Conditional Temperature-sensitive Restriction of *Pseudomonas* Bacteriophage CB3

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Restriction of *Pseudomonas* bacteriophage CB3 growth on some *Pseudomonas aeruginosa* hosts was studied. On restricting hosts, growth of this phage was severely inhibited below 32 C and hence was temperature-sensitive. Investigation of this phenomenon revealed that restricting hosts were not killed as a consequence of their infection under nonpermissive conditions. The ability of some hosts to restrict showed segregation in sexual crosses between restricting and nonrestricting hosts. However, the pattern of restriction among various hosts differed with the phage in question when other phages were compared with CB3. Temperature-shift experiments indicated that blockage of an early event in the phage lytic cycle occurred when restricting conditions were imposed on cells infected with CB3. This blockage could be eliminated by holding at permissive conditions until the cold-sensitive step was bypassed or by pulsing restricting cells for 5 min at 37 C.

There have been several reports of phage mutants which have lost the ability to propagate on any host at cold temperatures (5, 7, 8). However, in these systems, the inability to propagate at temperatures in the range of 18 to 25 C is dependent on a mutational event which occurred in the phage. With the *Pseudomonas* phage system described here, the inability to propagate at low temperatures is host-dependent and hence conditional and temperature-sensitive. In this instance, restriction of phage growth below 32 C is at least partially dependent on the genotype of the host; segregation of this trait can be observed for genetic crosses between male (FP⁺) and female (FP⁻). The objective of this report is to describe some of the biological properties of this system and to contrast these properties with the features of other restricting systems reported previously.

MATERIALS AND METHODS

Bacterial cultures. *Pseudomonas aeruginosa* strains R629, 1C, and 2 were received from B. W. Holloway, Faculty of Science, Monash University, Clayton, Victoria, Australia. R629 was a strain 1 × strain 2 sexual recombinant prepared by Holloway (12); it required tryptophan, isoleucine, and valine for growth and was FP⁺. Strain 1 was a leucine auxotroph and FP⁻. We prepared the sexual recombinants, strains Pa34 and Pa35, according to the procedure of Holloway (10). Strain Pa30 was from our collection. Bac-

terial culture and phage techniques were previously described (17, 18).

Media. Medium composed of 0.5% tryptone, 0.1% glucose, and 0.25% yeast extract (TGE) was used for cultivation of phage and bacteria. Prototrophic bacterial recombinants were selected on Vogel and Bonner minimal medium (20) supplemented with 1.8% glucose. To induce synchrony in phage growth experiments, the starvation buffer (SB) of Denhardt and Sinsheimer (6) was used. For these experiments, cells were routinely grown to mid-log phase in aerated TGE medium. Cells were centrifuged, washed once in SB, and suspended in SB at approximately 2×10^8 /ml. This was followed by vigorous aeration for 30 to 90 min. In these phage growth experiments, it was particularly important that cultures were not centrifuged in the cold.

Bacteriophages. PX7 and CB3 were described previously (18). CB10 was isolated and propagated in the same manner.

RESULTS

In a survey studying the phage type of *Pseudomonas*, host-dependent variations in phage susceptibility with the temperature of incubation were observed. The behavior of several of these phages when plated at 20 and 37 C is shown in Table 1. There was segregation for the restriction of CB3 phage growth at 20 C after the mating of male restricting strain R629 and female nonrestricting strain 1. We considered this observation indicative of the contribution of the host towards restriction in this *Pseudomonas* system. However, the variable restriction of the other two phages, PX7

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and CB10, indicate that the specificity (or susceptibility to restriction) is at least partially phage dependent. In this regard, it should be pointed out that the restricting bacterial strains were able to propagate other nonrestricted phages at 20 C.

Of the phages described here, CB3 was selected for more detailed study because it plated with an efficiency of one on both fully permissive strain 1 and conditionally restrictive strain 2 at the permissive temperature (37 C).

We next determined if phage produced on strain 2 at 37 C was modified during replication, thus allowing subsequent plating on this host at 20 C. When this was accomplished, lysates prepared on the strain 2 host still were unable to form plaques at 20 C on strain 2.

