Journal of Bacteriology, May 1987, p. 1847–1852
0021-9193/87/051847-06$02.00/0
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Vol. 169, No. 5

Cloning and Characterization of the *c1* Repressor of *Pseudomonas aeruginosa* Bacteriophage D3: a Functional Analog of Phage Lambda *c*I Protein

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Received 11 December 1986/Accepted 22 January 1987

We cloned the gene (*c1*) which encodes the repressor of vegetative function of *Pseudomonas aeruginosa* bacteriophage D3. The cloned gene was shown to inhibit plating of D3 and the induction of D3 lysogens by UV irradiation. The efficiency of plating and prophage induction of the heteroimmune *P. aeruginosa* phage F116L were not affected by the presence of the cloned *c1* gene of D3. When the D3 DNA fragment containing c1 was subcloned into pBR322 and introduced into *Escherichia coli*, it was shown to specifically inhibit the plating of phage lambda and the induction of the lambda prophage by mitomycin C. The plating of lambda *imm*^{434} phage was not affected. Analysis in minicells indicated that these effects correspond to the presence of a plasmid-encoded protein of 36,000 molecular weight. These data suggest the possibility that coliphage lambda and the *P. aeruginosa* phage D3 evolved from a common ancestor. The conservation of the functional similarities of their repressors may have occurred because of the advantage to these temperate phages of capitalizing on the potential of the evolutionarily conserved RecA protein to monitor the level of damage to the host genome.

The establishment and maintenance of lysogeny by temperate bacteriophages requires the continued presence of specific repressor proteins (13). For phage lambda of *Escherichia coli*, these functions are supplied by the product of the *c*I gene (25, 26). In *E. coli* the induction by DNA-damaging agents of the prophages of lambda and related viruses is initiated by the specific cleavage of the repressor of vegetative functions promoted by an activated form of the *recA* gene product (36, 38, 39). This cleavage takes place at a unique Ala-Gly bond within the repressor protein (29). We have isolated the *recA* gene of *Pseudomonas aeruginosa* PAO and demonstrated that its protein product is capable of mediating the induction by DNA-damaging agents of prophage lambda from *recA* mutants of *E. coli*, as well as prophage D3 from *recA* mutants of *P. aeruginosa* (27, 28). Our data suggest that the *P. aeruginosa* RecA protein mediates this induction by a mechanism similar to that of the *E. coli* RecA protein (28).

D3 is a temperate bacteriophage of *Pseudomonas aeruginosa* which was originally described by Holloway et al. (19). The D3 virion is complex, with a polyhedral head and a prominent tail with six knoblike projections (33). It contains a linear double-stranded DNA molecule of approximately 60 kilobase pairs in size (33). The prophage integrates into the *P. aeruginosa* PAO genome (9) and is inducible to lytic growth by UV irradiation (20). This induction requires that the lysogenized host have a RecA$^+$ phenotype (28) and leads to the formation of specialized transducing particles (9).

Egan and Holloway (14) demonstrated that the establishment and maintenance of lysogeny by phage D3 were dependent upon the expression of three genetic loci (*c1*, *c2*, and *c3*) within the D3 genome. Recently, Gertman et al. (16) determined that the insertion of IS*222* into a specific location in the D3 genome leads to loss of the ability of the phage to grow temperately. They identified the location of this insertion by restriction endonuclease analysis. Here we describe the cloning and characterization of the gene encoding the D3 repressor of vegetative function. Since the original mutations of Egan and Holloway (14) are no longer available for comparison, we have chosen the designation *c1* for this gene to indicate its functional analogy to the phage lambda *c*I gene.

(Portions of this work were conducted by T. A. Kokjohn in partial fulfillment of the requirements of the Ph.D. degree from Loyola University of Chicago, Chicago, Ill., 1987.)

MATERIALS AND METHODS

Bacteria and bacteriophage. The bacterial and bacteriophage strains used in this study are described in Table 1.

Media and culture conditions. Bacteria were maintained on Luria broth or L agar (32). Plasmid-containing cells were selected with 50 μg of ampicillin (Ap) per ml of medium for *E. coli* and 500 μg of carbernicillin (Cb) per ml of medium for *P. aeruginosa*. Tetracycline (Tc) was used at 10 μg/ml.

