GENE TRANSFER IN *CAULOBACTER CRESCENTUS*: POLARIZED INHERITANCE OF GENETIC MARKERS

AUSTIN NEWTON AND EILEEN ALLEBACH

Department of Biology, Princeton University, Princeton, New Jersey 08540

Manuscript received October 2, 1974

Revised copy received December 17, 1974

ABSTRACT

Recombination frequencies were determined for 15 independently isolated auxotrophs of *C. crescentus* crossed pairwise in all possible combinations. The results indicate that the mutants may be grouped into at least two types: "fertile" strains, which recombine with all other mutants at frequencies ranging from less than 10^{-6} to 3×10^{-2} , and "nonfertile" strains which recombine with fertile strains at high frequencies and with other nonfertile strains at low or negligible frequencies. Several lines of evidence indicate a polarized inheritance of markers. Two of these are (1) the preferential inheritance of unselected markers from the nonfertile parent in fertile $\times$ non-fertile crosses, and (2) the consistent ordering of markers based on the frequency at which the mutants recombine with each of the three fertile strains. Although the evidence is not conclusive at this point, the results are most consistent with conjugation as the mechanism of gene transfer in these bacteria.

C*CAULOBACTER CRESCENTUS* is a gram-negative bacterium that provides an attractive model system for the study of several developmental problems, particularly ones relating to temporal and spatial control. The basic steps in the life cycle (Figure 1) were determined by STOVE and STANIER (1962) and by POINDEXTER (1964) and the developmental aspects of the cell cycle have been considered more recently (NEWTON 1972; SHAPIRO, AGABIAN-KESHISHIAN and BENDIS 1971). An asymmetric dividing cell (A) produces two different cells, a motile swarmer cell (B) and non-motile stalked cell (C). The stalked cell divides repeatedly to give the stalked cell and a new swarmer cell, while the swarmer cell cannot divide until it completes a series of developmental steps that lead to formation of a stalked cell: loss of motility, loss of susceptibility to adsorption of RNA phages and stalk formation (SCHMIDT 1966; SHAPIRO, AGABIAN-KESHISHIAN and BENDIS 1971; NEWTON 1972).

A thorough study of development in *C. crescentus* requires both biochemical and genetic analyses of the system. Biochemical techniques used with other microorganisms have been applied directly to these cells, but studies of genetics in these bacteria have begun only recently. Earlier reports of recombination at frequencies of approximately 10^{-5} have appeared (RUBY 1967; SHAPIRO, AGABIAN-KESHISHIAN and BENDIS 1971 and JOLLIICK and SHERVISH 1972), and they indicate that cell contact is needed for gene transfer. We have also observed recombination between auxotrophic mutants in *C. crescentus*, strain CB15, and

FIGURE 1.—Cell cycle of *C. crescentus*. Shown are the dividing cell (A), swarmer cell (B) with flagellum and pili, and stalked cell (C).

we present results in this paper that further characterize the mating system. They show the presence of at least two, and possibly three fertility types, a polarized inheritance of genetic markers and recombination frequencies ranging from 10^{-6} to 3×10^{-2} , depending upon the genetic markers involved.

MATERIALS AND METHODS

Bacterial strains and media: The prototrophic parent strain for all of the mutants discussed is *C. crescentus*, CB15 (ATCC 19089). The broth medium contains 2 g of bacto-peptone (Difco), 1 g of yeast extract (Difco) and 0.2 mg of MgSO_4 per liter at pH 7. The minimal medium is the one used by POINDEXTER (1964) and contained 0.2% glucose as the carbon source. Cells were grown with vigorous shaking at 30° .

Isolation of mutants: Exponentially growing cultures of strain CB15 in broth medium were taken at a density of approximately 3×10^8 cells/ml and irradiated with ultraviolet light long enough to reduce the viable count 1000-fold. The surviving cells were grown in minimal medium that contained a mixture of amino acids and bases, subjected to penicillin (50,000 U/ml) selection in unsupplemented minimal medium (DAVIS 1948) and spread on broth plates. After two days of incubation at 30° , these plates were printed to minimal medium agar plates to identify auxotrophic mutants. The requirement for each mutant was determined by replica printing to a series of plates which contained pools of several amino acids and bases.

