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Construction of a Genetic Map for *Caulobacter crescentus*

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RP4-mediated conjugation has been used to transfer large fragments of chromosomal material in *Caulobacter crescentus*. In this system, conjugation proceeds from multiple origins, and haploid recombinants are recovered at frequencies of 10^{-6} to 10^{-7} per donor cell. The data from five-factor crosses were subjected to computer-assisted crossover analyses as a rapid method to determine marker order. Using this information and data from additional two- and three-factor crosses mediated by RP4 or the generalized transducing bacteriophage φCr30, we constructed the first genetic map for *C. crescentus*.

The dimorphic bacterium *Caulobacter crescentus* has been proposed as an elegant system for the study of temporal gene expression (18). Unlike differentiation in most other bacteria, differentiation in *Caulobacter* is not a response to nutritional deprivation but is a geneticall programmed sequence of events that occur during the course of normal logarithmic growth. During growth, a succession of the following three clearly differentiated cell types is seen: stalked cells, swarmer cells, and predivisional cells. Each sessile stalked cell elongates and forms a flagellum and several pili at the pole opposite the stalk during the transition from stalk cell to predivisional cell. Asymmetric cell division then yields a stalked cell similar to the original stalked cell and a motile swarmer cell which possesses the flagellum and pili. The stalked cell repeats the cycle directly, whereas the swarmer cell must first lose its flagellum and pili and synthesize a stalk. This morphological transition to a stalked cell is obligate for DNA replication, which in turn is required for cell division (17, 19).

Genetic studies of this differentiation process have been hampered by the lack of an efficient system for genetic mapping. Previous reports of recombination in *Caulobacter* (10, 11, 15) have described procedures that require heavy mutagenesis to confer donor ability, an aspect that makes donor isolation laborious and renders the isolates of questionable value for genetic analysis by virtue of the multiple mutations introduced during this type of treatment (8). These problems have been obviated by the demonstration that promiscuous drug resistance factors, such as RP4, transfer conjugally from *Escherichia coli* to *C. crescentus* at a high frequency and promote significant chromosomal mobilization in *C. crescentus* (1, 5). Inc P-1 R-factors enabled the rapid construction of linkage maps for several gram-negative bacteria for which no native fertility factor was available (3, 4, 12, 13, 21, 22). Therefore, we undertook the construction of a genetic map for *C. crescentus* by using RP4-mediated conjugation.

One problem in the construction of a genetic map is the determination of the relative map order of a large number of markers. This problem is particularly acute at the outset, when no linkage relationships have been established. To deal with this problem, we designed a computer program to analyze the crossover events resulting from five-factor crosses and to calculate the most probable relative order for these factors. This program allowed us to benefit from the additional data derived from multifactor crosses without having to undertake the complex crossover analyses required of a cross involving five unmapped markers.

A second problem lies in the verification of cotransfer frequencies obtained by conjugation. Cotransfer frequencies are a function of the physical proximity of two loci, yet factors such as marker interference, plating efficiency, and proximity to an origin of transfer can distort these values. Consequently, we used generalized transduction to verify chromosomal cotransfer frequencies and to resolve the order of closely linked markers.

In this paper we demonstrate the usefulness of RP4 in conjunction with the generalized transducing bacteriophage φCr30 for chromosomal linkage studies in *C. crescentus*. This work resulted in the first linkage map for this organism.

MATERIALS AND METHODS

Strains. The parental strain (CB15) was *C. crescentus* ATCC 19089, which was obtained from A. Newton. This strain is prototrophic and undergoes the

