Chromosome Mapping in *Acinetobacter calcoaceticus*

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RP4-mediated conjugation was used to map the loci of 23 different mutations on a circular linkage group in *Acinetobacter calcoaceticus* EBF65/65. The resulting genetic map indicated that the chromosomal organization of *A. calcoaceticus* differed from that of members of the enteric group of bacteria and was similar to *Pseudomonas* in showing an absence of clustering of functionally related genes.

INTRODUCTION

Transformation and transduction are both of limited value during the initial stages of mapping the chromosome of a previously unstudied micro-organism. The demonstration that the P incompatibility group plasmid RP4 could be used to mediate conjugal transfer of chromosomal genes in *Acinetobacter calcoaceticus* (Towner & Vivian, 1976*a*) therefore provided, for the first time, a method for mapping large segments of the genome in this species. Other P incompatibility group plasmids, but not all, are also capable of mobilizing chromosomal genes in *A. calcoaceticus* (Towner & Vivian, 1977). Some preliminary determinations of linkage have already been made and it has been demonstrated that the linkage map of *A. calcoaceticus* is circular (Towner & Vivian, 1976*b*).

The frequency of RP4-mediated chromosomal gene transfer is not sufficiently high to allow normal interrupted mating techniques to be used. This paper describes methods used to obtain more extensive linkage data from the low-frequency conjugation system available, with the aim of establishing the relative location of a wide selection of markers on the genetic map.

Although the events resulting in chromosomal gene mobilization by RP4 are not yet fully understood, the results presented in this paper are sufficient to allow some preliminary conclusions to be reached about the organization of chromosomal genes in *A. calcoaceticus* compared with other well-documented groups of micro-organisms.

METHODS

Media. Minimal medium (MM) as described by Towner & Vivian (1976*a*) was used for the detection of auxotrophic mutants and, with appropriate supplements, for the characterization of such mutants and the isolation of recombinants. Supplements were stored as sterile solutions and were added to MM immediately before use at the following final concentrations: individual amino acids, 50 µg ml⁻¹; individual vitamins, 1 µg ml⁻¹.

Basal salts medium (BSM) was based on that of Livingstone *et al.* (1972) and contained (g l⁻¹): KH₂PO₄, 2; (NH₄)₂SO₄, 1; MgSO₄·7H₂O, 0·4; adjusted to pH 6·9 with dilute NaOH and solidified when necessary with 1·5 % (w/v) Difco Bacto-Agar.

Complete medium used for maintenance of stock cultures and for crosses was Oxoid Tryptone Soya Agar (TSA).

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Isolation of mutants. The mutants used are listed in Table 1. All were derived from the prototrophic strain EBF65/65 (Towner & Vivian, 1976a).

Mutagenesis was performed with either ultraviolet light, ethyl methanesulphonate (Sigma) or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Sigma) using conditions based on those found by Ahlquist, Fewson & Ritchie (1975) to be effective in *A. calcoaceticus* NCIB 8250. Multiply marked mutants were isolated following repeated cycles of mutagenic treatment, while various different combinations of markers were produced as a result of crosses.

Spontaneous mutants resistant to streptomycin or rifampicin were isolated by spreading 0.1 ml of a dense suspension of bacteria on plates supplemented with either dihydrostreptomycin sulphate (2 mg ml⁻¹; Sigma) or rifampicin (100 µg ml⁻¹; Sigma). The occasional rare colony which appeared was picked off and purified.

Millipore mating technique. The technique of Towner & Vivian (1976a, 1977) was used for all crosses. For matings involving the mandelate utilization mutant 61c, the selective medium used was BSM, supplemented as appropriate, instead of MM.

RESULTS

Analysis using four-factor crosses

Linkage was first studied in four-factor crosses similar to those used by Hopwood (1959). Crosses between pairs of double auxotrophs were analysed by isolating recombinants on four different selective media, each containing a different non-parental combination of two of the four growth requirements. In crosses of this type it was therefore possible to recover 9 of the 16 possible combinations of parental markers and consequently to obtain a considerable amount of information from a single cross.