Crosses: Two auxotrophic strains were crossed by mixing equal volumes of broth cultures of exponentially growing cells at a density of approximately 3×10^8 cells/ml. The mixture was incubated without shaking for 60 min at 30° , diluted where required in broth medium and 0.1 ml spread to minimal agar plates. These plates were usually incubated for 5 days at 30° before counting the numbers of recombinants. 0.1 ml of an undiluted culture of each strain was also spread on a separate minimal agar plate as a control for reversion. Reversion frequencies for all markers are low, and less than 5×10^{-8} for most of the strains used in these studies.

Scoring of unselected markers: The inheritance of the unselected streptomycin or rifamycin markers was scored by purifying prototrophic recombinants on minimal agar medium and picking isolated colonies with sterile toothpicks to another minimal agar plate which served as a

master plate. The master plate was then replica printed to broth plates with either streptomycin (24 µg/ml) or rifamycin (10 µg/ml).

RESULTS

Isolation of mutants: Isolation of auxotrophic mutants of *C. crescentus* is possible using ultraviolet light mutagenesis followed by a modification of the penicillin selection technique developed by DAVIS (1948). More recently we have found that the selection step can be omitted by plating the survivors of irradiation directly on broth plates and screening for mutants by replica printing to minimal agar plates. Presumptive nutritional mutants are recovered at an average frequency of 7×10^{-4} in this procedure. Approximately 70 independent auxotrophic mutants have been isolated and partially characterized to date. We have chosen 15 of these strains for a detailed analysis of recombination; they are listed in Table 1.

Characteristics of genetic transfer: Recombination between strains was tested by mixing equal numbers of exponentially growing cells, incubating them for 60 min and plating appropriate dilutions on minimal agar plates (see MATERIALS AND METHODS); recombination frequencies are expressed as the number of prototrophic recombinants divided by the total number of cells (including both parents) spread on the selective plate. Longer or shorter times of incubation before diluting and spreading the cells did not significantly change the recombination frequency. All of our results agree with those reported previously (RUBY 1967; SHAPIRO, AGABIAN-KESHISHIAN and BENDIS 1971; and JOLICK and SHERVISH 1972) to show that recombinants are stable (unpublished), and that culture filtrates will not replace either of the two strains (Table 2).

The above conditions were adopted to standardize the procedure used in all crosses. There are, however, two lines of evidence that suggest much of the

TABLE 1
Mutants isolated from C. crescentus Cb15

Strain	Mutation	Requirement
C1	<i>cys-301</i>	cysteine or methionine
C2	<i>cys-302</i>	cysteine
C6	—	unidentified
C7	<i>his-301</i>	histidine
C10	<i>his-302</i>	histidine
C11	<i>ade-301</i>	adenine
C12	<i>cys-303</i>	cysteine or methionine
C13	<i>met-101</i>	methionine
C14	<i>met-302</i>	methionine
C17	<i>his-303</i>	histidine
C19	<i>ade-302</i>	adenine
C20	<i>ade-303</i>	adenine
C24	<i>glt-301</i>	glutamate or arginine
C25	<i>leu-301</i>	leucine
C26	<i>cys-304</i>	cysteine or methionine

TABLE 2

Substitution of culture filtrates in C. crescentus crosses

Cross	Cells	Filtrate	Fold dilution	Recombinants
1	C1 + C12		10 ²	282
2	C1	C12	10 ²	0
			0	0
3	C12	C1	10 ²	0
			0	0
4	C1 + C19		10 ²	870
5	C1	C19	0	0
6	C19	C1	0	0
7	C1 + C24		10 ²	78
8	C1	C24	0	0
9	C24	C1	0	0