DNA isolation. D3 DNA was isolated from the virion by the method of Hinkle and Miller (18). Plasmid DNA was isolated by the large-scale alkaline lysis method of Maniatis et al. (30). Density equilibrium centrifugation was carried out in a TL-100 ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.) at 100,000 rpm with a TLA 100.2 fixed-angle rotor.

Cloning of the D3 *c1* gene. Plasmid pME292 (22–24), a 6.8-kilobase-pair *P. aeruginosa* plasmid derived from pVS1 (24) which is maintained at approximately two copies per chromosome in *P. aeruginosa* PAO25 (23), was used to clone the D3 repressor. Cloning into the unique *Hin*dIII site

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TABLE 1. Bacterial strains and phages

Strain or phage	Plasmid	Relevant genotype[a]	Source or reference
E. coli	—[b]		
AB1157	—	*argE3 his-4 leuB6 proA2 supE44 trp-1 thi-1*	10
χ1488	—	*his-53 hsdR2 ilv-277 lysC65 purE41 thi-1 minA1 minB2*	11
HB101	—	*recA13 hsdS20 leu proA2 supE44 thi-1*	7
RM1154	pBR322	As for AB1157	This study
RM1157	pKML11	As for AB1157	This study
RM1158	pKML12	As for AB1157	This study
RM1160	pBR322	As for RM1154 but λR lysogen	This study
RM1163	pKML11	As for RM1157 but λR lysogen	This study
RM1164	pKML12	As for RM1158 but λR lysogen	This study
RM1184	—	As for AB1157 but λR lysogen	This study
RM2330	pKML1101	As for AB1157	This study
P. aeruginosa			
PAO1	—	Prototrophic	32
PAO25	—	*argF10 leu-10*	15
RM17	FP2[c]	*leu-38 str-2* D3 lysogen	9
RM247	—	As for PAO25 but D3 lysogen	This study
RM2130	pME294	As for PAO25	This study
RM2131	pKML5	As for PAO25	This study
RM2132	pKML6	As for PAO25	This study
RM2327	pKML7	As for PAO25	This study
RM2328	pKML8	As for PAO25	This study
RM2333	pKML6	As for RM2132 but D3 lysogen	This study
RM2334	pME294	As for RM2130 but D3 lysogen	This study
RM2335	pME294	As for RM2130 but F116L lysogen	This study
RM2336	pKML6	As for RM2132 but F116L lysogen	This study
RM2337	—	As for PAO25 but F116L lysogen	This study
Phage lambda			
λR	—	*R5am*	27
mms813	—	*vir*	27
λ207	—	*c*I *ind*	F. W. Stahl
λ *imm*[434]	—	*imm*[434]	27
JMC307	—	*b*1453 *J6am c*I857	F. W. Stahl
P. aeruginosa phage			
D3	—	Wild-type	19, 32, 33
D3c	—	*c1*-3	This study
F116L	—	Wild-type	19, 32, 33

[a] Genotype symbols and abbreviations are as specified by Bachmann (2).
[b] —, None present.
[c] FP2 is a fertility plasmid of *P. aeruginosa* (32).

of pME292 inactivates kanamycin resistance. Selection for the plasmid is made by Cb^r (22). pME294 is identical to pME292 except that the former is carried in the cell at approximately 15 copies per chromosome in *P. aeruginosa* PAO25 (2).

*Hin*dIII-digested phage D3 DNA and pME292 DNA were mixed and ligated with T4 ligase (30), and the ligated DNA was transformed into PAO25 by the method of Mercer and Loutit (31). Two hundred independent Cb^r clones were isolated and screened for their immunity to infection by D3c. Resistant clones were purified, and plasmid DNA from several independent clones was isolated. A portion of each sample was digested with *Hin*dIII. Each clone was shown to contain the same 9-kilobase-pair insert. The remainder of the DNA from these preparations was used to transform PAO25. Greater than 99% of the transformants generated were immune to D3c as judged by cross-streaking (32). A restriction map of the plasmid (pKML6) from one of these clones was prepared. All restriction enzymes were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and were used according to the recommendations of the manufacturer.