TABLE 1. List of strains

Strain	Genotype	Derivation and/or reference
SC117	*ilvB101*	Spontaneous in CB15
SC119	*purA101*	Spontaneous in CB15
SC130	*hisB104*	Spontaneous in CB15
SC135	*hisD105*	Spontaneous in CB15
SC143	*serA101*	Spontaneous in CB15
SC146	*argG105*	Spontaneous in CB15
SC165	*hisB107*	Spontaneous in CB15
SC175	*flaZ102*	Spontaneous in CB15 (9)
SC243	*flaC110*	Spontaneous in CB15 (9)
SC284	*flaH135*	Spontaneous in CB15 (9)
SC308	*aroC103 flaZ102*	Aro derivative of SC175
SC309	*metD106 flaZ102*	Met derivative of SC175
SC310	*serA104 flaZ102*	Ser derivative of SC175
SC328	*metF107*	Spontaneous in CB15
SC373	*argG105 purB104*	Pur derivative of SC146
SC374	*purB104*	Spontaneous in CB15
SC375	*metB112*	Spontaneous in CB15
SC382	*cysB102*	Spontaneous in CB15
SC383	*leuA109*	Spontaneous in CB15
SC384	*trpA102*	Spontaneous in CB15
SC392	*proA103*	Spontaneous in CB15
SC396	*pheB102*	Spontaneous in CB15
SC436	*cysC104*	Spontaneous in CB15
SC451	*proC104*	Spontaneous in CB15
SC458	*hisD117*	Spontaneous in CB15
SC459	*metB115*	Spontaneous in CB15
SC476	*glt-129 flaH135*	Glt derivative of SC284
SC477	*pheB105 flaC110*	Phe derivative of SC243
SC480	*aroB112 flaH135*	Aro derivative of SC284
SC495	*argG105 ilvB123 rif-103*	Rif in SC502
SC496	*hisD105 str-103*	Str in SC135
SC502	*argG105 ilvB123*	Ilv derivative of SC146
SC503	*argG105 metF116*	Met derivative of SC146
SC537	*argG105 hisB121 metF116*	His derivative of SC503
SC538	*argG105 hisD122 metF116*	His derivative of SC503
SC545	*lysA103*	Spontaneous in CB15
SC547	*argG105 hisD105 rif-103 str-103* (RP4)	Recombination between SC624 and SC496
SC562	*argG105 metF116 serA112*	Ser derivative of SC503
SC596	*ts-104*	Spontaneous in CB15
SC624	*arG105 ilvB103 rif-103* (RP4)	(5)
SC689	*argA107*	Spontaneous in CB15
SC754	*trpA102 rif-119*	Rif in SC384
SC755	*pheB102 str-114*	Str in SC396
SC764	*aroC103 flaZ102 str-118*	Str in SC308
SC770	*pheB105 flaC110 str-122*	Str in SC477
SC787	*trpA102 rif-119* (RP4)	Contact of J53 (RP4) with SC754
SC808	*aroC103 flaZ102 str-118* (RP4)	Contact of J53 (RP4) with SC764
SC824	*proC104 rif-130*	Rif in SC451
SC827	*proC104 str-141*	Str in SC451
SC828	*cysB102 str-142*	Str in SC382
SC844	*argG105 cysB102 rif-103 str-142* (RP4)	Recombination between SC624 and SC828
SC857	*serA104 flaZ102 str-144*	Str in SC310
SC859	*proA103 str-140*	Str in SC392
SC867	*proC104 str-141* (RP4)	Contact of J53 (RP4) with SC827
SC893	*flaZ102 argG105 rif-103* (RP4)	Recombination between SC624 and SC857
SC894	*leu-109 str-146*	Str in SC383
SC896	*argG105 hisB121 metF116* (RP4)	Contact of J53 (RP4) with SC537
SC897	*argG105 metF116 hisD122* (RP4)	Contact of J53 (RP4) with SC538
SC898	*argG105 metF116 serA112* (RP4)	Contact of J53 (RP4) with SC562
SC905	*hisB104* (RP4)	Contact of J53 (RP4) with SC130
SC906	*hisB104 str-150*	Str in SC130
SC908	*metB112* (RP4)	Contact of J53 (RP4) with SC375
SC911	*lysA103* (RP4)	Contact of J53 (RP4) with SC545
SC912	*argA107* (RP4)	Contact of J53 (RP4) with SC689

