Acridine Sensitivity of Bacteriophage T2H in *Escherichia coli*

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Normally acridine-sensitive, *Escherichia coli*-T2H complexes are rendered acridine-resistant if the infecting bacteriophage mutant is either *pr* or *q*. If these *pr* or *q* mutants are treated to produce sensitive revertants, one obtains a mutation at any of several dye-sensitizing (*ds*) sites in the early enzyme region of the T2 map. The *ds* mutants are nonspecific suppressors because they reduce the resistance of complexes containing either *pr* or *q* to proflavine. The *ds* mutants are not identical in action, since some make *pr* or *q* sensitive to proflavine and quinacrine, and others, to proflavine alone. Two *ds* mutants have *r* to *r*⁺ mutation patterns which differ, depending upon whether or not the *ds* is coupled with *r*7 (an *r*II mutant). The mutation patterns of *r*⁺ to *r* are the same for both *ds* mutants and for wild type. We suggest that dye sensitization may consist of alterations of early enzymes so as to produce slightly different forms of deoxyribonucleic acid which are in turn dye-sensitive.

Acridine sensitivity is a property of cells infected with the T-even coliphages (8) which is relieved by the presence of mutations in either of two bacteriophage genes, *pr* or *q*, in T2H (9) or *ac* and *q* in T4D (6). The *pr* gene, when in its mutant form, causes infected cells to be less permeable to acridines than when in its wild-type configuration (10, 18). The action of the *q* gene has been defined to the extent that it affects the maturation of phage [*q* does not affect cell permeability (18)]; the *q* mutation permits production of phage heads in dye, whereas the wild-type allele does not (16). Thus, the similar phenotypes identified by acridine resistance represent different bacteriophage functions—regulation of cell permeability by the *pr* gene and alteration of a step in mature phage production by the *q* gene.

Initially, it was our intention to study the *pr* gene by determining the gene size. In addition, we did routine mutagenesis experiments to look for *pr*⁺ revertants among the *pr* mutant stocks. The apparent wild-type revertants resulting from the mutagenic action of nitrous acid treatment all proved to be double mutants containing the original *pr* mutation and one of several distantly linked suppressor-like mutations which we labeled *ds*. Similar pseudorevertants were also found in mutagenized stocks of *q*. The *ds* mutants reduce the proflavine resistance of both the *pr* and *q* mutants, and hence are not gene-

specific suppressors. We mapped the *ds* mutants and tried to analyze their actions. It is primarily these studies on the *ds* mutants with which this paper is concerned.

MATERIALS AND METHODS

Bacterial strains. *Escherichia coli* S (Hershey) and B/1,5 (Luria) were employed in single-step growth experiments in liquid cultures and in replica plating experiments by the velvet imprinting technique (1, 12). K-12 (λ) was used to screen for *r*⁺ revertants after mutagenesis of *r*7 stocks with proflavine.

Bacteriophage. All *pr* and *q* mutant stocks were selected originally (9) from wild-type T2 (*pr*⁺*q*⁺*ds*⁺) by plating on proflavine- or quinacrine-supplemented agar plates. (Cells infected with either *pr* or *q* are in general resistant to either proflavine or quinacrine.) The occasional plaques which appeared were picked and purified. These resistant stocks (*prds*⁺ or *qds*⁺) served as the source for new stocks which were again sensitive to acridines. The pseudo wild-type strains recovered from nitrous acid treatment of *prds*⁺ and *qds*⁺ were labeled *prds* or *qds*. A large variety of other genotypes were used to produce the map showing the location of the *ds* markers. The order of several of these known markers was shown by Baylor, Hessler, and Baird (4). The location of the T2 amber mutants has been confirmed by complementation tests with homologous T4 ambers by both spot testing and growth tube experiments. To avoid repetitive use of symbols in this paper, whenever *pr*, *r*, *q*, etc. are used, it is assumed that they represent phage which are wild-type with respect to every other marker. When desig-

nation of the wild-type form is necessary for clarity, it will be included. The use of the term "wild-type phage" exclusively refers to $pr^+q^+r^+ds^+ \dots x^+$.