The fragment of phage D3 DNA inserted into pKML6 was purified and introduced into the *Hin*dIII site of pBR322 (6) to allow for introduction of the cloned D3 fragment into *E. coli* HB101 by the method of Davis et al. (12). Selection was made for Ap^r Tc^s clones. Clones with the D3 DNA inserted in opposite orientations were selected.

Bacteriophage methods. Methods for the preparation of lysogens, determination of immunity properties by cross-streaking, and determination of the efficiency of plating (EOP) have been described previously (28, 32). Lysates of bacteriophage lambda were prepared with tryptone agar as described by Kokjohn and Miller (28). Lysates of bacteriophage D3 were prepared by the method of Cavenagh and Miller (9) with L agar. Prophages of D3 and F116L were induced by UV irradiation, and lambda prophage was induced with mitomycin C by the methods of Kokjohn and Miller (27, 28).

Minicell analysis of pKML11 and pKML1101. Minicell analysis was carried out as described by Kokjohn and Miller (28) with the minicell-producing strain χ1488. Cells were labeled with [^{35}S]methionine (specific activity, 1,114 Ci/mmol; New England Nuclear Corp., Boston, Mass.).

TABLE 2. Effects of cloned D3 *cI* gene on EOP

Strain	Plasmid	Prophage	EOP (fold change)[a] with:	
			D3c	F116L
PAO25	—[b]	—	1.0	1.0
RM247	—	D3	2×10^{-2}	1.0
RM17	FP2	D3	2×10^{-3}	1.0
RM2130	pME294	—	0.7	1.0
RM2131	pKML5[c]	—	1×10^{-2}	ND[d]
RM2132	pKML6	—	2×10^{-3}	0.8
RM2327	pKML7	—	2×10^{-3}	ND
RM2328	pKML8	—	2×10^{-3}	ND

[a] Fold decrease (if any) of EOP relative to EOP of strain PAO25.
[b] —, None present.
[c] pKML5, pKML6, pKML7, and pKML8 are independent clones of the D3 *cI* gene in pME292.
[d] ND, Not done.

RESULTS

Cloning of D3 *cI* gene. A *Hin*dIII digest of phage D3 DNA was inserted into pME292 and used to transform *P. aeruginosa* PAO25. Isolated clones were cross-streaked (32) against a lysate of D3c (10^9 PFU/ml), and clones which appeared immune to infection by this clear-plaque mutant were used for further study. The EOP of D3c and the heteroimmune phage F116L on several of these clones was determined (Table 2). The presence of the cloned D3 fragment in PAO25 reduced the EOP of D3c >500-fold, to a level comparable to that observed with a true D3 lysogen. The D3c plaques observed on the strains containing the cloned fragment were extremely small and very turbid, indicating that the cloned fragment could suppress the clear-plaque phenotype of D3c. The cloned D3 DNA had no effect on the EOP of F116L. The construction contained in RM2132 was chosen for further study and designated pKML6.

Purified pKML6 DNA was labeled and hybridized to D3 and lambda DNA under conditions of high stringency (30). Under these conditions, the insert showed homology to D3 but not to lambda DNA (data not shown). A restriction map of the insert was prepared (Fig. 1). This map indicates that the cloned DNA fragment in pKML6 is derived from the same region of the phage genome determined by Gertman et al. (16) to contain genes necessary for turbid plaque formation.