TABLE 1—*Continued*

Strain	Genotype	Derivation and/or reference
SC913	*ts-104 str-153*	Str in SC596
SC999	*purA107* φCr30^r (RP4)	Contact of J53 (RP4) with SC432
SC1000	*purA106* φCr30^r (RP4)	Contact of J53 (RP4) with SC426
SC1003	*serA101 str-171*	Str in SC143
SC1013	*purB104 str-177*	Str in SC374
SC1017	*argG105 str-178*	Str in SC146
SC1111	*purA107*	Recombination between SC999 and SC1017
SC1112	*purA106*	Recombination between SC1000 and SC1017
SC1200	*metD106 flaZ102 str-147 amp-101*::Tn*5*	(5a)
SC1203	*purB104 str-177 ksg-101*	Kasugamycin resistance in SC1013
SC1378	*metB115 rif-145*	Rif in SC459
SC1532	*argA107 str-194*	Str in SC689

normal differentiation process described by Shapiro (17). *E. coli* strain J5-3 *pro met* λ$^+$ (RP4 Tc Ap Km) was obtained from H. M. Meade. *Caulobacter* donors were constructed by plate mating with this strain on PYE medium (8), followed by streaking onto selective medium (PYE containing 2 μg of tetracycline per ml) to isolate individual tetracycline-resistant clones. The nutritional mutants used in this study (Table 1) were isolated without mutagenesis by using the fosfomycin and D-cycloserine procedures described by Johnson and Ely (8). The isolation procedure for the motility mutants has been described previously (9). The uncharacterized temperature-sensitive mutant was a spontaneous isolate of a fosfomycin enrichment selection. Drug-resistant mutants were spontaneous isolates that were picked from plates containing the concentrations of antibiotics described below. All multiply marked strains were constructed by successive selections in the absence of mutagenesis or by recombination during mating. The bacteriophage φCr30 is one of the generalized transducing phages described by Ely and Johnson (6).

Media. Donor and recipient cultures were grown overnight in PYE broth as described previously (8). The defined medium used was M2 (8) supplemented with 0.3% glucose. The nutritional supplements used were as follows: 1 mM arginine, 1 mM thiosulfate (for *cysB* and *cysE*), 0.2 mM histidine, 1 mM lysine, 0.2 mM methionine, 1 mM phenylalanine, 1 mM tyrosine, 1 mM tryptophan, 1 mM proline, 0.2 mM adenosine, and 0.2 mM serine. Since histidine, methionine, and serine are inhibitory for *Caulobacter*, 10 mM L-alanine was added along with these amino acids to reduce the inhibitory effect (Ferber and Ely, manuscript in preparation).

Antibiotic-resistant mutants were isolated on supplemented PYE plates. Rifampin (10 μg/ml), novobiocin (5 μg/ml), and streptomycin (200 μg/ml in PYE; 50 μg/ml in M2) were obtained from the Sigma Chemical Co.

Filter mating. Equal volumes of stationary-phase R$^+$ donor and R$^-$ recipient cultures were mixed and collected on a membrane filter (pore size, 0.45 μm) as described by Ely (5). The cells were incubated on a PYE plate for up to 12 h at 33°C, suspended in M2 containing glucose, and plated onto an appropriate selective medium. After 4 to 5 days, recombinant clones were transferred with sterile toothpicks to master plates of the same selective medium. These master plates were subsequently replica-plated onto defined and differential media to determine the inheritance of unselected markers. Routinely, duplicate experiments involving 250 to 500 colonies were analyzed to determine the frequency of recombinant phenotypes.

Computer-assisted crossover analysis. A crossover analysis of multifactor crosses involving several unselected markers was used to determine the relative order of several linked markers. We designed a program to instruct the computer to (i) determine all possible orders for five markers, (ii) calculate the number of crossovers required of each order to generate the distribution of recombinants obtained from a particular experiment, (iii) list the orders with respect to probability (the lowest number of total crossovers required was considered to be the most probable), and (iv) calculate the cotransfer frequencies of all pairwise combinations of markers. Therefore, when the computer was provided with the genotype of each parent in a cross and the number of recombinants found for each recombinant class, it determined the most probable order for and the linkage relationships of the markers involved in the cross.

Fine-structure mapping by transduction. The preparation of the transducing bacteriophage lysates and the transduction procedure have been described by Ely and Johnson (6). Cotransductional mapping was limited to closely linked markers, as indicated by conjugal linkage mapping. Routinely, 200 to 300 transductants per cross were analyzed for unselected markers by replica-plating stable transductants onto appropriate selective media.