Table 2 summarizes the results of a cross of this type between strains c467(RP4) *ile-1 cys-3* and c460 *trp-2 arg-8*. Recombinants which appeared were scored for the two unselected markers on each medium by replica-plating to plates lacking, in turn, each of the supplements contained in the original selective medium. Genetic instability amongst recombinants was not observed (this would have been initially recognized by the failure of recombinants to replicate to the same medium as the original master plate).

The results in Table 2 show that the frequencies of the same genotype on different selective media were, in general, in fairly good agreement, indicating that differential recovery of particular genotypes on different media was not an important source of bias in the data.

In crosses of this type, four of the recovered genotypes differ from either the donor or the recipient by a single marker and the other five by two markers. Of these five, four (genotypes 6 to 9, Table 2) represent two pairs of complementary genotypes. The genotypes differing from the recipient by a single marker were much more frequent than those differing from the RP4-carrying donor by a single marker. This observation was consistent with the idea of polarized chromosome transfer from the RP4-carrying donor strain. Additional evidence for this was that one member of each of the complementary pairs of genotypes was much more frequent than the other (i.e. in Table 2, genotype 6 > genotype 7, and genotype 9 > genotype 8). This evidence would not be significant by itself in view of the small numbers in these classes of recombinants, but it substantiated the previous conclusion.

Further information was obtained from the data by using the seven values in the right-hand column of Table 2, each representing the average frequency of a different pair of complementary recombinant genotypes, to calculate relative frequencies of recombination between each of the six pairs of markers in the cross (Table 3). If all six frequencies had been approximately equal, no linkages would have been detected. However, recombination between *ile-1* and *cys-3* was much rarer than between the other pairs of markers (Table 3), indicating that they were closely linked compared with the other markers. Similarly, *arg-8* was more closely linked (with a lower relative frequency of recombination) to *cys-3* and *ile-1* than to *trp-2*. These estimates were only relative since there was no estimate of the frequency of the pair of progeny classes having the parental arrangement of markers.

Table 1. *Mutants of A. calcoaceticus* EBF65/65

Strain no.	Genotype	Strain no.	Genotype
c48	<i>ile-1 met-1</i>	c460	<i>arg-8 trp-2</i>
c411	<i>arg-3 his-1</i>	c461	<i>trp-4 ala-1</i>
c413	<i>lys-1 str-2</i>	c465	<i>arg-10 trp-2 his-1</i>
c415	<i>his-2 str-2</i>	c467	<i>ile-1 cys-3</i>
c417	<i>trp-1 str-2</i>	c474	<i>tyr-2 arg-10 trp-2 his-1</i>
c424	<i>met-4 his-1</i>	c478	<i>phe-1 thi-2</i>
c425	<i>leu-1 his-1</i>	c486	<i>his-1 ile-1</i>
c426	<i>trp-2 his-1</i>	c492	<i>arg-10 trp-2</i>
c432	<i>arg-3 thi-1</i>	c493	<i>phe-1 trp-2</i>
c450	<i>arg-8 his-1</i>	c495	<i>phe-1 his-1</i>
c452	<i>arg-8 trp-2 his-1</i>	c4121	<i>rif-3 trp-2 his-1</i>
c458	<i>his-6 ala-1</i>	c4123	<i>str-2 rif-3 trp-2</i>
c459	<i>cys-3 ala-1</i>	61c*	<i>ile-1 met-1 mdl-1</i>

* 61c is a mutant of c48 isolated by Dr C. A. Fewson (Department of Biochemistry, University of Glasgow) which utilizes L-mandelate in addition to D-mandelate as a sole carbon source.

Table 2. *Analysis of a four-factor cross*

Cross NP87 c467(RP4) *ile-1 cys-3 trp-2*⁺ *arg-8*⁺ × c460 *ile-1*⁺ *cys-3*⁺ *trp-2* *arg-8*

After mating on a Millipore filter, the bacteria were resuspended in 3 ml quarter-strength Ringer's solution and 0.1 ml portions were spread on plates of each of four selective media containing, respectively, isoleucine and tryptophan (selecting *cys*⁺ and *arg*⁺), isoleucine and arginine (selecting *cys*⁺ and *trp*⁺), cysteine and arginine (selecting *ile*⁺ and *trp*⁺) or cysteine and tryptophan (selecting *ile*⁺ and *arg*⁺). Recombinants which appeared were further classified by replica-plating to plates lacking, in turn, each of the original supplements.