Crosses 1, 4 and 7 were control crosses made in the normal manner (MATERIALS AND METHODS) with cells of both strains present and 0.1 ml of a 10²-fold dilution plated to minimal glucose agar. The other crosses were carried out in the same way except that the culture indicated was first sterily filtered (Millipore) and an equivalent volume of the filtrate substituted for that culture. Similar results were obtained in C12 × C26, C19 × C25 and C19 × C26 crosses.

gene transfer observed under these conditions takes place on the minimal agar plate and not during the 60 min of incubation before spreading the cells. Firstly, when cultures of two strains were incubated together for 0 min instead of 60 min, there was little change in the recombination frequency, and when the strains were diluted before mixing and spread individually on the selective plate, there was only a moderate reduction in the recombination frequency (Table 3). Secondly, if either of two strains was streptomycin-sensitive while

TABLE 3

Evidence for plate mating

Cross	Recombination frequencies (× 10 ³)*		
	A	B	C
C1 × C19	2.3	1.2	0.63
C12 × C19	25.†	22.‡	11.‡
C19 × C6	22.‡	26.	4.6
C12 × C6	4.5		1.4
C12 × C25	2.1		1.8
C12 × C26	18.		10.
C19 × C26	13.		12.

* Crosses were performed in three different ways: A, as described in MATERIALS AND METHODS, with a 60-min incubation of the mating mixture before diluting and spreading to minimal plates; B, as in A, with 0 min incubation of the mating mixture before diluting and spreading to minimal plates; and C, where the two cultures were diluted separately (usually 10²- or 10³-fold) and spread in succession to a minimal plate. Recombination frequencies are the results of one experiment except where noted.

† Average value from three experiments.

‡ Average value from two experiments.

TABLE 4

Effect of streptomycin in selective medium on recombination

Cross*	Minimal		Minimal + Streptomycin	
	Dilution	No. recombinants†	Dilution	No. recombinants†
C12S × C14R	0 ×	390	0 ×	0
C12R × C14S	0 ×	523	0 ×	0
C12R × C14R	0 ×	337	0 ×	258
C1S × C14R	0 ×	278	0 ×	0
C1R × C14S	0 ×	309	0 ×	1
C1R × C14R	0 ×	332	0 ×	307
C12S × C25R	10 ² ×	265	0 ×	0
C12R × C25S	10 ² ×	178	0 ×	1
C12R × C25R	10 ² ×	195	10 ² ×	256
C19S × C25R	10 ² ×	328	0 ×	0
C19R × C25S	10 ² ×	328	0 ×	0
C19R × C25R	10 ² ×	293	0 ×	(confluent)

* Matings between streptomycin-sensitive and streptomycin-resistant auxotrophs were performed as indicated and plated to minimal agar which contained either no streptomycin or 24 µg/ml streptomycin. Strains were either sensitive (S) or resistant (R) to streptomycin.

† Number of recombinant colonies per plate.

the other one was streptomycin-resistant, no recombinants were recovered when the cells were spread directly on plates that contained streptomycin (Table 4). The streptomycin-resistant markers were inherited at a high frequency in crosses with these same strains, however, when prototrophic recombinants were first selected on minimal agar plates which did not contain streptomycin (see section below). This may suggest that a relatively long incubation of both strains on the minimal agar is required before mating occurs, at least using these conditions. Recombinants between streptomycin-sensitive and -resistant strains have been observed on streptomycin-containing plates when the two mutants were first incubated together for 24 hours on a broth medium (RUBY 1967).