Media. The medium used contained: tryptone (Difco), 10 g; NaCl, 5 g; and distilled water, 1 liter; the pH was 7.0. The bottom-layer agar had the following composition: agar (Difco), 11 g; tryptone, 10 g; NaCl, 8 g; glucose, 1 g; and distilled water, 1 liter. Top-layer agar was the same as bottom-layer agar, except that 7 g agar was used. Bacteriophage stocks were made from agar lysates which were taken up in phosphate buffer. The phosphate buffer contained: 1% gelatin, 1 ml; $\text{Na}_2\text{HPO}_4 \cdot 7\text{HOH}$, 5.7 g; KH_2PO_4 , 1.5 g; NaCl, 4 g; K_2SO_4 , 5 g; and distilled water, 1 liter. After autoclaving, 10 ml of 0.1 M $\text{MgSO}_4 \cdot 7\text{HOH}$ and 10 ml of 0.01 M $\text{CaCl}_2 \cdot 2\text{HOH}$ were added. The final pH was 7.0. Acetate buffer contained: $\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{HOH}$, 10 g; acetic acid, 1.2 ml; and distilled water, 1 liter. This mixture was diluted 10-fold and was used at pH 5.2.

Methods. Crosses were performed with B/1,5 as host cell; phage inputs of five of each parent per bacterium were used and the growth period was 30 min at 38 C, after 10 min of adsorption. Details of crosses have been previously described (4, 9).

Proflavine mutagenesis was as described by Hessler (10): 4 $\mu\text{g}/\text{ml}$ of the acridine is present during the first 16 min of the growth cycle, but is diluted 100-fold into tryptone broth for a post-treatment 60-min growth cycle.

For photoinactivation, a 5-ml suspension of progeny from an untreated or an acridine-treated culture was exposed to visible light, and surviving phages were assayed at intervals (10).

The pseudo wild stocks were obtained by nitrous acid mutagenesis of stocks of pr or q mutants which were exposed to 0.05 M KNO_2 in acetate buffer (pH 4.5) for 10 min (2). These treated phage were then put through a single cycle of growth to eliminate heterozygotes, and the progeny were plated on S and incubated overnight at 38 C. The plaques were transferred by velvet onto dye-supplemented plates (3.5 μg of proflavine or 40 μg of quinacrine per ml) which had S cells (preincubated 2.5 hr) in the top-layer agar. The replica plates were incubated overnight at 38 C to permit development of the transferred plaques. Parental plaques (pr or q) were clear; pseudo wild plaques were very turbid.

The velvet transfer technique was used also to screen wild-type stocks for the new pr mutants used in mapping the pr gene. The pr^+ds plaques were distinguishable from wild only on the very best transfers. The genotype was verified by backcrossing with $prds^+$.

RESULTS

Unique quality of the pr gene. Ten new dye-resistant mutants (pr) were isolated from wild-type stocks which had been mutagenized with nitrous acid or proflavine. A series of crosses was performed among these mutants and with two existing pr mutant stocks of separate spontaneous origin (9). None of the 12 pr mutants recombined with one another to a level of at

least less than 1 in 500. One possible explanation for the failure of the pr mutants to recombine is that all other mutations in the pr gene are lethal. Another explanation is that the pr gene resembles the h gene (3, 20) in the sense that the proflavine-resistant phenotype requires a unique configuration, and that alterations are expressed phenotypically as wild type. The latter possibility has proven untestable because of the high incidence (1 in 1,000) of pseudo wild phages, which occurred far above the expected level of back mutation of pr to pr^+ , when pr was treated with nitrous acid.

