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Operator-Promoter Functions in the Threonine Operon of *Escherichia coli*

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The prophage curing properties of secondary-site lysogens of coliphage λ have been studied. The site of integration in the original lysogen (L79) is within the operator-promoter region of the *thr* operon. As a result, expression of the *thr* enzymes is reduced, and the strain is a leaky threonine auxotroph. Heat pulse curing of strain L79 and a *thr*$^+$ lysogenic revertant (L79-20) showed that heat pulse curing of both lysogens was *int* and *xis* dependent and occurred by correct excisions of the prophage. The heat pulse curing restored strain L79 to prototrophy whereas strain L79-20 synthesized the *thr* enzymes constitutively and at high levels. This indicates that the reversion mutation in strain L79-20 occurred outside of the prophage and within the operator-promoter region of the *thr* operon. In contrast, spontaneous curing of both lysogens occurred by both correct and incorrect excisions. Spontaneously cured derivatives of strain L79-20 gave rise to three classes of regulatory mutants affecting operator and promoter functions of the *thr* operon.

A cluster of three genes, *thrA*, *thrB*, and *thrC*, specifies the four enzymatic activities involved in threonine biosynthesis in *Escherichia coli* (11, 12). *thrA* codes for the aspartokinase I (EC 2.7.2.4)-homoserine dehydrogenase I (HDH-I, EC 1.1.1.3) complex, *thrB* codes for homoserine kinase (HK, EC 2.7.1.39), and *thrC* codes for threonine synthetase (EC 2.9.9.2) (9-12). Since the synthesis of all three enzymes is coordinate (10) and multivalently repressed by threonine and isoleucine (2, 10), it appeared probable that the three *thr* genes were components of a threonine operon (13). In this study, we present evidence for operator and promoter functions as controlling elements of the threonine operon.

Previously we described (3) a secondary-site *E. coli* lysogen of bacteriophage λ in which the prophage was located near the threonine operon. The presence of the prophage in the original lysogen (L79) caused a pleiotropic effect in that three of the *thr* operon enzymatic activities tested (aspartokinase I, HDH-I, and HK) were absent in extracts of this lysogen. Genetic and biochemical evidence indicated that the prophage is located outside of the operon to the left of *thrA*, in a location where it prevents efficient expression of the structural genes. In addition, lysogenic Thr$^+$ revertants that synthesize the enzymes constitutively were isolated.

Since the prophage was inserted to the left of *thrA*, as the chromosome is usually represented, and diminished expression of *thrA*, *thrB*, and *thrC*, it seemed likely that it was located within the operator-promoter regions of the threonine operon, consequently disrupting promoter function and making the cells threonine auxotrophs. Lysogenic Thr$^+$ revertants in which the prophage remains at the same site could arise by mutation either within the prophage or between the prophage and *thrA*. By curing the lysogenic Thr$^+$ revertants of the prophage and examining the regulatory properties of the *thr* operon in the cured strains, we have obtained evidence that in one of the lysogenic revertants examined the mutation arose outside of the prophage. In addition, it has been possible to isolate regulatory mutants in the operator-promoter region of the *thr* operon as spontaneous prophage deletions that remain Thr$^+$.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are described in Table 1. λ*vir*^{r} strains were isolated as described by Shimada et al. (7).

Culture media. Vogel-Bonner liquid minimal medium (15) supplemented with 0.2% glucose and 1 μg of thiamine and biotin per ml was used. Where indicated, DL-threonine and DL-isoleucine were supplemented at concentrations of 200 and 100 μg/ml, respectively. LB5 broth (1) supplemented with 10 mM $MgSO_4$ was used as complete medium. For plating, the above media were supplemented with 1.5% agar.

Prophage curing procedures. Spontaneous curing

TABLE 1. *Bacterial strains*

Strain	Genotype	Source and comment
RW592	*thi* Δ(*gal bio att* λ*uvrB*)	R. Weisberg
L79	*thi* Δ(*gal bio att* λ*uvrB*) *thr* (λ*cI*857)	Derivative of strain RW592; λ*cI*857 integrated near the *thr* operon; see reference 3
L79-20	*thi* Δ(*gal bio att* λ*uvrB*) *thr*$^+$ (λ*cI*857)	Thr$^+$ lysogenic revertant of strain L79; *thr* operon enzyme expressed constitutively; see reference 3
L79 λ*vir*^{r}		λ*vir*^{r} mutant of strain L79
L79-20 λ*vir*^{r}		λ*vir*^{r} mutant of strain L79-20

of the prophage was accomplished by growing strain L79 or L79-20 at 30 C and plating on LB5 agar for incubation at 42 C. The Thr$^+$ or Thr$^-$ character of survivors was determined by replica plating. Heat pulse curing was accomplished as described by Shimada et al. (7).

