ISOLATION OF SPONTANEOUSLY DERIVED MUTANTS OF *CAULOBACTER CRESCENTUS*

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ABSTRACT

Caulobacter crescentus has a penicillinase which precludes the use of penicillin for mutant enrichment. However, two other antibiotics, fosfomycin and D-cycloserine, can be used to enrich for *C. crescentus* mutants. In enrichment procedures for *C. crescentus* auxotrophs, spontaneously derived mutants occur at a frequency of 5–10% among the survivors of an enrichment procedure. Consequently, large numbers of mutants are readily obtained without any need for mutagenesis. These mutants are heterogeneous both with regard to the type of mutation and to the nutritional requirement. A similar procedure has been used to isolate temperature-sensitive mutants.

CAULOBACTER CRESCENTUS is a stalked bacterium which differentiates into three clearly distinguishable cell types during its normal growth cycle (STOVE and STANIER 1962). Stalked cells differentiate to become predivisional cells with a flagellum and pili. Asymmetric cell division ensues, giving rise to a stalked cell like the original and a motile swarmer cell. The stalked cell immediately starts a second cycle; whereas the swarmer cell must first become a stalked cell by synthesizing a stalk and losing its flagellum and pili. Because of this unique cell cycle, *C. crescentus* has been proposed as a model for the study of cellular differentiation (SHAPIRO, AGABIAN-KESHISHIAN and BENDIS 1971) and has been the subject of a number of studies in recent years (for review, see SHAPIRO 1976).

Mutants of *C. crescentus* that affect the control of morphogenesis have been isolated (NEWTON, OSLEY and TERRANA 1975; KURN, AMMER and SHAPIRO 1974). However, only limited analyses of these mutants have been possible. For detailed study it is necessary to isolate large numbers of mutants for genetic and biochemical analyses. Most Caulobacter mutants have been obtained by direct screening after extensive mutagenesis with either N-methyl-N′-nitro-nitrosoguanidine or ultraviolet irradiation (JOLLICK and SCHERVISH 1972; NEWTON and ALLEBACH 1975). Recent studies with some of these mutants have revealed the presence of large numbers of unselected mutations (JOHNSON, WOOD and ELY, unpublished; ELY and JOHNSON, unpublished). Therefore, we attempted to develop techniques to isolate spontaneously derived mutants which presumably would differ from the parental strain by only a single mutation.

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Preliminary experiments with penicillin showed that *C. crescentus* has an efficient penicillinase that can interfere with mutant enrichment procedures using penicillin. In order to find an alternative to pencillin, we screened 8 additional antibiotics for differential killing of growing and nongrowing cells and found two, fosfomycin and D-cycloserine, which were effective. We report here the procedures developed to isolate spontaneously derived mutants with the aid of these two antibiotics and some of the results obtained from the use of these procedures.

MATERIALS AND METHODS

Strains: The *Caulobacter crescentus* wild-type strain CB15 was obtained from AUSTIN NEWTON and SC1 (*arg-101*) was isolated after penicillin enrichment as a spontaneously derived auxotroph from CB15. *C. crescentus* PC7, PC10 and PC17 were obtained from NEWTON and ALLEBACH (1975); WS5 and WS13 were obtained from JOLLICK and SCHERVISH (1972). The prototrophic strains SB2625 (ELY, FANKHAUSER, and HARTMAN 1974) and K-12 were used for experiments with *Salmonella typhimurium* and *Escherichia coli*, respectively. Phage ϕCr30, ϕCbK, and ϕCr40 are described in JOHNSON, WOOD and ELY (1977).

Growth conditions: Complex medium was a modification of the peptone yeast extract (PYE) medium of POINDEXTER (1964) and consisted of 0.2% peptone (Difco), 0.1% yeast extract, 0.8mM $MgSO_4$, and 0.5mM $CaCl_2$. The addition of Ca^{++} is necessary since Ca^{++} is an absolute requirement for growth of *C. crescentus* and is not always present in sufficient quantities in the other components. PYE plates were made by the addition of 1% agar. Defined medium (M2) was a modification of the minimal medium of POINDEXTER (1964). To prepare M2 media, 1.74g Na_2HPO_4, 1.06g KH_2PO_4, and 0.5g NH_4Cl were added to 1 liter of high-quality deionized, distilled water prepared in an Ultrascience Hi-Q all-glass still. (Ordinary deionized or distilled water contains some inhibitor of *C. crescentus* growth and results in an 8 to 10 hr doubling time as compared to the 3 hr doubling time obtained with high quality water). The solution was autoclaved and after cooling, 10 ml each of 50mM $MgSO_4$, 50mM CaCl and 1 mM $FeSO_4$ in 0.8mM EDTA, pH 6.8 were added aseptically. (The $MgSO_4$ and $CaCl_2$ solutions can be autoclaved, but the $FeSO_4$ must be filter sterilized to avoid oxidizing the iron). These three minerals were the only ones needed for optimal growth of *C. crescentus* and replaced a more complicated mineral solution. Plates were made by addition of 1% Bacto-agar (Difco) to the media described above. However, for M2 plates the $CaCl_2$ was omitted, since a 1% solution of Bacto-agar already contains approximately 0.5mM Ca^{++} (WUTHIER and GORE, personal communication) and since the addition of $CaCl_2$ often resulted in a precipitation in the plates. Liquid cultures were aerated in bubbler tubes. *C. crescentus* was grown at 33° and *S. typhimurium* and *E. coli* were grown at 37°. Optical density measurements were made with a Klett-Summerson colorimeter using a green (no. 54) filter.