RESULTS

Characteristics of RP4-mediated conjugation in *C. crescentus*. Initial studies with RP4 in *C. crescentus* showed that conjugation could occur both between strains of *C. crescentus* and between strains of *C. crescentus* and *E. coli* (5). During filter matings, RP4 was transferred between *C. crescentus* strains at a frequency of approximately 10^{-1} per donor cell. In all crosses used throughout these studies, colonies that were recombinant for chromosomal mutations

TABLE 2. Computer-assisted linkage and crossover analysis of a multifactor cross in *C. crescentus*[a]

Recombinant phenotype	Distribution
Aro^+ Ilv^+ Rif^s Arg^- Fla^- Str^s	2
Aro^+ Ilv^+ Rif^s Arg^+ Fla^+ Str^r	23
Aro^+ Ilv^+ Rif^s Arg^+ Fla^- Str^r	95
Aro^+ Ilv^+ Rif^s Arg^+ Fla^+ Str^s	39
Aro^+ Ilv^+ Rif^s Arg^+ Fla^- Str^s	175

[a] In this analysis the donor was SC624, the recipient was SC764, the counterselected marker was *ilvB*, the selected marker was *aroC*, the unselected markers were *argG*, *rif*, *str*, and *flaZ*, and the selective medium was M2 containing glucose and 1.0 mM arginine. The percentages of cotransfer with the selected marker were as follows: *argG*, 1.0%; *flaZ*, 19.0%; and *str*, 65.0%. As determined by this analysis, the most probable order was *argG-str-aroC-flaZ-ilvB* (minimum number of crossovers for the phenotyic distribution, 668). The second most probable order was *argG-flaZ-aroC-str-ilvB* (minimum number of crossovers, 672). The third most probable order was *argG-aroC-str-flaZ-ilvB* (minimum number of crossovers, 714).

occurred at a frequency of approximately 10^{-6} to 10^{-7} per donor cell, indicating that chromosome mobilization occurred from multiple sites. Recombinants from conjugation experiments were stable through repeated purifications regardless of the presence of RP4. Purified recombinants containing RP4 possessed chromosomal donor abilities indistinguishable from those of transconjugants that received an RP4 plasmid directly from *E. coli*. The ability of RP4 to mediate significant mobilization of all of the chromosomal markers tested enabled us to identify conjugational linkage groups and encouraged us to develop methods to allow the determination of gene order among groups of linked markers.

Linkage and crossover analyses. To determine linkage relationships, we performed a series of crosses with pairs of strains carrying multiple mutations. Mutations with a low frequency of reversion (less than 10^{-8}) were chosen as selected and counterselected markers for the recipient and donor strains. Multifactor crosses provided us with a linkage analysis of five or more markers in a single experiment and allowed the determination of gene order by multifactor crossover analysis.

A typical multifactor cross (Table 2) involved the use of a single unselected donor auxotrophic mutation in conjunction with several unselected nonnutritional mutations in both the donor and the recipient. A stable auxotrophic mutation was used to counterselect the donor strain. When possible, an unselected donor mutation known to be unlinked to the selected marker was used to identify rare donor revertants, thereby preventing the inclusion of these revertants in the calculation of linkage values (e.g., *rif* in strain SC624). Linkage values represented the percentage of instances that a donor unselected marker was coinherited with the selected marker by the recipient, and these values were expressed as percentages of cotransfer.

The major drawback in an analysis of five-point crosses for the determination of gene order is the amount of time required to determine the gene order manually. There are 60 possible gene orders for five genes. To eliminate this extensive manual work, we developed a method of gene order analysis which was done by computer.

