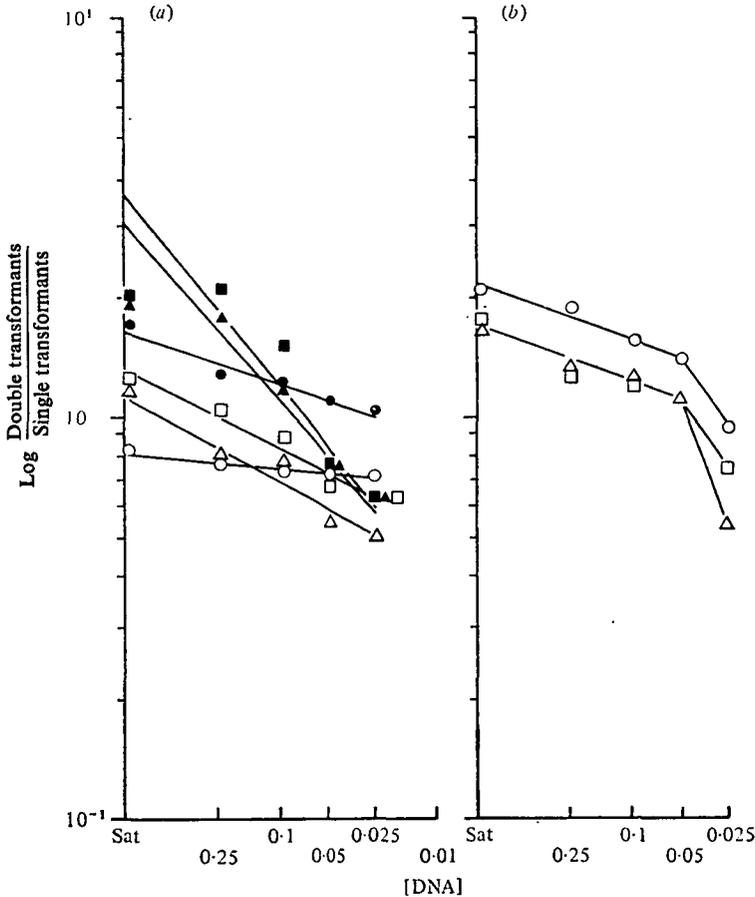
Fig. 3. Plots of DNA concentration against the log of the ratio of double transformants to single transformants obtained from dilution curves using strain SIII-I as recipient to strain Cl3-derived donor DNA. (a) ○, *tet-A ery-r2*; ●, *str-r41 opt-r2*; △, *tet-A ery-r41*; ▲, *tet-A opt-r2*; □, *ery-r2 str-r41*; ■, *ery-r2 opt-r2*. (b) ○, *amiA-r1 str-r41*; △, *amiA-r1-ery-r2*; □, *amiA-r1 opt-r2*.

Table 7. *Values of the slopes representing distances between markers and their application to the chromosome map*

Linkage group	Slope
tet-A ery-r2	1·6
tet-A str-r41	10·5
ery-r2 str-r41	9·5
tet-A opt-r2	22·0
ery-r2 opt-r2	24·5
str-r41 opt-r2	7·0
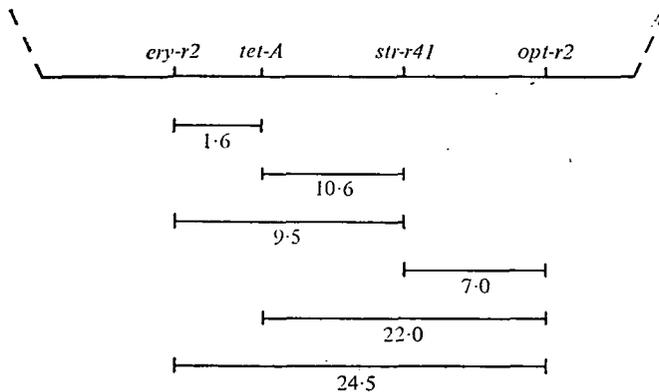
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