Detection of penicillinase: Cultures of CB15 were grown in PYE in approximately 1×10^8 cells per ml and various concentrations of penicillin G were added. Optical density and viable cell measurements were made at intervals. The concentration of penicillin remaining in the culture medium was assayed by spotting drops of the culture medium onto lawns of penicillin-sensitive *S. typhimurium* as indicator bacteria. Resultant zones of inhibition were compared to those produced from drops of standard solutions of penicillin G.

Assay for selective killing by antibiotics: Sensitivity to the various antibiotics was determined by spot tests on indicator lawns of wild-type *C. crescentus*. Subsequently, either 10 or 100 μg per ml of the effective antibiotics were added to CB15 log phase cultures in M2 + glucose medium. Optical density and viable counts were taken at intervals to measure the degree of killing. Tolerance of nongrowing cells to the test antibiotic was measured by addition of similar concentrations to an arginine auxotroph, SC1, which had been incubated for 12 hrs (4 generation times) in M2 + glucose medium lacking arginine. Viable counts were compared to those obtained with SC1 incubated in the same medium in the absence of any drugs.

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Fosfomycin was a gift from DR. DAVID HENDLIN of Merck, Sharp and Dohme, vancomycin and bacitracin were gifts from DR. ROBERT J. HOSLEY of Eli Lilly and Co., ampicillin was a gift from DR. KENNETH E. PRICE of Bristol Laboratories, and netropsin was a gift from DR. E. L. PATTERSON of Lederle Laboratories. D-cycloserine, novobiocin, and nalidixic acid were obtained from Sigma Chemical Co., Penicillin G. was obtained from Calbiochem.

RESULTS

Penicillinase activity: Preliminary experiments using penicillin to enrich for mutants of *C. crescentus* suggested that *C. crescentus* strains CB15 and CB13 have a penicillinase activity. Addition of pencillin G (100 units per ml) had little effects on the growth of a culture of *Caulobacter* CB15 but did cause a transient inhibition of cell division and the formation of elongated cells (NEWTON, OSLEY and TERRANA 1975). When the concentration of penicillin was increased to 500 units per ml, the optical density of the culture continued to increase for approximately ½ doubling and then remained constant while the viability of the culture decreased. After approximately 24 hours an increase in optical density was observed, and an assay for penicillin, as described in MATERIALS AND METHODS, showed no antibiotic activity. Thus, *C. crescentus* was able to detoxify fairly high concentrations of penicillin. Addition of 10,000 units of penicillin per ml caused the viability of the culture to be reduced by a factor of 10^5 within 12 hrs. Under these conditions, a significant amount of the penicillin was not inactivated and growth never resumed. Similar experiments with *C. crescentus* CB13 showed that the strain also has a pencillinase activity and that it is more effective than that of CB15.

A CB15 culture known from control experiments using fosfomycin (see below) to contain approximately 100 spontaneously occurring mutants per ml was tested to determine whether cultures could be enriched for mutants using high concentrations of penicillin. The culture was grown for 4 hrs in minimal medium to stop the growth of the mutant cells and then incubated for 6 to 12 hrs with 10,000 units of penicillin per ml. The treatment resulted in a reduction of the surviving cells by a factor of 10^5, so that if the mutants survived the treatment they would have become a significant fraction of the total population. However, none of the surviving clones was auxotrophic, indicating that all of the mutant cells in the culture had been killed during the penicillin treatment. Furthermore, control experiments with a culture of an auxotrophic mutant showed that penicillin caused up to a 40-fold decrease in the viability of nongrowing cells in the time required to eliminate the growing cells. Similar experiments with *S. typhimurium* showed that effective killing of growing cells occurred within 90 min when 10,000 units per ml of penicillin was used and that death of nongrowing cells was negligible over this time period. Therefore, penicillin is not practical for enrichment procedures with Caulobacter.

