Non-random Origin of Partial Revertants of a Pleiotropic Phage Resistant Variant of *Caulobacter crescentus* CB15

By EVELYN T. FARRELL AND PETER R. SMITH

Department of Microbiology, University College, Galway, Ireland

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A variant of *Caulobacter crescentus* CB15, strain GU1, was isolated on the basis of its resistance to phage ϕ CbK. Strain GU1 was also resistant to phages ϕ Cb12r and ϕ Cb13, developed filaments in liquid culture and showed altered sensitivity to the inhibitors methylene blue, sodium deoxycholate and ampicillin. Analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis of the membranes of strains CB15 and GU1 showed major differences. Partial revertants of the GU1 phenotype were isolated in which only the inhibitor sensitivities were changed. The origin of these partial revertants was investigated by the fluctuation analysis of Luria & Delbruck (1943). The results suggest that these partial revertants, which were stably inherited, were non-random in origin.

INTRODUCTION

In attempts to investigate the *Caulobacter* cell cycle, non-motile, phage resistant and morphological mutants have been isolated. Many of these mutants exhibited pleiotropic changes of phenotype (Fukuda *et al.*, 1976; Kurn & Shapiro, 1975; Schmidt, 1968). Reversion studies on some of them yielded partial revertants with novel phenotypes which did not occur in mutant or parent cultures (Fukuda *et al.*, 1976; Schmidt, 1968). Similar results have been reported in reversion studies on colicin tolerant mutants in *E. coli* (Rolfe *et al.*, 1974). Gorini & Beckwith (1966) have argued that the emergence of such novel phenotypic characters indicates that these partial revertants are due to suppressor mutations. Thus, they predict that such revertants would be produced in a random manner. In this paper we report the isolation and partial characterization of a pleiotropic phage resistant mutant of *Caulobacter crescentus* and the origin of partial revertants of the mutant phenotype.

METHODS

Strains. All strains were derived from *Caulobacter crescentus* CB15 which was a gift from Dr L. Shapiro, Albert Einstein College, New York. The peptone yeast extract (PYE) medium of Poindexter (1964) was used throughout. All incubation was carried out at 30 °C and broth cultures were agitated on a rotary shaker. Phage ϕ CbK was a gift from Dr Shapiro and phages ϕ Cb12r (ATCC 19089-B1) and ϕ Cb13 (ATCC 19089-B2) were obtained from the American Type Culture Collection.

Isolation of phage resistant variants. Portions (0.1 ml) of 12 h broth cultures of CB15 were spread on plates that had been surface-seeded with 10^9 plaque-forming units of phage ϕ CbK. The plates were incubated for 48 h and no more than one colony per plate was subcultured for further testing.

Morphology. All strains were examined for morphological changes by phase-contrast microscopy using a Nikon model L-Ke microscope.

Determination of sensitivity to inhibitors. Sensitivity to ampicillin (Beecham), sodium deoxycholate (BDH) and methylene blue (Gurr, London) was determined by plating approximately 500 colony-forming units (c.f.u.) from a 24 h broth culture on twofold serial dilutions of the inhibitors in PYE agar. Sensitivity to ampicillin was also determined in PYE broth. The lowest concentration of the drug that inhibited colony

formation or macroscopically visible turbidity was determined after 48 h incubation. This concentration was referred to as the minimum inhibitory concentration (m.i.c.).

Isolation of revertants of strain GU1. Variants with decreased sensitivity to methylene blue and sodium deoxycholate were isolated by plating 10^6 to 10^7 c.f.u. from a 24 h broth culture on twofold serial dilutions of the inhibitors in agar. Colonies which appeared on plates containing the m.i.c. or a higher concentration of the inhibitors were isolated. The alteration of the sensitivities of the putative revertants was checked by plating 500 c.f.u. of strain GU1 and of the variants on identical twofold serial dilutions of the inhibitors in PYE agar.

Fluctuation analysis of reversion in strain GU1. A 12 h broth culture of bacteria was diluted into 500 ml of prewarmed PYE broth to a final concentration of approximately 10^2 c.f.u. ml⁻¹. Twenty 10 ml portions were distributed to culture tubes and one 200 ml portion was placed in an Erlenmeyer flask. All cultures were incubated until their A_{680} reached 0.1. Cultures which grew at abnormal rates were discarded. Samples (0.1 ml; approximately 10^7 c.f.u.) of the 20 individual cultures were spread on the surface of selective plates, as were 20 0.1 ml samples from the bulk culture. Selective plates contained the minimum concentration of the inhibitors that prevented colony formation when inoculated with the strain under test at 500 c.f.u. ml⁻¹. Random colonies were isolated from these selective plates and tested for altered sensitivity to the inhibitors by the method described above.