Growth of CB3 at 20 and 37 C on fully permissive strain 1 and on conditionally restrictive strain 2 in broth culture is shown in Fig. 1. Growth at 37 C is shown in Fig. 1A. On both hosts, 95% of the phage was adsorbed in 5 min, and the latent

period was approximately 35 min. On the average, a burst size of 75 was observed for strain 1C, whereas a burst size of 50 was observed for strain 2. Growth at 20 C is shown in Fig. 1B. At this temperature, adsorption was less rapid than at 37 C, with 90% of the phage adsorbed to either host in 10 min. On permissive strain 1C, a latent period of 140 min was observed, with burst sizes averaging 45 to 50. However, with conditionally restrictive strain 2 at 20 C, little phage production was observed. This finding confirms results obtained by spotting indicator lawns incubated at various temperatures.

We next sought to determine the threshold temperature at which CB3 infection of strain 2 was inhibited. To accomplish this, one-step growth curve determinations were run as before at various temperatures, and the phage titer per milliliter of culture at the end of the rise period was plotted after correction for unadsorbed phage (Fig. 2). Phage growth was severely restricted on strain 2

TABLE 1. Host range and cold-sensitive restriction of *Pseudomonas* phages^a

Phage	<i>P. aeruginosa</i> strain											
	Pa30		R629		Pa34		Pa35		2		1C	
	20 C	37 C	20 C	37 C	20 C	37 C	20 C	37 C	20 C	37 C	20 C	37 C
PX7.....	+	+	+	+	+	+	+	+	—	+	+	+
CB3.....	—	+	—	+	—	+	+	+	—	+	+	+
CB10.....	—	+	+	+	+	+	+	+	—	+	+	+

^a Confluent lysis, +; neither lysis nor discrete plaque formation at 20 C, -.

FIG. 1. Growth of CB3 on permissive and conditionally restrictive *P. aeruginosa* at (A) 37 and (B) 20 C.

at incubation temperatures of 32 C and lower. As the growth temperature was raised from 20 to 32 C, progressively increased phage titers occurred, indicating a gradual loss of restriction and subsequent phage production. However, the most significant increase in phage growth apparently occurred between 32 and 37 C.

Since a few phage were usually produced under restrictive conditions at 20 C, single-burst experiments were done to distinguish between the production of a small number of phage by all of the infected bacteria and the production of normal bursts by only a fraction of the originally infected bacteria. The results of two of these determinations are shown in Table 2. In the two separate experiments, A and B, the number of infected bacteria per sampling tube was varied. Phage was

TABLE 2. Occurrence and burst size of CB3 produced under restrictive conditions

Determination	Expt A	Expt B
Multiplicity of infection....	0.12	0.12
No. of samples (0.5 ml)	50	50
No. infected bacteria per sample.....	0.78	1.91
Total no. of infected bacteria.....	39	95
No. of phage yielder tubes..	5/50	13/50
No. of phage in yielder tubes.....	28, 9, 3, 1, 7	8, 3, 9, 11, 4, 2, 21, 6, 2, 2, 4, 2, 5
Infected bacteria producing phage.....	13%	14%

FIG. 2. Threshold temperature for CB3 restriction.

adsorbed in buffer followed by antiserum neutralization and the distribution of portions into sampling tubes at 20 C. These experiments indicated that phage produced under restrictive conditions is made by a fraction of the originally infected cells. In another series of experiments, the multiplicity of infection was increased to as high as 10. When the proportion of multiply infected cells increased, fewer phage yielders were observed in the single-burst experiments and the viability of cells infected under restrictive conditions was significantly reduced. Thus this system is not subject to the multiplicity of activation phenomenon resulting in increased numbers of phage yielders, as shown in other restricting systems.