Genotype of selected progeny					No. of recombinants obtained for each selection				Average frequency
					<i>ile</i> ⁺ <i>trp</i> ⁺	<i>ile</i> ⁺ <i>arg</i> ⁺	<i>cys</i> ⁺ <i>trp</i> ⁺	<i>cys</i> ⁺ <i>arg</i> ⁺	
1.	+	+	+	+	19	14	21	18	18
2.	<i>ile</i>	+	+	+	—	—	1	1	1
3.	+	<i>cys</i>	+	+	6	2	—	—	4
4.	+	+	<i>trp</i>	+	—	72	—	125	98.5
5.	+	+	+	<i>arg</i>	292	—	272	—	282
6.	<i>ile</i>	+	<i>trp</i>	+	—	—	—	4	} 2.5
7.	+	<i>cys</i>	+	<i>arg</i>	1	—	—	—	
8.	<i>ile</i>	+	+	<i>arg</i>	—	—	1	—	} 9.5
9.	+	<i>cys</i>	<i>trp</i>	+	—	18	—	—	
Total					318	106	295	148	

Table 3. *Determination of relative frequencies of recombination*

Data from cross NP87 (Table 2). The sum of the four components (taken from the right-hand column of Table 2) represents the relative recombination frequency for each of the six pairs of markers in the cross (Hopwood, 1959).

Components	<i>ile-1</i> <i>cys-3</i>	<i>ile-1</i> <i>trp-2</i>	<i>ile-1</i> <i>arg-8</i>	<i>cys-3</i> <i>trp-2</i>	<i>cys-3</i> <i>arg-8</i>	<i>trp-2</i> <i>arg-8</i>
	1	18	18	18	18	98.5
	4	4	4	1	1	282
	2.5	282	98.5	282	98.5	2.5
	9.5	2.5	9.5	9.5	2.5	9.5
Total	17	306.5	130	310.5	120	392.5

This value would appear in the denominator of the ratio of recombinants to total progeny for each pair of loci if an absolute recombination percentage were calculable.

A hundred crosses of this type were used to confirm and extend the preliminary conclusions on linkage reached by Towner & Vivian (1976*b*). However, because of the small numbers of recombinants obtained in some matings, it was not possible to obtain significant results for some combinations of markers. For this reason more extensive analysis was carried out using the co-inheritance frequencies of unselected markers and pooling the results from several matings.

Analysis using co-inheritance frequencies

Most crosses performed were of the four-factor type described above. On each of the four plating media, one donor marker was selected, one was counter-selected, and the other two were non-selected. After classification of recombinants with respect to the two unselected markers on each medium, crosses were further analysed by calculating the percentage co-inheritance frequency of each of the unselected donor markers with respect to the selected donor marker. The results which were obtained were used as a measure of linkage between pairs of markers (although these results do not necessarily bear any relationship to the actual physical distance between the markers).

The approach used was to study two markers, selecting each in turn with the other unselected. The order of the two markers could then be deduced from the asymmetry in the inheritance of the non-selected marker, selection of the proximal marker giving a lower co-inheritance frequency than selection of the distal marker (Hopwood, 1972). In some cases, results obtained using different selections were in conflict. This was usually due to the location and resulting influence of the marker against which selection was originally made.

The technique described above was used to obtain linkage data for 150 different combinations of the 23 markers studied, using the two different RP4 donor types described by Towner & Vivian (1976*b*); each combination was studied in several crosses and the results were pooled.