Phage adsorption. The kinetics of phage adsorption were determined by the method of Schmidt & Stanier (1965).

Analysis of membrane proteins. Membranes were isolated by the method of Galdiero (1973) and analysed by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis using the technique of Maizel (1969).

RESULTS

Origin of strain GU1

One hundred and fifty phage ϕ CbK resistant variants of *C. crescentus* CB15 were isolated without the aid of mutagenesis. One hundred and forty-nine of these were also resistant to phages ϕ Cb13 and ϕ Cb12r. Of these 149, one strain, GU1, showed altered cell morphology. Approximately 20% of the cells in PYE broth cultures of GU1 were filaments of 10 to 70 μ m in length. Cultures of *C. crescentus* CB15 may produce filaments in PYE broth but only do so in old (3 to 4 d) stationary phase cultures. The filaments produced in old cultures of the wild-type have a characteristic loose spiral morphology (Poindexter, 1964) which is absent from those produced by strain GU1. Strain GU1, on the other hand, is filamentous at all stages of growth. Examination of the membrane proteins of strain GU1 and its parent CB15 by SDS-polyacrylamide gel electrophoresis revealed at least three major differences in band pattern (Fig. 1). Examination of the membrane proteins of strain CB15 grown with sub-inhibitory concentrations of ampicillin or NaCl, conditions which induce filamentation in this strain, showed them to be similar to those of CB15 not GU1. Analysis of the membrane proteins of other variants of CB15 which were also resistant to ϕ CbK, ϕ Cb12r and ϕ Cb13 showed them to be similar to those of CB15. This suggests that the changes detected in the protein banding pattern of GU1 are not a necessary consequence of filamentation or of phage resistance.

Strain GU1 also showed altered sensitivity to inhibitors. With respect to ampicillin, strain GU1 was 2.5 times more resistant than CB15 in solid medium and twice as sensitive in liquid medium. With respect to sodium deoxycholate and methylene blue in solid media, strain GU1 was twice as sensitive as the parent strain CB15. The reproducibility of twofold differences in sensitivity to inhibitors was tested by carrying out 20 independent determinations of the relative sensitivity of CB15 and GU1 to the inhibitors. The reproducibility was 100% with respect to ampicillin and 90% with respect to methylene blue and deoxycholate. Thus, these changes in sensitivity cannot be artefacts of the test procedure. The phenotype of GU1 (Table 1) has remained constant over 4 years storage, culturing and testing.

Fig. 1. Densitometer tracings of membrane protein patterns on SDS-polyacrylamide gel electrophoresis of *Caulobacter crescentus*. (a) Strain CB15 grown in PYE with ampicillin ($150 \mu\text{g ml}^{-1}$). (b) Strain CB15 grown in PYE. (c) Strain GU1 grown in PYE. Revertants of strain GU1 showed patterns that were identical with that from GU1.

Table 1. *Phenotypes of C. crescentus strains CB15 and GU1*

Phenotype	Strain CB15	Strain GU1
ϕCbK	Sensitive	Resistant
ϕCb12r	Sensitive	Resistant
ϕCb13	Sensitive	Resistant
50 % ϕCbK adsorption	35 min	100 min
Filaments	Absent	Present
Ampicillin in broth*	400	200
Ampicillin agar*	300	750
Deoxycholate*	1000	500
Methylene blue*	0.5	0.25

* Figures refer to minimum inhibitory concentrations of inhibitors in $\mu\text{g ml}^{-1}$.

Table 2. *Distribution of the numbers of resistant bacteria in 20 samples of cultures of C. crescentus strains CB15 and GU1*

Strain	Source		Selective agent†		
			MB	DOC	KAN
CB15	Same culture	Mean $\bar{x}$	288	122	70
		Variance V^*	1374	173	186
		$V/\bar{x}$	4.7	1.4	2.6
CB15	Similar cultures	Mean $\bar{x}$	308	45	324
		Variance V^*	14846	1757	8147
		$V/\bar{x}$	48.2	39	25
GU1	Same culture	Mean $\bar{x}$	92	72	109
		Variance V^*	52	145	259
		$V/\bar{x}$	0.57	2.0	2.3

* Corrected for sampling error (Luria & Delbruck, 1943).

† MB, Methylene blue; DOC, sodium deoxycholate; KAN, kanamycin.