Enzyme assays. Cells were grown and extracts were prepared as described previously (3). HK was assayed by the method of Theze et al. (10). HDH-I was assayed by the method of Patte et al. (6). Since both HDH-I and HDH-II activities are present in extracts (14), HDH assays were performed in the presence and absence of 0.04 M DL-threonine to determine the amount of threonine-inhibitable activity. Tryptophan synthetase assays were performed by the method of Smith and Yanofsky (9). Protein determinations were performed by the method of Lowry et al. (5). Specific activities are expressed as micromoles of substrate consumed or product formed per minute per milligram of protein.

RESULTS

Spontaneous and heat pulse curing of bacteriophage. Removal of prophage from *E. coli* strains lysogenic for λcI857 is readily accomplished by culturing the lysogens at 42 C (7). Under these conditions, excision of prophage occurs spontaneously, presumably generated by events reciprocal to integration (correct excision) and by incorrect excisions leading to deletion of some of the host genetic material. Thus, as seen in Table 2 (spontaneous curing), incubation of strains L79 *vir*^{r} and L79-20 *vir*^{r} at 42 C resulted in both Thr$^+$ and Thr$^-$ phenotypes among the nonlysogenic survivors. Presumably the Thr$^-$ colonies arose when part of the *thr* operon was excised.

Heat pulse curing by a 6-min treatment at 42 C favors expression of phage *int* and *xis* functions (7, 8, 16) and thus increases the proportion of correct excisions at the Δ'P.P'Δ site. This allows production of normal phage and restores the original deoxyribonucleic acid sequence in the host chromosome. Table 2 shows that the curing frequency generated by a heat pulse was 100-fold higher than the spontaneous curing frequency and, additionally, greater than 99% of the heat pulse-cured derivatives of both lysogens are Thr$^+$. These data support the idea that heat pulse curing favored correct excisions by the prophage. If a significant number of incorrect excisions occurred, some would generate deletions of the *thr* genes and render the cells Thr$^-$. The fact that heat pulse curing increased the curing frequency 100-fold indicates that *int* and *xis* are responsible and should, therefore, favor correct excisions at the Δ'P.P'Δ site.

Repression of HDH-I and HK in heat pulse-cured derivatives of strains L79 vir^r and L79-20 vir^r. Table 3 shows the ability of threonine and isoleucine to repress HDH-I and HK activities multivalently in heat pulse-cured derivatives of strains L79 λ*vir*^{r} and L79-20 λ*vir*^{r}, respectively. If the heat pulse curing results in correct excisions of the prophage, then strain L79 derivatives should become Thr$^+$ (which they do; Table 2) and should regain normal *thr* operon regulatory properties. Table 3 shows that HDH-I and HK are repressible in all four of the independently isolated heat pulse-cured strain L79 derivatives tested. In addition, all remain *trpR*$^+$ as evidenced by the tryptophan synthetase B protein levels. These data are consistent with the conclusion that heat pulse curing normally results in a restoration of the original deoxyribonucleic acid sequence in the chromosome.

If, in fact, heat pulse curing resulted in correct excisions at the Δ'P.P'Δ site, such strains may provide an indication of the location of the constitutive mutation in strain L79-20. If the mutation is located within the prophage, correct excision of the prophage would eliminate the mutation, and the cured strains should display regulatory properties similar to strain RW592. If, on the other hand, the mutation is located outside of the prophage,

TABLE 2. *Spontaneous and heat pulse curing of lysogens 79 and 79-20*

Curing method	Strain	Curing frequency[a]	Cured colonies of phenotype:[b]		% Thr$^+$
			Thr$^+$	Thr$^-$	
Spontaneous	79 λ*vir*^{r}	1.13×10^{-5}	107	187	36
	79-20 λ*vir*^{r}	1.61×10^{-5}	31	90	26
Heat pulse	79 λ*vir*^{r}	1.3×10^{-3}	800	3	99.6
	79-20 λ*vir*^{r}	2.4×10^{-3}	196	1	99.5

[a] (Number of survivors at 42 C/ml)/(number of survivors at 30 C/ml) on LB5 media.
[b] Plates were replica plated to minimal plates to determine the number of Thr$^+$ and Thr$^-$ colonies.