Recombination frequencies: Table 5 is a matrix that gives the results of crosses between all of the auxotrophic strains listed in Table 1. Most recombination frequencies are the averages of two or more experiments. Inspection of these data suggests that the strains tested can be divided into at least two groups. The first set of strains, C1, C12 and C19, we provisionally call "fertile". These mutants recombine with almost all other strains, including the other two fertile strains, at frequencies ranging from 10⁻⁶ to 3 × 10⁻². The second group of strains, which we call "nonfertile", is made up of the remainder of the mutants; they either fail to recombine with one another (frequency less than 3 × 10⁻⁷) or they recombine at frequencies several hundredfold lower than they would recombine with a fertile strain. The assignment of strains to a fertile and a nonfertile class may be an oversimplification. Mutants like C2 and C17, which do recombine at low but

TABLE 5
Recombination matrix*

	C1	C2	C6	C7	C10	C11	C12	C13	C14	C17	C19	C20	C24	C25	C26
C1	—	0	145	5	2.1	8.2	52	.31	1.4	9	36	4.7	38	57	170
C2	0	—	1.4	2.0	20	4.1	500	.3	0	4.5	290	.6	9.8	.094	7.1
C6	145	1.4	—	.06	0	0	700	0	0	9.5	1500	.075	0	0	0
C7	5	2.0	.06	—	0	.04	2.7	0	0	6	9.6	.42	.054	0	2.2
C10	2.1	20	0	0	—	0	4.6	0	0	0	4	0	.015	0	0
C11	8.2	4.1	0	.04	0	—	220	0	.011	.80	0	0	0	0	0
C12	52	500	700	2.7	4.6	220	—	1.5	.68	13	3700	8.5	15	450	530
C13	.31	.3	0	0	0	0	1.5	—	0	2.4	4.5	0	.034	0	0
C14	1.4	0	0	0	0	.011	.68	0	—	0	2	.03	.036	0	0
C17	9	4.5	9.5	6	0	.80	13	2.4	0	—	1.3	.09	2.4	3.1	8.9
C19	36	290	1500	9.6	4	0	3700	4.5	2	1.3	—	5.2	500	1000	1650
C20	4.7	.60	.075	.42	0	0	8.5	0	.03	.09	5.2	—	.04	.4	.045
C24	38	9.8	0	.054	.015	0	15	.034	.036	2.4	500	.04	—	0	.018
C25	57	.094	0	0	0	0	450	0	0	3.1	1000	.4	0	—	0
C26	170	7.1	0	2.2	0	0	530	0	0	8.9	1650	.045	.018	0	—

* Recombination frequency $\times 10^5$; frequencies less than 10^{-7} are reported as "0".

FIGURE 2.—Order of mutants determined by frequency of recombination with C1, C12 and C19. The data from Table 5 were used to order all strains that recombined with the three fertile strains according to increasing frequency of recombination. One strain was placed under another when the difference in frequency was not sufficient to be certain of their relative order. Data from the C12 $\times$ C7 and C12 $\times$ C25 crosses were not used since they represent the results of single crosses (Table 5).

significant frequencies with strains that are otherwise nonfertile, may represent a third fertility type (see DISCUSSION).

Figure 2 shows all of the strains arranged in an order determined by the frequency of recombination with C1, C12 and C19. As indicated in the figure, the difference in frequencies was sometimes too small to be certain of relative order. C1 is less fertile than C12 and C19, but the general ordering of mutants in the three arrays is similar; the group of mutants showing the highest frequency of recombination is the same for each of the fertile strains. One evident exception to the general consistency of the ordering is the low frequency of recombination between C19 and C17. This type of discrepancy could be explained by linkage between *ade-302* and *his-303*.

Inheritance of unselected markers: Since we cannot as yet perform interrupted mating experiments with *C. crescentus*, it is not possible to test directly whether the ordering shown in Figure 2 results from a polarized transfer of markers from the fertile strains to the nonfertile strains. An indirect test for conjugation, however, is to see whether unselected markers are inherited preferentially from the nonfertile parent. For these experiments we performed reciprocal crosses between fertile and nonfertile strains in which one of the pair was resistant to either streptomycin or rifamycin and the other strain was sensitive to the drug. The two markers were chosen because the inhibitors affect synthesis of different polymers and they are not genetically linked, at least not on the chromosome of *Escherichia coli* (TAYLOR and TROTTER 1972).