Origin and location of dye-sensitive pseudo wild mutations. The pr and q mutant stocks of spontaneous origin were treated with nitrous acid to induce mutation to the wild-type form. These "revertant" stocks were crossed with wild type. The progeny were plated on S and incubated overnight at 38 C. The plates were then imprinted by velvet to S cells on dye-supplemented plates so that parental and recombinant phenotypes could be distinguished (Fig. 1). All five pseudo wild stocks were found to consist genetically of the appropriate parental pr or q mutant, plus a dye-sensitizing (ds) mutant. The pr or q markers give clear transfer plaques on dye plates, whereas the wild-type and pseudo wild plaques give poor transfers. When ideal results were obtained, the order of decreasing clarity of transfer plaques was: $prds^+ > prds > pr^+ds^+ > pr^+ds$. The pseudo wild stocks were also crossed with several other mutants, and with one another as well, to construct a linkage map (Fig. 2).

The map has been constructed from multifactor crosses, each of which contained as an internal control two markers of known location from the following: $r1$, $rm1$, $r2$, $r7$, $am3$, $am7$, $am11$, $ts9$, $ts16$, as well as pr or q . Of the five ds mutants definitely located, all are between $r7$ and $r1$ in the early enzyme region of the map (7). There are four more ds mutants which, though not precisely located by preliminary crosses, can be tentatively placed in the early enzyme region. No ds mutants were found outside of the early enzyme region.

Quantitative analysis of the ds phenotype. The quantification of the distinction of $prds$ or qds from resistant and sensitive phenotypes has proven complex. The greatest differences are found when S cells are used as host. Extensive comparative studies of relative phage yields of B/1,5 cells plus acridines infected with (i) $prds$ or $prds^+$ or (ii) ds or wild type permit some distinction between the two pairs of phage parents, since the ds -containing complexes generally had a lower phage yield than ds^+ ones.

FIG. 1. (A) Portion of a photograph of agar plate upon which were plated progeny from a cross between $r2pr^{+}ds^{+}$ and $r^{+}prds4$; a = $r^{+}prds^{+}$ recombinant plaque; b = $r^{+}prds4$ parental type; c = $r2pr^{+}ds^{+}$ parental type. (B) Photograph of portion of replica plate made by velvet transfer of plaques in Fig. 1A; a, b, and c arrows point to corresponding plaques in 1A. (C) Portion of photograph of agar plate containing progeny of cross between $r2pr^{+}ds^{+}$ and $r^{+}prds2$; a = $r2prds^{+}$ recombinant plaque; b = $r^{+}prds2$, c = $r2pr^{+}ds^{+}$ parental types. (D) Portion of photograph of replica plate made by velvet transfer of plaques in 1C; a, b, and c arrows point to corresponding master plaques in 1C. Plating cells throughout were *Escherichia coli* S. Replica plates contained 3.5 $\mu\text{g/ml}$ of proflavine, and plates were preincubated for 2.5 hr before velvet imprint, overnight after imprinting, 38 C.

FIG. 2. Partial map of T2H bacteriophage showing location of new *ds* mutants in relation to known markers. Map lists recombination frequencies established by multifactor crosses which contained as an internal control two known markers; *pr* = proflavine resistant; *r* = rapid lysis; *am* = amber; *ts* = temperature-sensitive; *ds* = dye-sensitizing. Numbers in parentheses refer to homologous T4 gene containing a complementary amber mutation.

However, infected S cells in liquid culture, as on dye replica plates, did yield larger differences in numbers of progeny phage (Table 1), corresponding to our qualitative estimates as expressed in Table 2.

Presence of any *ds* marker in the genome leads to reduced phage yield in proflavine, as judged from replica plate results, regardless of which of the resistant markers (*pr* or *q*) are present also (Table 2). The *ds* mutants, therefore, are not

acting like specific suppressors, since the *pr* or *q* functions are different; rather, the phenotypes are additive. The particular mutants *prds4* and *prds2* have been studied by Silver (*personal communication*) by the fluorometric technique (18) to see whether the uptake of dye by cells infected with *pr* was altered by the presence of a mutant at a *ds* site. No significant differences were found between *prds*⁺ and *prds* in B/1,5 cells; S cells have not been investigated yet.

TABLE 1. Comparison of average yield of bacteriophage T2H per infected *Escherichia coli* S cell in the continual presence of proflavine during a 35-min growth cycle^a

Expt	Proflavine μg/ml	Bacteriophage genotype		
		prds ⁺	prds4	pr ⁺ ds ⁺
1	0	100	210	285
	1	100	140	40
	2	61	92	12
2	0	186	124	—
	0.5	170	85	—
	2	85	30	—

^a In tryptone broth at 38 C.