The method of analysis was based on the assumption that those phenotypic classes which required the least number of crossover events would occur most frequently. Therefore, the most likely gene order would be the one which required the fewest crossovers to generate all of the progeny of a cross. Figure 1 shows the portions of the donor and recipient chromosomes involved in the crossover events required for the generation of the various recombinant progeny from the cross between SC624 and SC764. The computer-selected most probable order (Table 2) required no quadruple crossovers to generate the observed phenotypic distribution. The second most probable order would have required two quadruple crossovers, and the third and all other possible gene orders would have required increasingly higher numbers of unlikely quadruple crossover events to generate the same phenotypic distribution. Thus, computer analyses of five-factor crosses provided a determination of the relative orders

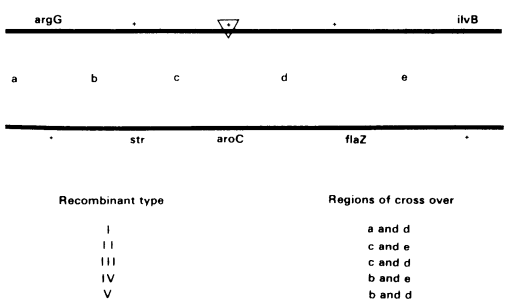

FIG. 1. Gene arrangement requiring the least number of crossover events for the generation of recombinant progeny in the cross between SC624 and SC764 (Table 2). The top line represents a portion of the donor (SC624) chromosome. The recombination of the indicated donor marker ($aroC^+$) with the recipient chromosome (bottom line) was the selected event. Cotransfer and recombination of additional donor markers gave rise to five recombinant types. The computer-selected most probable order shown in this figure was the only order that required solely double crossover events to generate each of the recombinant types.

TABLE 3. Cotransfer of *metB*, *argA*, and *hisB* by conjugation[a]

Donor	Recipient	Selected marker	Counter-selected marker	Unselected marker	% Cotransfer of selected and unselected markers
SC908	SC1532	*argA*	*str*	*metB*	98
SC912	SC1378	*metB*	*str*	*argA*	85
SC908	SC906	*hisB*	*str*	*metB*	91
SC905	SC1378	*metB*	*str*	*hisB*	81
SC905	SC1532	*argA*	*str*	*hisB*	61
SC912	SC906	*hisB*	*str*	*argA*	75

[a] Donor strains containing RP4 were mated with the recipients as described in the text.

of markers with cotransfer frequencies less than 80%. However, the computer was not able to distinguish reliably the relative order of markers with higher cotransfer frequencies due to infrequent recombinational events between these markers. Nevertheless, computer-analyzed five-factor crosses provided most of the data required to construct the framework of the first map for *C. crescentus*.

Once the general map position of a given marker was identified, two-point crosses were used to determine the linkage relationships to additional surrounding markers. In most cases, sensitivity to the antibiotic streptomycin was used to counterselect the donor strain (Table 3). These results were often sufficient to determine gene order. In some cases, further analyses were needed to determine gene orders more precisely.

Transductional verification of linkage and map order. When conjugational cotransfer frequencies were greater than 45%, linkage could be confirmed by transduction. Typical values are shown in Table 4. We did not detect transductional linkage between markers showing conjugal linkage values less than 45%. The comparative cotransfer frequencies derived from the conjugation and transduction data shown in Tables 3 and 4 are illustrated in Fig. 2. To verify the relative order of *argA*, *hisB*, and *metB*, transductional data were required in addition to the conjugational linkage values. The transductional data showed that *argA* and *hisB* had the lowest linkage values and, therefore, that *argA* and *hisB* were the distal markers. This, the map order is *argA-metB-hisB*. Similar analyses were carried out for the other regions of the chromosome where close linkage was observed. From these results we were able to construct a linkage map (Fig. 3) consisting of a single set of contiguous linkages. Linkage was not established between the two most distal markers, *cysB* and *ilvB*.

TABLE 4. Cotransduction of *metB*, *argA*, and *hisB*[a]

Donor	Recipient	Selected marker	Unselected marker	% Cotransfer
SC375	SC689	*argA*	*metB*	73
SC689	SC459	*metA*	*argA*	44
SC375	SC130	*hisB*	*metB*	46
SC130	SC375	*metB*	*hisB*	37
SC130	SC689	*argA*	*hisB*	8
SC689	SC130	*hisB*	*argA*	15

[a] Bacteriophage grown on the donor strains were used to transduce the recipient.