Induction of D3 prophage in the presence of pKML6. The induction of D3 prophage by UV irradiation is dependent on a $RecA^+$ phenotype (28). If the induction mechanism of prophage D3 is the destruction of the *cI* repressor upon exposure to a DNA-damaging agent, it should be possible to titrate this effect by increasing the concentration of D3 *cI* repressor in the cell. Such a case has been shown to be true for the induction of lambda prophage in *E. coli* (4, 29, 37). When the cloned lambda cI is introduced into a lambda lysogen, the increased concentration of repressor protein produces subinduction conditions for the prophage (4) upon exposure to an inducing agent. Increasing the dosage of the D3 repressor gene by introducing pKML6 into the lysogenic strain should therefore inhibit induction of the D3 prophage by UV irradiation. Isolates of RM247 were prepared containing pKML6 (RM2333) and pME294 (RM2334). The ability of UV irradiation to induce lytic growth of the resident prophage in each of these strains was analyzed (Fig. 2A). The presence of pKML6 specifically inhibited the induction of the D3 prophage by UV irradiation. The induction of the heteroimmune prophage F116L was not inhibited by the presence of pKML6 (Fig. 2B).

Effects of cloned D3 *cI* gene on induction of lambda prophage in *E. coli*. The D3 DNA insert in pKML6 was subcloned into the *Hin*dIII site of pBR322 and transformed into *E. coli* HB101 with selection for Cb^r Tc^s clones. Plasmids (pKML11 and pKML12) with the D3 DNA insert in opposite orientations with respect to the tetracycline promoter (6, 34) were selected. These plasmids were introduced into lambda lysogens of *E. coli* AB1157, and the induction of the prophage was attempted with mitomycin C (27). pKML11 inhibited the induction of the lambda prophage, whereas the presence of pKML12 and pBR322 had no effect on this process (Table 3). It would appear that the D3 *cI* gene product is capable of protecting the lambda prophage from induction by activated *E. coli* RecA protein.

EOP of phage lambda on strains containing pKML11 and pKML12. If the D3 *cI* gene product was acting nonspecifically by interacting with activated *E. coli* RecA protein, the ability of phage lambda to infect and grow lytically in strains of *E. coli* containing clones of the D3 *cI* gene should not be affected. When this was tested (Table 4), it was found that the EOP of wild-type lambda on strains containing pKML11 was dramatically reduced. This inhibition appeared specific for wild-type lambda, because both virulent mutants of lambda and imm^{434} phage were not affected by the presence of the plasmid. A mutant of phage lambda carrying the temperature-sensitive mutation *c*I857 was inhibited at both the permissive and nonpermissive temperatures. A deletion subclone of pKML11, pKML1101, was prepared by cleavage of CsCl-purified pKML11 DNA with *Sal*I and religation (Fig. 1). This *Sal*I site was shown to be adjacent to a site of insertion of IS*222*, which produces the clear-plaque phenotype in D3 (16). pKML1101 had no inhibitory effect on the EOP of lambda.

Identification of proteins encoded by pKML11 and

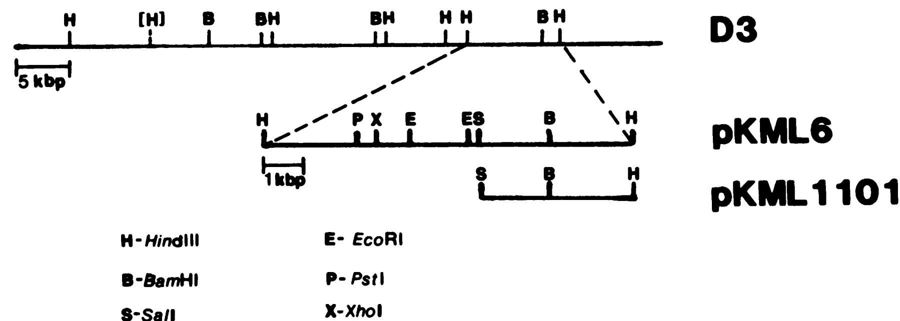

FIG. 1. Restriction endonuclease map of phage D3. pKML6 contains the *cI* gene. pKML1101 is a *Sal*I deletion which inactivates the *cI* gene. *Hin*dIII site in brackets is present in some but not all strains of D3.