TABLE 2. Qualitative differences in phenotypes of acridine-resistant and acridine-sensitive bacteriophage T2H^a

Bacteriophage genotype	Proflavine, 3.5 μg/ml	Quinacrine, 40 μg/ml
Wild type.....	++ ^b	+
pr ⁺ q ⁺ ds4.....	0	0
pr ⁺ q ⁺ ds5.....	0	0
pr.....	++++	++++
prds2.....	++	++
prds4.....	++	++
prds5.....	++	++
q.....	++++	++++
qds4.....	++	++
qds5.....	++	++++
qds8.....	++	++++

^a Based on velvet transfer to dye-supplemented agar plates; *Escherichia coli* S.

^b Degrees of transfer: ++++ = clear; +++ = slightly turbid; ++ = turbid; + = very turbid; 0 = none. The differences between ++ and + type plaques are sometimes very subtle, requiring that phenotypes be confirmed by genetic crosses. See Fig. 1 and 4.

Our experimental evidence for the nonspecificity of the *ds* mutation as a suppressor of *pr* is derived from photoinactivation studies which compared *prds*⁺, *prds*, and wild type. Hessler has established that the photosensitivity of phage progeny depends on the amount of dye present at the time of phage maturation (10). These studies corroborated those of Silver (18), which demonstrated that the *pr* mutant controls cell permeability. Hessler (10) has shown that phage progeny produced in the presence of dye differ in their resistance to light inactivation. If the *ds* mutation, *ds4* in this case, increased the permeability of cells infected with a *pr* (reduced permeability) mutant, one would expect greater uptake of dye by *prds*-infected cells than by

pr-infected ones. Using acriflavine in a subinhibitory concentration (0.01 μg/ml) throughout the growth cycle, we obtained essentially comparable phage yields from *pr*, *prds4*, and wild type. Photoinactivation of the phage progeny showed that the two genotypes which contained the *pr* mutation had the same photoinactivation resistance, significantly higher than that of wild type (Fig. 3). From this, we conclude that *ds4* did not suppress the *pr* function which controls the intracellular level of dye.

The *ds* mutants are not identical in their effect on dye sensitivity. At least two, *ds5* and *ds8*, although proflavine-sensitive with *q* in the genome, are quinacrine-resistant (Fig. 4), whereas *ds2* and *ds4* when present in the genome suppress both the *pr* and *q* mutations in either dye. Although the acridines are structurally similar, they seem to differ in their actions. Proflavine is mutagenic on T2, whereas quinacrine has not proven mutagenic in our hands. Proflavine (and acriflavine) renders both infective centers and progeny phage photosensitive (10); quinacrine does not (Hessler, unpublished data). The failure of quinacrine

FIG. 3. Result of photoinactivation of progeny from infected *Escherichia coli* B/1,5 cells grown in 0.01 μg/ml of acriflavine for 60 min. Similarity of survival curves for *prds*⁺ and *prds4* indicates that cells infected with those phages took up similar amounts of dye. Wild type-infected cells had only one-tenth as many survivors among photoinactivated progeny. In all cases, untreated and acriflavine-supplemented, the average yield of phage was over 200 per bacterium.

FIG. 4. Individual differences shown by two different *ds* mutations. (A) Portion of a photograph of an agar plate containing progeny of a cross between *r7qds4* and *r⁺qds8*; a = *r7qds4* parental type; b = *r⁺qds8* parental type; c = *r⁺qds⁺ds⁺*, recombinant. (B) Portion of photograph of replica plate which contained 3.5 µg/ml of proflavine in bottom-layer agar. (C) Portion of replica plate which contained 40 µg/ml of quinacrine in bottom-layer agar. Notice that *r7qds4* plaques are equally turbid on both dyes, whereas the *r⁺qds8* (b) is very turbid on proflavine, but indistinguishable from *r⁺qds⁺ds⁺* (c) on quinacrine.