Isolation of auxotrophs: In order to develop a method to enrich for Caulobacter auxotrophs, we screened several additional antibiotics known to affect cell wall biosynthesis or reported to have a differential killing effect on growing and nongrowing cells (Table 1). Of the eight additional antibiotics tested, two, D-cyclo-

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TABLE 1

Effects of various antibiotics on growing and nongrowing Caulobacter

Antibiotic	Sensitivity* Growing cells	Nongrowing cells
Ampicillin	R	R
Bacitracin	R	R
D-cycloserine	S	R
Fosfomycin	S	R
Nalidixic Acid	S	S
Netropsin	S	S
Novobiocin	S	S
Penicillin G	R	R
Vancomycin	S	S

* S indicates sensitivity to the antibiotic and R indicates resistance when cells are growing in M2 medium.

serine and fosfomycin, killed growing cells while nongrowing cells retained viability. D-cycloserine is an analogue of D-alanine and presumably prevents D-alanine from being incorporated into the peptide cross-linkages in the mucopeptide layer of the cell wall (ZYGMUNT 1962). It has been used previously to enrich for auxotrophic mutants of *E. coli* (CURTISS *et al.* 1965). Fosfomycin inhibits the pyruvyltransferase activity necessary for cell wall biosynthesis (KAHAN *et al* 1974) and to our knowledge has not been used previously to enrich for nongrowing cells. Concentrations of up to 1000 μg per ml of D-cycloserine or fosfomycin resulted in a reduction of the viability of cultures growing in defined medium by a factor of 10^5 in 12 to 20 hrs, while similar experiments with nongrowing cells resulted in negligible loss of viability over the same time period. Concentrations of 100 μg/ml of D-cycloserine or 500 μg/ml fosfomycin proved to be the minimal concentrations for effective killing.

In order to isolate auxotrophs of *C. crescentus*, unmutagenized cultures were shifted from PYE broth to a minimal salt glucose medium, and either 100 μg D-cycloserine per ml or 500 μg fosfomycin per ml were added after approximately one doubling in the minimal medium. After 13, 15, and 18 hrs incubation the cultures were centrifuged to remove the antibiotic, the pellet was resuspended in PYE broth and plated on PYE plates. Any plates with small numbers of colonies were replicated onto M2 plates to identify the mutant clones. In typical experiments 5 to 10 percent of the survivors were auxotrophs, while as many as 54% were auxotrophs in extreme cases. From these experiments, we estimate that auxotrophs are present at a frequency of 10^{-6} to 10^{-7} in *C. crescentus* cultures after growth in complex medium.

For comparison, similar experiments were performed with *S. typhimurium* and *E. coli*. The procedure for *S. typhimurium* and *E. coli* was identical except that only 100 μg/ml fosfomycin was used. Fosfomycin or D-cycloserine was added after about 4 hrs in minimal medium and the culture was washed and plated after an additional 4 hrs and 20 hrs, respectively. The killing of growing

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cells with either fosfomycin or D-cycloserine was as efficient as with penicillin. None of the three antibiotics resulted in any significant killing of nongrowing auxotrophs. However, the frequency of spontaneous mutants among *S. typhimurium* or *E. coli* survivors was more than an order of magnitude less than among *C. crescentus* survivors, indicating that *C. crescentus* has a spontaneous mutation frequency which is at least ten-fold higher than that of *S. typhimurium* or *E. coli*.

Characterization of C. crescentus *auxotrophs*: The auxotrophs isolated spontaneously from *C. crescentus* are quite heterogeneous. For instance, in one experiment employing parallel treatments with fosfomycin and D-cycloserine, 131 auxotrophs having seventeen different nutritional requirements were isolated. Thus, a single culture can contain a wide variety of spontaneously derived mutants. Table 2 provides a list of the mutants characterized to date. Most of the mutants have requirements similar to those found with auxotrophs of other species of bacteria. However, several of them appear to be unique and suggest that amino acid biosynthetic pathways may be somewhat unusual in Caulobacter.

TABLE 2

Caulobacter auxotrophs

Requirement	Number isolated
arginine	13
arginine and asparate	1
aromatics	6
aspartate	2
aspartate, arginine, or lysine	1
aspartate, arginine, or glutamine	1
aspartate, glutamate, or histidine	1
aspartate, arginine, glutamate, or histidine	1
aspartate, arginine, glutamate, or proline	1
aspartate, arginine, glutamate, histidine or proline	7
glutamate	1
glutamate or histidine	1
guanosine	1
histidine	14
isoleucine, or methionine, or threonine	5
isoleucine and valine	14
leucine	8
leucine or valine	1
leucine, valine, or isoleucine	1
methionine	10
methionine or cysteine	1
phenylalanine	1
proline	2
purine	3
serine	5
threonine	2
tryptophan	1
unknown	7

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The mutant strains have been characterized with regard to phage sensitivity and have a pattern of sensitivity identical to that of the wild type (Table 3). In contrast, mutants isolated by screening survivors of extensive mutagenesis with either nitrosoguanidine or ultraviolet light often have altered patterns of phage sensitivity. These results suggest that the spontaneously derived mutants contain a single mutation while mutagenized strains contain several additional mutations. These latter strains will be difficult to use for genetic studies since most of them are resistant to the Caulobacter transducing phage ϕCr30 (Table 3 and JOHNSON, WOOD and ELY 1977).