The fraction of phage yielders occurring at 20 C can be determined another way (Table 3). For these experiments, cells were infected in SB at 37 C and then diluted into broth or SB at 20 C. Unadsorbed phage was determined by filtration and subtracted from the total plaque counts before computation of the per cent surviving infective centers. For infected cells diluted into TGE broth, a progressive decline in infectious centers occurred to a minimal number, approximating the number of phage yielders obtained in the single-burst experiments. This was followed by the production and release of free phage, which was confirmed by examining filtrates of infected cultures. Interestingly, the onset of the production of free phage by the surviving phage yielders corresponded to the end of the latent period at 20 C observed for CB3 infection of permissive strain 1C. When the infected bacteria were diluted into buffer at 20 C instead of into nutrients as shown on the right of Table 3, phage genome decay, as evidenced by loss of plating as infectious centers,

TABLE 3. Persistence of restricted CB3 genomes at 20 C in cells infected in TGE broth and SB^a

Postadsorption incubation time <i>min</i>	Per cent surviving infective centers	
	TGE	SB
0	100	100
30	59	97
60	45	86
90	23	75
120	18	56
150	52	55
180	86	57
210	120	31
140	150	19

^a Cells were infected in SB at 37 C and shifted to TGE or SB at 20 C.

was minimal for the first 30 min at 20 C with a slow decline thereafter. However, prolonged incubation did not result in the production of phage. We interpreted these results collectively to indicate that restriction is promoted by the presence of growth nutrients. Also, assuming genome decay in restricting cells had gone to completion, the average burst size of yielder cells could be computed by dividing the number of infectious centers at the end of the recovery period by the number present at the decay curve minimum. With the data presented here, this amounted to an average burst of 8 phage per infected bacterium.

In view of the results shown in Tables 2 and 3, we were prompted to question what happened to the cells which were originally infected but failed to register as infectious centers after exposure to 20 C or failed to register as phage yielders at 20 C. To study this, we infected conditionally restrictive strain 2 at various incubation temperatures and plated for the survival of viable bacteria at both 20 and 37 C. The results of typical experiments are in Table 4. These data indicated that the per cent survival is greater for cells plated at 20 C following infection than for cells plated at 37 C regardless of the temperature of infection. Thus, bacterial cells were not killed as a consequence of their infection at restrictive temperatures.

To determine the nature of the restrictive event occurring in CB3-infected strain 2 cells at 20 C, we next did temperature-shift experiments. It was considered that a cold-sensitive block in the temporal sequence of phage production could be detected by infecting cells at 37 C and then transferring them at various times during the latent period to 20 C. Similarly, experiments were done

in which cells were infected at 20 C, transferred to 37 C for a brief period, and returned to 20 C to indicate that the infecting phage genome was not immediately destroyed upon entry under restricting conditions (Table 5). The results at the left of Table 5 show phage production after infection at 37 C and a shift to 20 C at various times. The zero-time shift data in the first row, wherein nutrients were added to infected cells after the shift to 20 C, again show the restriction typical of CB3 infection of the conditionally restrictive host at 20 C. However, when nutrients were added at 37 C and the infected cells were held at this temperature for 5 min, phage production equivalent to that occurring on the fully permissive strain 1C host was observed at 20 C. Longer incubations at 37 C before the shift to 20 C promoted slightly increased burst sizes. Furthermore, after shifts to 20 C, no decrease occurred in the number of infective centers during the remainder of the latent period. This is in contrast to data shown in Table 3; according to this data, bacteria infected at 20 C showed a progressive decay in infectious centers.

The right side of Table 5 contains data representative of infection at 20 C in SB followed by a short pulse at 37 C in the presence of nutrients. Once again, it is apparent that short exposure to nutrients at 37 C prior to return of the infected cells to 20 C allowed the restrictive event in the conditionally restrictive host to be bypassed. The control for these experiments was infected in SB at 20 C, shifted up to 37 C with nutrients added, and held at 37 C until lysis occurred.

Holloway (11) showed an effect of growth conditions on *Pseudomonas* phage restriction and modification. In view of his observations, we considered it appropriate to determine the effect

TABLE 4. *Bacterial survival after CB3 infection at 20 or 37 C^a*

Infection temp ^b (C)	Plating temp (C)	Per cent survivors
37	37	38
37	20	45
20	37	68
20	20	93

^a Phage was adsorbed in SB for 5 min at 37 C and for 20 min at 20 C, followed by dilution into and plating from TGE broth onto the surface of TGE agar plates.