TABLE 3. Effect of *ds* (dye-sensitizing) loci or external loci on the frequency of mutation of *r7* to *r⁺* in bacteriophage T2H^a

Phage genotype	<i>r⁺</i> frequency (× 10 ⁻⁷) ± SE ^b		Extent of increase	No. of experiments
	Untreated	Proflavine-treated		
<i>ds⁺</i>				
<i>r7</i>	2.0 ± 0.9	51.0 ± 16.4	25×	17
<i>r7h</i>	2.0 ± 1.3	50.1 ± 13.0	25×	7
<i>r7pr</i>	3.2 ± 2.2	31.0 ± 6.7	10×	8
<i>ds2</i>				
<i>r7</i>	0.5 ± 0.3	87.0 ± 14.0	174×	3
<i>r7h</i>	0.2 ± 0.1	10.3 ± 2.7	50×	3
<i>ds4</i>				
<i>r7</i>	3.2 ± 1.4	7.9 ± 3.2	2.5×	7
<i>r7pr</i>	6.2 ± 3.5	11.5 ± 5.6	1.9×	6

^a Infected B/1,5 (*Escherichia coli*) was treated for 16 min with 4 µg of proflavine per ml, and then was diluted 100-fold into tryptone broth for a 60-min growth cycle at 38 C. Burst size of dye-treated cells was usually at least 70% that of untreated cells.

^b ±SE = standard deviation between experiments.

to produce photosensitive progeny may be due to a less effective penetration or a difference in specificity of binding sites. In vitro studies of acridines show similarities between quinacrine

and proflavine. Each binds to deoxyribonucleic acid (DNA) by intercalation between base pairs, as well as by external stacking along the DNA molecule (13, 14), and both show similar reactions when dye-DNA complexes are exposed to changes in ionic strength or to denaturation stresses (5).

Functional differences among the ds mutants. The efficiency of proflavine as a mutagen was studied in detail to compare genomes containing *ds* and *ds⁺* alleles. We compared *ds4* and *ds2* in a well-controlled manner, described below.

We studied the reversion frequency of the *r7* mutation to *r⁺*, as well as the mutation rate for *r⁺* to *r*, in stocks with and without *ds*. The *h* and *pr* markers were employed as control mutant sites, as they are located outside the early enzyme region and are unlikely to be concerned with DNA synthesis. Great care was taken to acquire stocks with low background mutant levels. Every genotype selected for mutagenesis was tested whenever necessary to confirm genetic identity. Proflavine treatment of *r7* stocks containing *h*, *pr*, or *ds2* produced significant increases in the *r⁺* revertants (Table 3). The differences among *h*, *pr*, and *ds2* in the extent of *r⁺* revertant increases are not significant, although the incidence of mutants in *ds2* was considerably higher. With the *ds4* stocks, however, there was no significant increase in the number of *r⁺* revertants above that of the dyeless control culture; the *ds4* genomes were not mutagenized by our

TABLE 4. Comparison of mutation rate from r^+ to r in T2H bacteriophage stocks containing ds^+ , $ds2$, and $ds4^a$

Expt	Genotype of bacteriophage	No. of progeny scored				Extent of mutant increase
		Untreated		Proflavine		
		r ⁺	r	r ⁺	r	
1 (4 μg/ml)	pr ⁺ ds ⁺	1,477	2	4,146	48	10×
	pr ⁺ ds2	1,998	2	9,660	33	3.4×
	pr ⁺ ds4	1,220	0	8,058	39	6×
2 (8 μg/ml)	prds ⁺	6,071	5	7,823	47	8×
	prds4	5,268	5	8,683	32	4×

^a *Escherichia coli* B/1,5 cells. Proflavine concentrations listed were present from 0 to 16 min. Cultures were diluted 100-fold for 60-min growth periods in tryptone broth at 38 C.

treatment. T tests showed the $r7ds^+-r7ds4$ difference significant at the 99% level and the $r7prds^+-r7prds4$ difference significant at the 95% level.