TABLE 3. Effect of cloned D3 *cI* gene on induction of λR prophage by mitomycin C

Strain	Plasmid	Phage produced (PFU/ml)		Induction ratio
		Spontaneous	Induced[a]	
RM1184	—[b]	1×10^5	1.8×10^9	1.8×10^4
RM1160	pBR322	6×10^4	2.2×10^8	3.6×10^3
RM1163	pKML11	4×10^5	1.6×10^6	4.0
RM1164	pKML12	9×10^3	5.0×10^7	5.5×10^3

[a] Cells were incubated with mitomycin C at 5 μg/ml for 150 min.
[b] —, None present.

pKML1101. pKML11 and pKML1101 were introduced into the minicell-producing strain χ1488 to allow the identification of plasmid-specific proteins (Fig. 3). pKML11 produced a protein of 36,000 molecular weight, which was not encoded by pKML1101.

DISCUSSION

We have isolated a 9-kilobase-pair fragment of the D3 genome which carries the *cI* gene. The product of this gene represses lytic functions of the phage, and the cloned fragment imparts immunity to superinfection by phage D3 to cells containing it. The product of the *cI* gene has been tentatively identified as a protein of approximately 36,000 molecular weight. Expression of the gene product of the cloned *cI* gene in *E. coli* is dependent upon the orientation of the inserted D3 DNA fragment in pBR322. pKML11 expresses the gene, whereas pKML12 does not. The inserts in pKML11 and pKML6 both have the same orientation with respect to nearby external promoters normally used in their respective vectors for expression of drug resistance (6, 22, 34). This suggests that the D3 *cI* gene may be using an external promoter for expression and, therefore, may not be subject to the same regulation of expression as it is when part of the intact D3 genome.

D3 lysogens are inducible by UV irradiation in Rec^+ strains of *P. aeruginosa* (20, 28). Mutations in the *P. aeruginosa recA* gene eliminate this induction (28). The *P. aeruginosa recA* gene product is also capable of allowing induction of lambda prophage from *recA* mutants of *E. coli* (27, 28). For *E. coli*, the induction of lambda prophage after exposure to DNA-damaging agents is known to be the result of the specific cleavage of the lambda *c*I repressor promoted by an activated form of the RecA protein (29, 36, 38, 39). The data available to date suggest that the *P. aeruginosa* RecA protein may be responsible for the induction of D3 prophage by a similar mechanism.

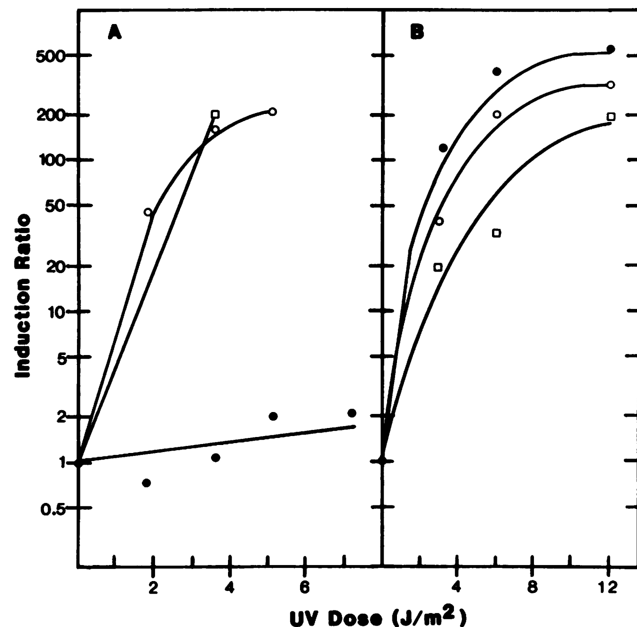

FIG. 2. UV induction of prophage. Induction experiments were carried out as described in the text. (A) D3 lysogens. Symbols: ○, strain RM247; ●, RM2333(pKML6); □, RM2334(pME294). (B) F116L lysogens. Symbols: ○, strain RM2337; ●, RM2336(pKML6); □, RM2335(pME294).