The types of mutations found in some of these mutants were determined by reversion studies as described by HARTMAN *et al.* (1971). Some mutants did not revert and were presumed to be extended deletions. Others reverted only spontaneously and were presumed to be negative frameshifts, while the remainder were reverted by nitrosoguanidine or by ICR 191 or by both and were presumed to be either base substitutions or positive frameshifts, respectively. Thus, we concluded that we are obtaining a variety of mutational types as a result of spontaneous mutation.

Isolation of temperature-sensitive mutants of C. crescentus: In order to analyze the *C. crescentus* cell cycle, it is important to have conditional mutants which affect the cell cycle. Some temperature-sensitive mutants have been isolated by direct screening after extensive mutagenesis (NEWTON, OSLEY and TERRANA 1975). However, use of fosfomycin or D-cycloserine would allow the isolation of spontaneously derived temperature-sensitive mutants which cause a cessation of growth. We have found that the procedures used to isolate Caulobacter auxotrophs can be used to isolate temperature-sensitive mutants from cultures growing in rich media after a shift to the restrictive temperature. D-cycloserine is less

TABLE 3

Phage resistance patterns of some Caulobacter histidine auxotrophs

Strain	Selected marker	Mutagenic treatment*	Phage resistance†		
			ϕCr30	ϕCbk	ϕCr40
CB15	*his*‡	(wild type)	S	S	S
SC121	*his*	spont.	S	S	S
SC122	*his*	spont.	S	S	S
SC135	*his*	spont.	S	S	S
PC7	*his*	UV	R	S	S
PC10	*his*	UV	R	R	S
PC17	*his*	UV	R	R	S
WS5	*his*	NG	R	S	R
WS13	*his*	NG	S	S	S

* UV indicates irradiation with ultraviolet light, NG indicates treatment with N-methyl-N′-nitro-nitrosoguanidine, and spont. indicates that the mutants were obtained without any mutagenic treatment.

† R indicates resistance and S indicates sensitivity to the test phage in spot tests.

‡ Strains were histidine (*his*) requiring mutants found among the auxotrophs obtained from the various procedures (SC strains from this paper; PC strains from NEWTON and ALLEBACH 1975; and WS strains from JOLLICK and SCHERVISH 1972).

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efficient than fosfomycin, perhaps because of the presence of D-alanine in the growth medium. Spontaneously occurring temperature-sensitive mutants occur less frequently than the auxotrophic mutants but can be obtained at a frequency of 10^{-3} from among the survivors, suggesting that they occur at a frequency of 10^{-8} to 10^{-9}. Several temperature-sensitive mutants have been isolated to date and at least one of these appears to be a cell cycle mutant.

DISCUSSION

C. crescentus appears to have a penicillinase which precludes the use of low levels of penicillin for mutant enrichment procedures. Both fosfomycin and D-cycloserine can be used in place of penicillin for enrichment procedures with *C. crescentus, S. typhimurium* or *E. coli.* These antibiotics provide efficient killing of growing cells while the loss of nongrowing cells is negligible under the same conditions. Thus, they should be valuable for the isolation of auxotrophic and temperature-sensitive mutants in other species of bacteria which are resistant to penicillin.

The spontaneous mutation frequency for *C. crescentus* appears to be about tenfold higher than that of *S. typhimurium* LT-2, so that mutants are readily obtained in the absence of mutagenesis. Furthermore, these mutations appear to include frameshifts and deletions as well as point mutations. Thus, the use of mutagens for *C. crescentus* is unnecessary whenever suitable enrichment or direct selection procedures are available. This result is particularly fortunate since base analogues such as 2-aminopurine and 5-bromouracil, which are often employed as mild mutagens for *S. typhimurium* and *E. coli,* have no mutagenic effect on *C. crescentus* (ELY and JOHNSON, unpublished). Also, in contrast to other Caulobacter auxotrophs, the spontaneously derived mutants obtained after enrichment with fosfomycin or D-cycloserine appear to be the result of single mutational events and have normal patterns of phage resistance.

The Caulobacter auxotrophs obtained in these studies are particularly interesting, since mutants with a number of unexpected phenotypes have been obtained. Experiments are currently under way to determine the type of defects present in these mutants. In addition, all of the mutants are being used to construct a genetic map for Caulobacter and the temperature-sensitive mutants will be used to analyze the Caulobacter cell cycle.

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