We further compared $ds4$ and $ds2$ with wild type for the general mutation r^+ to r . The extent of increase in r mutants stimulated by proflavine was similar for wild type, $ds2$, and $ds4$ (Table 4). The increase in all three stocks was not significantly different from previously reported results on wild type (10). Therefore, we conclude that the presence of the $ds4$ site does not preclude proflavine-induced mutagenesis.

DISCUSSION

The ds mutations are nonspecific in their proflavine "sensitization" of either pr^- or q^- infected cells. All the ds mutations are in the early enzyme region, and they thus may affect the production of enzymes which control DNA synthesis, as well as other early functions. The $ds5$ and $ds11$ markers are very near $am3$, which is homologous with an amber (B22) in gene 43 of T4. A flaw in gene 43 results in no DNA synthesis (7). The $ds4$ marker is close to $am7$ (in T4 gene 39, whose function concerns the onset of DNA synthesis). The markers $ds2$ and $ds8$ fall in a region which has many mutants concerned with DNA synthesis (7).

The ds mutations may be in genes whose products contribute to DNA synthesis in particular, and the altered DNA produced by ds -bearing phage, although functional in normal media, may be impaired by acridines. An example of such impairment might be binding of acridines to sites not normally available on the DNA, thus slowing down DNA synthesis enough to give a reduced burst size.

It is plain that one simple explanation will not serve to encompass the actions of all the ds sites.

$Ds5$ and $ds8$, for example, each severely reduces the resistant q phenotype in proflavine, but they are nonfunctional in quinacrine.

The $ds4$ mutation, in contrast to $ds2$, reduces the capacity of $r7$ -bearing phage to yield r^+ revertants but does not affect production of r mutants by r^+ phages. In this case, the product of the $ds4$ -bearing genome may interact with that of the $r7$ gene to prevent the setting up of mutagenic conditions for the short period when dye is present. As a concrete example, suppose that $r7$ (an rII mutant) affects phosphorylation in B cells as well as in K [which Sekiguchi found for rII mutants of T4 (17)]. An explanation for the action of $ds4$ is that it upsets the normal balance of the $r7$ product in B, inducing a nonlethal alteration in the course of DNA synthesis. Proflavine which is present during the DNA synthetic period for an $r7ds4$ phage may then encounter conditions which do not permit dye intercalation and, hence, the additions and deletions of base pairs regarded as acridine mutagenesis (15). The routine dilution of the infected cells at 16 min washes out most of the dye and permits normal phage maturation. The $ds4$ function, we would argue, does not affect the function of the r^+ allele, and proflavine mutagenesis can therefore occur in r^+ds4 stocks.

Several recent studies provide some basis for our suggestion that a ds mutant could influence the way in which phage DNA reacts to acridines. Speyer (19) reported that a mutant in gene 43 of T4, a gene which controls DNA polymerase, has the properties of a mutator gene. That is, a mutant in gene 43 results in production of DNA polymerase which somehow renders new phage DNA more liable to mutate spontaneously. Yanofsky, Cox, and Horn (21) have reported on an *E. coli* mutator gene which confers on *E. coli* unusual capacity for mutation of a specific base pair, AT to GC. Further, Johnson and Bach (11) have

shown that both polyamines and quinacrine have effects on this *E. coli* mutator system. The polyamines reduce the spontaneous mutation rate of stocks carrying the mutator gene. Quinacrine suppresses the mutagenic action of 2-aminopurine on wild-type stocks of *E. coli*. Johnson and Bach think that it is the binding of the quinacrine molecules, as well as the polyamines, to the DNA molecule which is somehow responsible for the result observed. In both cases, it is suggested that the whole DNA molecule is affected, although the expression of the effect (reduction in mutation rate) may result from quite different mechanisms. If any of the *ds* mutants cause production of slightly altered DNA synthetic enzymes, these in turn might render DNA synthesis acridine-sensitive and result in reduced overall DNA synthesis. This would serve to explain the lower average phage yield of *prds*-infected cells which are grown in the continuous presence of the dyes. It should be experimentally possible to determine consistently low gross DNA production, and this may be one way of assessing a *ds* function.

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