^b Samples of a given cell suspension infected at 37 C were plated at 37 and 20 C. Therefore, in the absence of reversible adsorption confirmed by filtration, the effective multiplicity of infection was the same for cells used for both platings. Samples of a given cell suspension infected at 20 C, treated similarly, are also shown here.

TABLE 5. *Bypass of cold-sensitive restriction at 20 C in P. aeruginosa strain 2^a*

Adsorption in SB at 37 C ^b		Adsorption in SB at 20 C ^c	
Time at 37 C	Burst size at 20 C	Time of pulse at 37 C	Burst size at 20 C
<i>min</i>		<i>min</i>	
0	0.9	0	1.2
5	11	5	11
10	12	10	20
15	14		
30	16		

^a For these experiments, phage was adsorbed in SB at an input multiplicity of 0.1 phage per bacterium. Phage synthesis was initiated by dilution into TGE broth at 20 or 37 C.

^b Control kept at 37 C = 10.

^c Control kept at 37 C = 20.

of prior bacterial growth conditions on temperature-sensitive restriction. Data representative of CB3 infection of strain 2 cells grown at 37 C under aerated and static conditions and at 43 C under static conditions are shown in Table 6. Data for CB3 infection of fully permissive strain 1C grown under aerated conditions at 37 C is also included for comparison. With burst size as the criterion for optimal infection, these data showed that static growth of conditionally restrictive strain 2 promotes CB3 phage production in successfully infected bacteria under the permissive conditions of 37 C. In addition, prior growth of propagating cells at 43 C further enhances this effect. However, these data also indicated that conditions which optimize phage production per infected cell also

increase the number of abortive infections. Therefore, it may be that conditions which seem to promote phage production, in fact, merely select out those members of the bacterial population that are good phage yielders.

When cells were infected under restrictive conditions at 20 C, statically grown cells again showed more phage production per infected bacterium than strain 2 cells grown with aeration. As for 37 C infections, the number of abortive infections was greater for those cell preparations which showed larger bursts, thus apparently effecting some loss of restriction. However, the phage crop for the surviving phage yielders was significantly greater for statically grown cells than for aerated cells. Therefore, it may be that static growth conditions promote phage synthesis under restrictive conditions, although the data showed that conditions promoting synthesis do not reduce the number of abortive infections. In addition, when fully permissive strain 1C was grown under static conditions, no differential effect of temperature on phage production at 37 or 20 C was observed.

Exposure of *Salmonella typhimurium* to temperatures of 45 to 50 C for brief periods has been shown to result in the breakdown of the restriction mechanism (19). For this reason, we also determined the effect of sublethal heat treatments on the conditionally restricting host. When this was done, with exposure times and temperature relationships that did not preclude normal phage production under permissive conditions, no loss of restricting ability at 20 C was observed. We also tried to promote decreased restriction by exposure of cells to ultraviolet (UV) light before infection, as has been done for other systems (4, 16). UV dosage less than the amount required to kill 99.9% of the cells was without effect on restriction at 20 C. In addition, attempts to overcome restriction at 20 C by the preparation of streptomycin mutants as reported by Lederberg (15) were unsuccessful. These mutants, varying in their streptomycin resistance from 50 to 1,000 μg per ml, were tested at 20 C and were still observed to restrict the growth of CB3, although normal phage sensitivity at 37 C was observed as for the wild-type strain. Thus, the results of these later studies may militate against the presence of resident prophage as the basis for temperature-sensitive restriction, although this possibility cannot be excluded.

TABLE 6. Effect of growth conditions on the ability of bacteria to propagate CB3 at 37 and 20 C^a

Determination	Growth conditions			
	Strain 2			Strain 1 C (aerated at 37 C)
	Aerated at 37 C	Static at 37 C	Static at 43 C	
Infection at 37 C				
Abortive infections/ml	103	227	298	0
Zero-time infective centers (PFU/ml) ^b	241	114	46	346
Maximal rise infective centers (PFU/ml)	4,300	5,100	3,900	15,200
Burst size	19	45	85	57
Infection at 20 C				
Abortive infections/ml	213	240	257	0
Zero-time infective centers (PFU/ml)	91	60	57	344
Maximal rise infective centers (PFU/ml)	140	272	326	850
Burst size	1.5	4.5	5.8	25

^a Phage was adsorbed as in Table 4. Adsorption was terminated by dilution into TGE broth and unadsorbed phage were determined by membrane filtration (Millipore Corp., Bedford, Mass.). Abortive infections were calculated by subtracting the zero-time infectious centers from those obtained with the 1C host, with correction for unadsorbed phage. At 37 C, adsorption of phage was greater than 98%; at 20 C, adsorption of phage was greater than 86%, with an input multiplicity of 0.1 phage/10⁸ bacteria.