TABLE 6

Polarity of marker inheritance in fertile $\times$ nonfertile crosses

Unselected marker	Cross	Inheritance of drug markers (no. of recombinants)	
		Sensitive	Resistant
A. Streptomycin			
	C19S × C6R	20	84
	C19R × C6S	81	23
	C19S × C10R	4	52
	C19R × C10S	45	11
	C19S × C14R	8	104
	C19R × C14S	154	14
	C19S × C20R	24	76
	C19R × C20S	70	23
	C19S × C25R	15	85
	C19R × C25S	70	30
B. Rifamycin			
	C19S × C10R	16	89
	C19R × C10S	87	18
	C19S × C14R	8	104
	C19R × C14S	154	14
	C19S × C20R	12	86
	C19R × C20S	87	13
	C1S × C24R	37	63
	C1R × C24S	88	12
	C19S × C24R	106	108
	C19R × C24S	219	82
	C19S × C25R	229	159
	C19R × C25S	196	219

Crosses were performed as described (MATERIALS AND METHODS) using strains either sensitive (S) or resistant (R) to A, streptomycin and B, rifamycin. Inheritance of these markers was determined (MATERIALS AND METHODS) and the number of sensitive and resistant strains among the recombinants tested was expressed as shown above.

Table 6,A shows typical results with streptomycin as the unselected marker, and Figure 3 presents a schematic representation of the direction of polarity in this type of cross for all of the mutant pairs tested. In most of these cases the unselected streptomycin marker, sensitive or resistant, was inherited more frequently from the nonfertile strain than from the fertile strain.

When rifamycin was the unselected marker, polarity was strong in most crosses, e.g., in C19 $\times$ C10 and C19 $\times$ C14, but less so in others, e.g., in C19 $\times$ C24, where equal numbers of resistant and sensitive recombinants were recovered when the nonfertile strain was rifamycin-resistant. This result might be expected if the number of rifamycin-resistant recombinants were reduced because of a deleterious effect of this marker; we have some indication that this may be the case for growth on minimal medium (unpublished). In the C19 $\times$ C25 cross, however, there is an apparent reversal of polarity. We have no ex-

FIGURE 3.—Polarity of inheritance of streptomycin markers. Arrows indicate that reciprocal crosses were carried out between a fertile strain and a nonfertile strain and the arrows (inferred direction of gene transfer) point toward the strain from which recombinants preferentially (at least 70% of recombinants) inherited the unselected streptomycin marker. The only exceptions were the C12S × C10R and C12S × C24R crosses, where approximately equal numbers of sensitive and resistant recombinants were recovered.

planation for this result unless there is some linkage between the rifamycin region and *leu-301*, which is the mutation in C25.

DISCUSSION

Our study of recombination in *C. crescentus* is at an early stage and it is still too early to make any firm conclusions about the exact mechanism of gene transfer. In particular, the use of "fertile" and "nonfertile" above to designate the types of strains is not intended to suggest that a sex factor is necessarily responsible for the genetic transfer observed in this system. It does seem useful, however, to consider the properties of genetic transfer in *C. crescentus* that would be characteristic of mating systems in other gram-negative bacteria separately from those that are not. The following four observations are consistent with conjugation as the mechanism for gene transfer. (a) Filtrates of medium from one of the cultures used in a fertile cross will not replace the cells used in the same cross. (b) Auxotrophs may be grouped into at least two types, fertile and non-fertile. Fertile strains recombine with all mutants which have a different phenotype, often at very high frequencies. The nonfertile strains recombine with fertile strains, but they either fail to recombine with one another or they recombine at very low frequencies. This latter group of nonfertile strains may represent a third fertility class, e.g. similar to the F^+ mating type in *E. coli*. (c) The wild-type alleles of the fertile strains can be ordered according to how frequently these genetic markers are inherited in crosses with other strains: the relative order of these markers is generally the same for C2, C12 and C19. Thus, if conjugation is occurring, Figure 2 would correspond to the order of chromosome transfer and would indicate that the fertile strains arose independently

with their origins in the same region to give the same direction of transfer. (d) There is a preferential inheritance of unselected genetic markers from the non-fertile parent (Table 6 and Figure 3). The results using rifamycin as the unselected marker are not as clear as those using streptomycin. This point deserves further investigation using other unselected markers.