In most crosses where the total number of recombinants examined exceeded 30, it was possible to attach a degree of significance to each result by calculating a confidence interval for each co-inheritance frequency obtained. This was done by considering each recombinant as an independent trial (total of n independent trials) with a probability p of 'success' (i.e. co-transfer of unselected marker) and a probability $(1-p)$ of 'failure' (i.e. no co-transfer). If $n > 30$, the number of 'successes', x , has a distribution which is approximately normal. If x 'successes' are observed in n trials, ($n > 30$), the estimated standard error of the 'observed sample proportion' ($\hat{p} = x/n$) is therefore $\sqrt{[\hat{p}(1-\hat{p})/n]}$ and a $(100-\alpha)\%$ confidence interval for p is $p \pm z(\alpha)\sqrt{[\hat{p}(1-\hat{p})/n]}$ where $\pm z(\alpha)$ are the $\alpha\%$ points of the normal distribution curve.

Linkage map of A. calcoaceticus EBF65/65

The results obtained using the techniques described above are summarized in Fig. 1. It was possible to resolve the order of most of the markers examined and only a few ambiguities remain. These were mainly due to the absence of strains bearing the particular combinations of markers required to solve particular sequences, or to the small numbers of recombinants obtained in certain crosses making it difficult to attach any great significance to the results obtained.

It should be emphasized that although it was possible to obtain information on the sequence of markers from the type of analysis used in this study, it was not possible to use the information obtained to provide a measure of the distance between markers. This is due to the fact that in all bacterial systems so far investigated, zygotes formed are incomplete diploids and crossovers leading to selected progeny tend to be non-randomly arranged, being concentrated in the vicinity of the selected markers (see, for example,

Fig. 1. Linkage map of *Acinetobacter calcoaceticus* EBF65/65. Loci are arbitrarily spaced at equal intervals. The relative order of the bracketed loci is uncertain.

Hopwood, 1967). The loci shown in Fig. 1 have therefore been arbitrarily spaced at equal intervals.

DISCUSSION

The linkage map (Fig. 1) is the only data of this kind available at present for *A. calcoaceticus*. Although some of the marker positions are still uncertain and the study of linkages is still at a very early stage, it is already possible to reach some preliminary conclusions about the organization of chromosomal genes in this species compared with other organisms.

A feature of chromosome maps of some species of bacteria, such as *Escherichia coli*, *Salmonella typhimurium* and *Bacillus subtilis*, is the clustering of genes affecting sequential steps of certain biosynthetic or degradative pathways. However, such a gene arrangement does not seem to be common in *Pseudomonas aeruginosa* where genes concerned with related steps of biosynthetic pathways do not show close linkage (Fargie & Holloway, 1965). It was therefore of interest to determine whether *A. calcoaceticus* could be allocated to one or other of these two categories.

Perhaps the most intensively studied biosynthetic pathway is that for the synthesis of tryptophan. In all members of the enteric bacterial family so far studied, the genes for this pathway seem to be clustered in one operon on the chromosome, whereas members of the *Pseudomonas* genus seem to have three separate linkage groups (Crawford, 1975). Using transformation, Sawula & Crawford (1972) showed that the six *trp* genes of *A. calcoaceticus* were also dispersed in three regions, no two of which could be carried on the same transforming DNA molecule. However, although these authors were unable to determine the exact gene order within clusters, it was clear from the distribution that the *trp* gene arrangement was also quite unlike that of *Pseudomonas*. In this paper the positions of three *trp* loci selected at random were investigated and each of these was found to map at a different position, thereby supporting the conclusions of Sawula & Crawford (1972), although the loci studied have not yet been correlated with actual genes of the pathway.

A second pathway which is clustered in the enteric family and seems to have the charac-

teristic operon type of control is the histidine pathway (Ames, Hartman & Jacob, 1963). Only three *his* loci have so far been studied in *A. calcoaceticus*, but again these have been shown in this paper to map at completely different sites.

It therefore appears from the preliminary linkage results presented here that the chromosomal organization of *A. calcoaceticus* differs from the members of the enteric family and is more akin to *Pseudomonas* in, so far, showing an absence of clustering of functionally related genes. However, as discussed above, Sawula & Crawford (1972) showed that significant differences also seem to exist between these two genera.

It should now be possible for other workers to add further markers to the *A. calcoaceticus* map with relative ease and it will then be of considerable interest to examine the regulation of biosynthetic and degradative pathways in *A. calcoaceticus*, where the chromosomal organization differs from that of other genera.

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