Fluctuation analysis of partial revertants of strain GU1

When dense cultures of strain GU1 were plated on agar containing the m.i.c. of methylene blue (MB) and sodium deoxycholate (DOC) for that strain, colonies were formed by approximately 1×10^{-6} and 5×10^{-5} of the total c.f.u. plated, respectively. To shed more light on the origin and nature of these revertants the frequency of their occurrence was analysed by the fluctuation test of Luria & Delbruck (1943). Table 2 shows the results of plating 20 samples from a single 200 ml culture of GU1 and of CB15 on agar containing their respective minimum inhibitory concentrations of MB, DOC and kanamycin. With both strains the variance between the numbers of clones in the series of samples capable of forming colonies was close to their mean value indicating that the variation, as would be expected, is a result of chance. Table 2 also presents the results of plating samples of 20 independent cultures of strain CB15 on agar containing the minimum inhibitory concentrations of the same drugs. In these cases the numbers of clones in each culture capable of forming colonies showed a variance much larger than their mean as would be predicted by the theory of random mutation. Table 3 presents the results and analysis of plating samples of 20 independent cultures of strain GU1 on agar containing the minimum inhibitory concentrations of MB, DOC and kanamycin. The distribution of colonies on agar containing kanamycin showed a high variance but the distributions on agar containing MB and DOC did not.

Variants of strain GU1 that formed colonies on agar containing the minimum inhibitory concentrations of MB and DOC were isolated. Fifty independent variants were selected in this way from each selective medium, and their phenotypes were analysed (Table 4). None of these 100 strains showed gain of phage ϕ CbK sensitivity, loss of filamentation or reversion to wild-type CB15 SDS-polyacrylamide gel electrophoresis membrane protein bands. With respect to ampicillin sensitivity, 26% of variants selected on MB agar had a wild-type CB15 level of resistance and 28% of the variants selected on DOC agar had this phenotype. The phenotypes shown in Table 4 for these partial revertants were stable during a minimum of four cycles of plating, subculturing and testing. Microscopic examination of broth cultures of variants of strain GU1 selected on MB agar showed that 20% of them contained deformed cells termed 'fats'. No 'fats' were seen in cultures of variants selected on DOC agar or in any other cultures of GU1. In contrast, variants of strain CB15 selected for a twofold increase in resistance to MB or DOC showed no unselected phenotypic changes.

Table 3. *Distribution of the numbers of resistant bacteria in a series of similar cultures of C. crescentus* GU1

No. of cultures . . .	Selective agent†											
	MB				DOC				KAN			
	20	20	17	14	19	18	20	16	20	18	20	20
Culture no.												
1	5	10	11	46	290	78	100	106	93	162	225	21
2	12	2	12	34	310	89	95	72	69	89	314	54
3	0	11	28	52	236	54	79	73	101	78	345	90
4	10	6	22	36	226	66	107	61	116	130	190	71
5	8	15	20	28	259	68	76	80	205	86	452	62
6	6	8	48	48	288	71	131	67	81	100	294	397
7	8	6	33	28	243	56	108	92	180	69	250	108
8	14	7	47	24	324	62	89	52	135	67	220	27
9	6	33	13	31	284	77	114	57	346	72	326	108
10	7	6	43	26	271	89	119	90	100	90	480	131
11	9	12	28	35	292	62	100	73	91	384	390	136
12	11	15	29	34	256	70	80	78	85	157	188	27
13	6	9	26	30	258	67	82	50	100	163	374	204
14	6	5	33	34	253	79	83	56	150	91	302	181
15	13	20	29		213	77	85	51	67	66	492	125
16	8	12	40		244	92	83	65	109	52	392	186
17	12	5	23		270	97	114		112	78	294	71
18	20	10			294	53	109		136	480	258	81
19	5	8			321		72		45		286	179
20	12	18					102		106		421	185
Mean $\bar{x}$	8.9	10.9	28.5	34.7	275	81	101	70	121	131	324	122
V^*	8.6	35	95	32	1077	283	530	201	14641	12213	8147	7037
$V/\bar{x}$	0.96	3.2	3.3	0.93	3.9	3.4	5.2	2.8	33	93.2	25	57.6

* Corrected for sampling error (Luria & Delbruck, 1943).

† MB, Methylene blue; DOC, sodium deoxycholate; KAN, kanamycin.

Table 4. *Phenotypes of partial revertants of C. crescentus* strain GU1

Phenotype characters tested were: sensitivity to ϕ CbK, ϕ Cb12r and ϕ Cb13; SDS-polyacrylamide gel electrophoresis membrane protein patterns; filamentation; sensitivity to methylene blue (MB), sodium deoxycholate (DOC) and ampicillin (AMP). Characters unchanged from strain GU1 are not shown; characters changed to wild-type cb15 levels are indicated by capitals; characters changed to at least twofold higher resistance than wild-type cb15 are indicated by capitals followed by a plus sign.