^b PFU, plaque-forming units.

DISCUSSION

The conditional nature of our temperature-sensitive restriction is clearly indicated by the above experiments. In restricting hosts, the factor(s) responsible for restriction is constitutive and its production apparently is not affected by prior

growth conditions attendant to the production of infected cells. The fact that a given host may show temperature-sensitive restriction to one phage but not to another which is restricted similarly on other hosts also implies some degree of specificity in this phenomenon.

The conditional temperature-sensitive restriction of CB3 phage has points of similarity and dissimilarity to other previously described phage-bacterium restriction systems. The failure of CB3 to initiate productive infections under restrictive conditions could be accounted for by the repression of the CB3 phage genome by a repressor coded for by the presence of a related resident prophage (14). However, electron micrographs of concentrated lysates do not reveal the presence of intact phage, although the conditionally restricting host shows UV- or mitomycin-inducible lysis. The absence of recognizable phage in induced lysates and the failure of heat treatments, UV exposure, or mutation to streptomycin resistance to promote derestriction, however, do not preclude repression resulting from a resident, possibly defective, prophage. One could account for temperature-sensitive restriction if a noninducible or defective psychrophilic prophage, whose activity is expressed only below 32°C was present as has been shown for virulent psychrophilic phages infecting mesophilic bacterial hosts (18). Alternatively, the resident prophage could be mesophilic but producing a temperature-sensitive repressor. However, both of these explanations necessarily require that the prophage not be heat-inducible if its repressor is temperature-sensitive.

The restriction of improperly host-modified phage, as reported by Arber (1) as a causal basis for CB3 restriction, is not indicated because prior growth of CB3 in the restricting host under permissive or restrictive conditions did not alter its plating efficiency on the conditionally restricting host under permissive or restrictive conditions. Although host modification and restriction were observed by Holloway for other *P. aeruginosa* phages (11, 13), modification apparently is not involved in the restriction of CB3 phage.

Recently, a report appeared describing the restriction of coliphage T7 by *Shigella sonnei* (9). In this report, restriction without modification, which we observed for CB3 infection of *P. aeruginosa*, was described. However, with T7 infecting *S. sonnei*, infection of restricting bacteria resulted in the death of the bacteria, whereas with our system restricting cells survived the infection. Thus, if CB3 is directing its own destruction under restrictive conditions, this activity leaves host functions unimpaired.

Conditional restriction of *rII* mutants of T4

by *Escherichia coli* K-12 (λ) was reported by Benzer (2). In this system, functional complementation was observed between *rII* phage mutants, resulting in the production of both phage types in the restricting host. In our system, however, we were unable to obtain significant CB3 production under restrictive conditions with CB3 and other nonrestricted *Pseudomonas* phages (*unpublished data*).

Superficially, the inability of *P. aeruginosa* to produce CB3 at 20°C resembles the outcome of *S. dysenteriae* infected by P1 phage at 20°C (3). On the other hand, our system demonstrates that the outcome of infection under restrictive conditions is not the establishment of lysogeny. This observation does not preclude the possibility that CB3 may, in fact, behave as a virulent mutant of some previously temperate phage. However, if this were so, the nature of its virulence must be somewhat dependent upon the identity of the host bacterium.

Regardless of the points of similarity or difference between temperature-sensitive CB3 restriction and other systems, the dominant characteristic of our system, which sets it apart from others, is the conditional nature of this restriction; i.e., CB3 is restricted on some hosts only below 32°C, whereas on other hosts CB3 growth is permitted at all temperatures studied.

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