Two other characteristics of gene transfer in these bacteria are unexpected if conjugation were involved. Firstly, recombinants cannot be selected directly by plating the mating mixture on minimal plates which contain streptomycin unless *both* of the strains are resistant to the inhibitor. This can be explained either by the requirement for the two strains to incubate for a period on the minimal plate before gene transfer takes place or by the initial formation of a zygote which contains the cellular components of both parents and which is consequently killed by streptomycin. Secondly, recombination between two fertile strains is in some cases as frequent as it is between a fertile strain and a non-fertile strain. In other words, there is no reduced frequency of recombination similar to that observed in *E. coli* when two male strains are crossed. Although their role in gene transfer has not been established, *C. crescentus* does have pili to which RNA phages adsorb (POINDEXTER 1964; SCHMIDT 1966). Since pili are found on swarmer cells and not on stalked cells, the two developmental stages could correspond to donor and recipient states, respectively, in a strain capable of genetic transfer.

While comparisons of the above results with those obtained in other bacterial systems suggest conjugation as the simplest explanation for the polarized inheritance of genetic markers observed in *C. crescentus*, other mechanisms are conceivable. Culture filtrates will not substitute for cells in crosses (Table 2), but phage or DNA could be released from parental cells during incubation on the minimal plates and be responsible for gene transfer. Another possibility is the formation of zygotes. In all of these latter cases gene transfer would necessarily be followed by preferential elimination of certain genes or marker-specific recombination in order to explain the pattern of inheritance observed. The characteristics of prezygotic and postzygotic selection of markers have been discussed in detail by JACOB and WOLLMAN (1961). Interrupted mating experiments and zygotic induction of temperate phages are two ways of distinguishing between these alternatives.

We thank STEPHEN NEVILLE, LEWIS HAUT, SUZANNE DEGNEN and ELLEN EBERT for the isolation of mutants used in this work, and DEBORAH WYGAL who made the original observations of recombination in this laboratory.

This work was supported by Grant VC35C from the American Cancer Society and Grant GB18510 from the National Science Foundation.

LITERATURE CITED

- DAVIS, B. D., 1948 Isolation of biochemically deficient mutants of bacteria by penicillin. *J. Am. Chem. Soc.* **70**: 4267.
- JACOB, F. and E. L. WOLLMAN, 1961 *Sexuality and genetics of bacteria*. Academic Press, New York and London.
- JOLLIFF, J. D. and E. M. SHERVISH, 1972 Genetic recombination in *Caulobacter*. *J. Gen. Microbiol.* **73**: 403-407.

- NEWTON, R., 1972 Role of transcription in the temporal control of development in *Caulobacter crescentus*. Proc. Natl. Acad. Sci. U.S. **69**: 447-451.
- POINDEXTER, J. S., 1964 Biological properties and classification of the *Caulobacter* group. Bacteriol. Rev. **28**: 231-295.
- RUBY, C. L., 1967 Genetic exchange in *Caulobacter crescentus*. M.A. thesis, Indiana University.
- SCHMIDT, J., 1966 Observations in the absorption of *Caulobacter* bacteriophages containing ribonucleic acid. J. Gen Microbiol. **45**: 347-353.
- SHAPIRO, L., N. AGABIAN-KESHISHIAN and I. BENDIS, 1971 Bacterial differentiation in stalked bacteria. Science. **173**: 884-892.
- STOVE, J. L. and R. Y. STANIER, 1962 Cellular differentiation in stalked bacteria. Nature. **196**: 1189-1192.
- TAYLOR, A. L. and C. D. TROTTER, 1972 Linkage map of *Escherichia coli* strain K12. Bacteriol. Rev. **36**: 504-524.

Corresponding editor: D. SCHLESSINGER