Phenotype	Percentage of isolates of each phenotype	
	Selected on	
	MB agar	DOC agar
DOC	—	14
DOC+	—	6
MB	26	—
DOC, MB	22	18
DOC+, MB	26	20
DOC, MB+	—	4
DOC+, MB+	—	10
DOC, AMP	—	2
MB, AMP	4	—
DOC, MB, AMP	10	26
DOC+, MB, AMP	8	—
DOC, MB+, AMP	4	—

DISCUSSION

Strain GU1 has maintained a different phenotype from that of its parent CB15 through numerous subcultures during 4 years. Thus, its phenotypic change must be considered as a stable inherited change. Similar phenotypic changes have been reported for class II *amp* mutants in *E. coli* (Nordstrom *et al.*, 1970). These mutants show poor adsorption of phages T3 and T4, small variations in sensitivity to a variety of inhibitors, a twofold increase in resistance to ampicillin in solid media and increased sensitivity to ampicillin in broth media. These phenotypic changes have, in some mutants, been shown to be the consequences of mutations in *lpsA* or *lpsB* or *galU* genes, which lead to the production of an altered lipopolysaccharide.

No evidence is presented in this paper as to the causal lesions involved in the change of phenotypes from the wild-type strain CB15 to the variant GU1. The phenotypes of the partial revertants of GU1 do suggest that the control of the phenotypic characters of phage resistance, filament formation and membrane protein patterns, to which unselected phenotypic variants were not detected in the 100 strains tested, may be under a different control system from those of MB, DOC and ampicillin sensitivity. Unselected phenotypic variants were isolated in these latter properties in 78, 70 and 26% of the strains tested (Table 4). These partial revertants showed reproducible phenotypic changes which were stably inherited through many generations.

Luria & Delbruck (1943) predicted that if a particular phenotypic change was the result of an adaptation mechanism, then the distribution of the changed cells in a number of similar cultures would show a variance equal to the mean. If, on the other hand, such changes were caused by random genetic changes, then the variance would be 'abnormally high'. They further stated that this prediction would not be affected by the number of different mutational events that could give rise to the phenotypic change that was being selected. The essential condition for this prediction is that the initial number of bacteria in a culture must be so small that the number of mutations which occur during the first division cycle of the bacteria is small. This condition has been met in the experiments reported here where the initial number was approximately 10^2 c.f.u. per culture.

The results for strain GU1 (Table 3) show that the distribution of revertants, i.e. cells capable of colony formation on plates containing the m.i.c. of MB and DOC, in a series of similar cultures do not show the predicted abnormally high variance. The variance is, in fact, close to the mean. These results are not consistent with the predictions of an explanation of the origin of the partial revertants by random mutation. On the other hand, the distribution of cells in a series of similar cultures of CB15 capable of forming colonies on plates containing the m.i.c. for CB15 of MB and DOC does show an abnormally high variance. Thus, the failure to demonstrate a high variance for GU1 cannot be explained by reference to either the particular selective agents used or to the smallness of the twofold increase in resistance being selected. These conditions are identical in the experiments both with CB15 and with GU1. The distribution of cells in a series of cultures of strain GU1 capable of growing on agar containing the m.i.c. of kanamycin shows the high variance predicted by the random mutation theory. Thus, it is not possible to explain the low variance in the distribution of colonies of GU1 on agar containing MB and DOC by reference to some unknown property of strain GU1. This work suggests that the MB and DOC resistant variants of strain GU1, but not those of CB15, are produced by a mechanism other than that by which normal mutants originate. It is interesting that the GU1 variants produced by this abnormal mechanism show pleiotropic changes whereas the CB15 variants produced by a mechanism consistent with the random mutation theory showed no unselected pleiotropic changes.

The most reasonable conclusion is that the partial revertants represent the result of an adaptive inheritable change in GU1. Such a change may theoretically be inherited either in

the genotype or phenotype. Our current understanding of the flow of information in cells makes the hypothesis of adaptive changes in genetic information unattractive. The alternative of adaptive changes in phenotypes that are inheritable thus appears to be the most plausible explanation of the partial revertants. A similar hypothesis has previously been suggested to explain the development of heat resistance in *Streptococcus faecium* recovering from heat shock (Duitschaeffer & Jordan, 1974). It may not be a trivial coincidence that, in both the work reported here and in the work on *S. faecium*, the most probable site of the phenotypic change is in the membrane of the cell.

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