In *E. coli*, overproduction of a particular immunity-type repressor inhibits the induction of resident prophage of that specific immunity group only (14, 29, 37). Increasing the concentration of *c*I repressor by addition to the cell of the cloned *c*I gene does not saturate activated RecA protein, since prophages of other distinct immunity groups which are present are induced normally (4). Instead, it appears that the increased concentration of *c*I protein shifts the equilibrium between the monomeric and dimeric forms of the repressor to favor increased concentrations of dimer, which is less susceptible to cleavage by the activated RecA protein (35). Thus, inactivation of the overproduced *c*I repressor does take place upon exposure to an inducing treatment, but the decrease in effective repressor concentration is such that the lambda prophage is induced suboptimally. This phenomenon has been termed subinduction (4). D3 lysogens of *P. aeruginosa* harboring pKML6 are likewise incapable of inducing the resident prophage after exposure to UV irradiation. The

TABLE 4. Effect of cloned D3 *cI* gene on EOP of phage lambda

Bacterial strain	Plasmid	Prophage	EOP[a] of lambda phage strain:					
			λR (cI^+)	JMC307 (*c*I857) at °C		mms813 (*vir*)	λ207 (*ind*)	λ imm^{434} (imm^{434})
				30	43			
AB1157	—[b]	—	1.00	1.00	1.00	1.00	1.00	1.00
RM1184	—	λR	$<8 \times 10^{-6}$	$<2 \times 10^{-5}$	$<2 \times 10^{-5}$	1.00	$<5 \times 10^{-6}$	ND[c]
RM1154	pBR322	—	1.00	1.00	1.00	1.00	0.44	ND
RM1157	pKML11	—	$<8 \times 10^{-6}$	$<2 \times 10^{-5}$	$<2 \times 10^{-5}$	1.00	$<5 \times 10^{-6}$	1.00
RM1158	pKML12	—	0.25	ND	ND	1.00	ND	ND
RM2330	pKML1101	—	1.00	ND	ND	1.00	ND	ND

[a] EOP relative to EOP of AB1157.
[b] —, None present.
[c] ND, Not done.

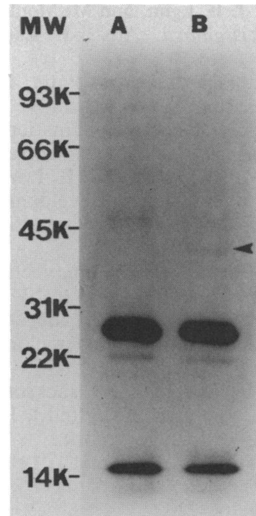

FIG. 3. Proteins encoded by pKML11 and pKML1101. Minicells were prepared from χ1488 derivatives containing pKML1101 (A) and pKML11 (B). The plasmid-encoded proteins were labeled with [^{35}S]methionine and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Migration of standard molecular weight markers is indicated to the left. Arrow indicates the protein unique to pKML11.

presence of the D3 *cI* clone in a D3 lysogen of *P. aeruginosa* may inhibit induction by a mechanism analogous to that of subinduction of phage lambda. Consistent with this hypothesis is the observation that lysogens of the heteroimmune phage F116L are induced normally in cells containing pKML6.

Phages D3 of *P. aeruginosa* and lambda of *E. coli* have several characteristics in common. They are both specialized transducing phages (9). Both appear to exercise a choice between lytic or temperate growth subsequent to infection of the cell, and their prophages integrate into unique sites in the chromosomes of their respective hosts (9). However, their receptors are different, and lambda does not infect *P. aeruginosa*, nor does D3 infect *E. coli* (data not shown). Whereas both are complex icosohedral phages, the morphologies of the two phage virions are different (1, 33). Their genomes, while sharing similar G+C content, have unique restriction maps and are quite different in size (1, 33). Southern hybridization, under conditions of high stringency, has revealed no large regions of DNA sequence homology.

The introduction of the cloned D3 *cI* gene into an *E. coli* lambda lysogen caused the inhibition of prophage induction by mitomycin C. This observation suggests that the D3 repressor protein acts in some fashion to protect the lambda *c*I protein from destruction by activated RecA. If the present model of the subinduction phenomenon is correct, it seems most plausible that the D3 *cI* repressor inhibits lambda prophage induction directly, perhaps by interacting with lambda *c*I protein to form heterodimers of the two phage repressors, thereby increasing the effective concentration of repressor dimers in the cell, which are less susceptible to inactivation by activated *E. coli* RecA protein. Alternatively, the D3 *cI* gene product could provide protection by directly inhibiting the activation of RecA protein. Whereas certain plasmids have been observed to inhibit the induction-promoting activity of the *recA441* allele (3), it seems unlikely that the D3 *cI* protein acts by this mechanism, since the D3 prophage is itself UV inducible. Such an effect would be expected to affect all UV-inducible phages simultaneously. The ability of cells containing the cloned *cI* gene to support the induction of heteroimmune prophages argues against a generalized inhibitory effect upon the activation of the RecA protein.