Markers with low transduction frequencies. *C. crescentus* chromosomal markers which are transduced by φCr30 with low efficiency have been observed previously (6). Mapping by conjugation indicated that the markers with low transduction frequencies all lay within the same region of the map. The transduction efficiencies of *purB*, *serA*, and *aroC* were 10^{-6} to 10^{-7} recombinants per PFU, whereas the transduction efficiencies of markers on the remainder of the map were 10^{-4} to 10^{-5}. Despite the diminished recovery of transductants in experiments in which selected markers in this region were

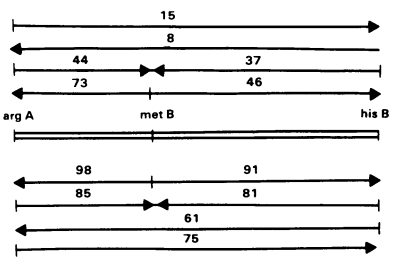

FIG. 2. Comparative transductional and conjugational linkage values used in the determination of the gene order of the markers *aroA*, *metB*, and *hisB*. The values above the double line are the percentages of cotransduction of the two markers indicated for each value. The arrowheads designate the selected marker in the experiment. The values below the double line are the percentages of conjugational cotransfer.

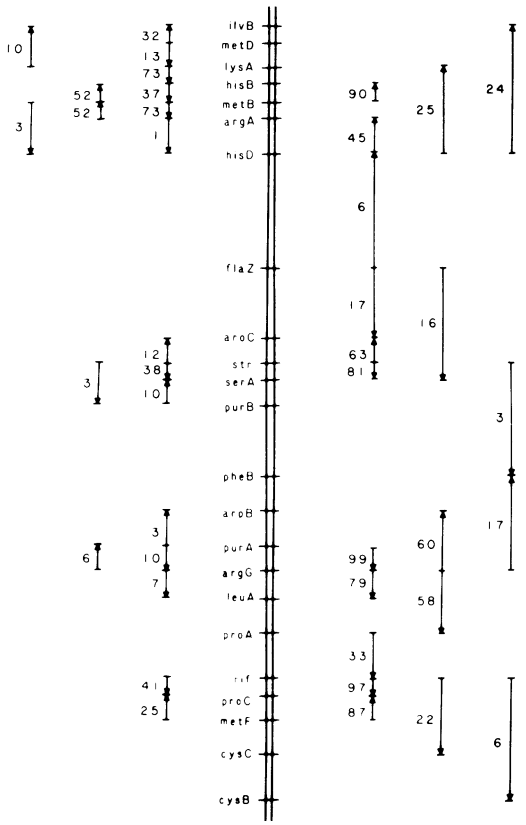

FIG. 3. Genetic linkage map for *C. crescentus*. The linkage values on the left were obtained by transduction, and those on the right were obtained by conjugation. Each value is the average of the values derived from two to five experiments. The selected marker of each cross is indicated by an arrowhead.

used, transductional linkage values correlated well with linkage values obtained by conjugation in this region. Thus, the low transduction frequencies appear to be due to some aspect of the transduction process and not due to an effect on recombination in the region.

DISCUSSION

We demonstrated that RP4-mediated chromosome mobilization in *C. crescentus* is a simple and efficient tool for the genetic analysis of this organism. This chromosome mobilization is similar in several respects to the chromosomal mobilization mediated by RP4 or R68.45 in several species of the *Rhizobiaceae* (2). A characteristic of R68.45-mediated conjugation is the mobilization of large segments of the donor chromosome. Estimates of the average donor segment sizes range from one-seventh of the chromosome in *Rhizobium leguminosarum* (3) to one-third of the chromosome in *Rhizobium meliloti* (13), with the demonstration that these segments were most frequently inherited in toto. We observed a similar phenomenon during RP4-mediated conjugation in *C. crescentus*. Conjugal cotransfer of markers spanning linkage distances many times greater than those attainable by cotransduction indicated that large segments of the chromosome were incorporated into R^- strains. This incorporation occurred with a minimum of recombination between distal markers since recombinants requiring multiple recombination events were found infrequently. The possibility that RP4 adversely affected recombination in *C. crescentus* was an unlikely explanation, since the transductional recombination frequencies of R^+ and R^- strains are similar. To explain the incorporation of larger chromosomal segments in *Rhizobium*, Beringer et al. (3) have postulated an "end effect" favoring preferential recombination of the ends of a transferred segment with the recipient chromosome. A similar mechanism may be operating in *Caulobacter*.