In addition to the effects on induction of lambda prophage, pKML11 was capable of producing apparent immunity in *E. coli* to wild-type and certain clear-plaque mutants of coliphage lambda. The cloned D3 *cI* gene may produce immunity to phage lambda by one of the following mechanisms. (i) This plasmid could contain D3 DNA coding for a phage-specific restriction-modification system. (ii) The presence of pKML11 could act to reduce the apparent phage titer by increasing the frequency of lysogenization upon initial infection by antagonizing a host-encoded function. (iii) The presence of the D3 *cI* gene product in *E. coli* AB1157 might inhibit the EOP of coliphage lambda by a direct interaction of the D3 repressor (or of at least a heterodimer of lambda *c*I and D3 *cI* proteins) with lambda DNA. The first two mechanisms would demonstrate nonspecific effects on the EOP of temperate phages, whereas the last mechanism would be expected to be specific for lambda.

We favor the last hypothesis. It is supported by the observation that the effects of the D3 *cI* gene in *E. coli* are specific for lambda phages having wild-type promoters. While *c*I$^+$ and *c*I857 lambda phages were inhibited by the presence of pKML11, lambda *vir* and *imm*^{434} were capable of normal EOP. These data suggest that the D3 *cI* repressor may be capable of specifically interacting with p_R and p_L of phage lambda to repress the lytic functions of the phage.

We consider the first two mechanisms unlikely because they are not supported by the phage-specific nature of the results obtained. A restriction system similar to that encoded by phage P1 (17) would inhibit the plating of any virus whose genome contained recognition sites for the restriction endonuclease. However, lambda *vir* and *imm*^{434} were capable of normal production of virus in the presence of pKML11 and thus were not restricted. Likewise, when a clone of the lambda *c*III gene is introduced into *hflA*$^+$ strains of *E. coli* (21), the increase in lysogen formation due to the antagonism of the HflA protein function is observed with phages other than lambda, including *imm*^{434}, which are sensitive to regulation by the HflA protein (5). The cloned D3 *cI* gene did not reduce the EOP of *imm*^{434} phage. In addition, one would not expect a clone of a *c*II- or *c*III-like gene to produce the observed suppression of the clear-plaque phenotype of lambda *c*I857 at the nonpermissive temperature.

It is clear that the *recA* gene has been disseminated widely and conserved throughout the eubacteria (27, 28). It has been suggested that lambda and certain other phages have evolved to capitalize upon the potential of the RecA protein to monitor the level of DNA damage to the host cell (27). It seems unlikely that the evolution of phages lambda and D3 has been convergent, since the closely related phages lambda, 434, and P22, which are all regulated by RecA, do not demonstrate cross-immunity. However, lambda phages of *E. coli* and D3 of *P. aeruginosa* may have evolved from a common ancestor in a manner similar to that proposed by Campbell and Botstein (8). The phages may have retained functional similarities in their repressor proteins because of the advantages of being able to respond to RecA surveillance of the host cell DNA. Thus, one may hypothesize that whereas the phages have long since diverged in many ways, radical change in repressor structure and function has been suppressed to retain responsiveness to the RecA protein.

ACKNOWLEDGMENTS

We thank A. J. Clark, D. Haas, H. Echols, R. E. Malone, and F. W. Stahl for strains, bacteriophages, and plasmids used in this study. We also thank H. Echols for his helpful advice during the course of this study.

This study was supported in part by a grant from the Dean's Development Fund of the Stritch School of Medicine and in part by cooperative agreement No. CR12494 from the Gulf Breeze Laboratory of the U.S. Environmental Protection Agency.

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