Since large pieces of the *C. crescentus* chromosome were transferred from apparently random points of origin, data from RP4-mediated conjugations were particularly well suited to computer-assisted analysis. By using five-factor crosses, we were able to determine the relative map positions of a large number of markers with a relatively small number of experiments. If recombination frequencies were high and small pieces of the donor chromosome were incorporated by the recipient, five-factor crosses would not have been practical since distant markers would appear to be unlinked. On the other hand, since large pieces of the donor chromosome were incorporated by the recipient, it was necessary to employ transduction to determine the gene order of closely linked markers. By combining these techniques, we were able to determine relative gene orders in a definitive manner and to use these orders to construct a genetic map for *Caulobacter*.

The transduction procedure with bacteriophage ϕCr30 is relatively simple, and because of the large size of its capsid (6), this phage should be able to transduce large pieces of the *C. crescentus* genome. Transductional linkage values always correlated well with the linkage values obtained by conjugation. Thus, the construction of a contiguous transductional linkage map is feasible, judging by the current rapid addition of new markers to the existing map and the fact that large stretches of the map already have been linked by transduction.

The phenomenon of low transduction frequencies for markers lying in a specific region of the chromosome (the *purB-serA-aroC* region) invites speculation regarding the nature of the DNA peculiar to that region. Newman and Mas-

ters (14) have concluded that the variation in transduction frequency among *E. coli* markers with the generalized transducing phage P1 is due to recombinational discrimination in the recipient cells between transduced DNA of some regions of the genome over others. UV-induced damage to the transducing particles eliminated the variation in frequency, presumably by overriding the recombination substrate specificity. However, the variations in transduction frequencies observed with φCr30 were observed even with routine 5-min UV irradiation of lysates, indicating that another mechanism, such as regional packaging specificity, may determine the φCr30 transduction frequency. In the generalized transducing bacteriophage P22 of *Salmonella typhimurium*, transducing DNA fragments may be formed by sequential encapsulation from a small number of preferred starting points in the host chromosome (7, 20). Genomic regions displaying low transduction frequencies may have a paucity of these preferred starting points. It is possible that the *purB-serA-aroC* region of the *C. crescentus* chromosome does not have many starting points for φCr30 packaging and, therefore, is transferred at lower frequencies in transduction experiments.

Caulobacters are bacteria that are well adapted to survival in nutritionally dilute environments (16). The sensitivity of *C. crescentus* to millimolar concentrations of certain amino acids in defined media is probably a function of this physiological adaptation. Consequently, the plating efficiencies of recombinants on defined media containing inhibitory amino acids introduce some variability in the estimation of linkage values. Incorporation of L-alanine in defined media containing such amino acids reduced the inhibition and enabled us to obtain more reproducibility in replicate experiments. However, problems with plating efficiencies still exist. The experimental results shown in Fig. 2 illustrate the type of linkage discrepancies observed in reciprocal experiments. In this case, when the *metB* marker was used nonselectively (i.e., the cross or transduction was plated onto minimal medium containing methionine and alanine), the values for linkage of this marker to the adjacent markers (*argA* and *hisB*) appeared to be higher than when the *metB* marker was used as the selected marker. Therefore, the recovery of auxotrophs appeared to depend upon the type of amino acid used in the medium. Another problem with defined media involved the recovery of two unselected amino acid auxotrophic markers. The presence of more than one amino acid in the media appeared to preclude the recovery of one or the other of the auxotrophic markers. In one experiment, we circumvented this problem by selecting the *ts-104* (SC913) marker on complete medium at the restrictive temperature and counterselecting the *argG metF* donor strain (SC896) with streptomycin. In this instance, we apparently obtained complete recovery of both cotransferred auxotrophic markers and obtained the expected linkage values. We are now investigating several strategies to perform most conjugations and transductions on complete media. The use of complete media should eliminate most problems concerning plating efficiencies and should allow us to make more accurate estimates of linkage.

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