TABLE 3. *HDH-I and HK activities of heat pulse-cured derivatives of strains L79 λvir^r and L79-20 λvir^r*

Strain	Growth media[a]	Sp act (×10^3)		% Threonine inhibition	Sp act (×10^3)	
		HDH	HDH + Thr[b]		HK	*trpB*
RW592	Minimal	2.23	0.62	72	4.49	1.60
	Thr + Ile	0.83	0.23	73	1.14	
L79-1 λ*vir*^{r}	Minimal	2.85	0.77	73	6.21	0.69
	Thr + Ile	1.10	0.22	80	0.91	
L79-2 λ*vir*^{r}	Minimal	2.73	0.78	71	6.36	0.88
	Thr + Ile	1.02	0.28	73	1.70	
L79-3 λ*vir*^{r}	Minimal	2.90	0.57	80	7.18	0.83
	Thr + Ile	1.16	0.23	80	1.45	
L79-5 λ*vir*^{r}	Minimal	1.95	0.56	71	5.49	0.79
	Thr + Ile	0.76	0.17	77	1.22	
L79-20	Minimal	1.47	0.40	73	1.75	
	Thr + Ile	2.02	0.38	81	2.00	
L79-20-1 λ*vir*^{r}	Minimal	7.99	2.51	69	23.90	0.98
	Thr + Ile	8.34	2.73	67	21.44	
L79-20-2 λ*vir*^{r}	Minimal	9.25	2.73	70	23.84	0.82
	Thr + Ile	8.73	2.68	69	22.92	
L79-20-3 λ*vir*^{r}	Minimal	7.79	2.33	70	20.02	0.71
	Thr + Ile	8.65	2.47	71	23.21	
L79-20-4 λ*vir*^{r}	Minimal	9.70	2.93	70	24.98	0.99
	Thr + Ile	9.17	2.68	71	21.43	
L79-20-5 λ*vir*^{r}	Minimal	9.45	2.83	70	26.24	1.05
	Thr + Ile	8.88	2.59	71	21.36	

[a] Thr + Ile, Minimal medium supplemented with 200 μg of DL-threonine and 100 μg of DL-isoleucine per ml.
[b] Assay performed in the presence of 0.04 M DL-threonine.

heat pulse-cured strains of L79-20 may show abnormal regulatory properties. Table 3 shows that the second alternative is consistent with the results. All five independently isolated L79-20 strains synthesize three to four times the normal amounts of *thr* operon enzymes. In addition, the enzymes are not repressible by threonine and isoleucine. These results support the conclusion that the mutation in strain L79-20 is located in the bacterial chromosome outside the prophage, probably between the prophage and *thrA*. When the prophage is cured by a heat pulse, the deoxyribonucleic acid sequence at Δ.Δ′ is restored, and the remaining

mutation directs high-level synthesis of the enzymes which is not repressible by threonine and isoleucine. In these heat pulse-cured strains, and in strain L79-20, the mutation acts as though it alters both operator and promoter functions of the *thr* operon. Thus, the mutation in strain L79-20 and its derivatives is probably in the operator-promoter region of the *thr* operon.

Repression of HDH-I and HK in spontaneously cured derivatives of strain L79-20. Since spontaneous curing of the prophage in strains L79 and L79-20 apparently resulted in a high number of Thr$^-$ derivatives (Table 2), it is possible that deletions of the bacterial chromosome may occur frequently under these conditions. Thus, it was thought possible to obtain different types of regulatory mutants by screening for spontaneous prophage deletions of strain L79-20 which remain Thr$^+$. The reasoning behind this hypothesis is that it may be possible to isolate Thr$^+$ derivatives of strain L79-20 that are not produced by correct excisions of the prophage but are the result of incorrect excisions that produced small deletions in the regulatory portion of the *thr* operon. Such deletions would be detected as long as the *thr* genes are expressed sufficiently to permit growth.

Table 4 shows the regulatory properties of five independently isolated spontaneous Thr$^+$ prophage deletions from strain L79-20. These strains fall into three classes. The first class, A, consists of mutants 6 and 10, which synthesize high levels of HDH-I and HK not repressible by threonine and isoleucine. In fact, these strains behave identically to the heat pulse-cured derivatives of strain L79-20 *vir*^{r} (Table 3), and mutants 6 and 10 may have arisen by spontaneous correct prophage excisions. Class B consists of mutants 2 and 4. These strains synthesize HDH-I and HK constitutively, at low levels. The third class, C, is represented by mutant 9, which displays low-level, constitutive expression of the *thr* genes but, in addition, is *trpR*$^-$. Thus, this strain contains a deletion that extends at least into *trpR* (3). It is not known whether this deletion fuses the *thr* operon with *trpR* or whether the deletion extends past *trpR* into some other gene or operon.

DISCUSSION

It was shown in a prior communication (3) that, in strain L79, the prophage is located between *trpR* and *thrA*. This integration caused a pleiotropic effect in that *thrA* and *thrB* enzyme activities were reduced markedly. Since the *thr* operon is transcribed from *thrA* to *thrC* (13) and since lysogenic *thr*$^+$ revertants could be obtained from strain L79 (3), it is probable that the prophage is located outside of the structural genes. The likely location of the prophage is within the operator-promoter region of the *thr* operon. Integration into this region could prevent ribonucleic acid polymerase-promoter interactions and thus cause the polar effect observed.

Lysogenic *thr*$^+$ revertants could then arise by one of two events. First, a mutation within the prophage (increasing promoter function and allowing read-through into the *thr* operon) could occur. This mutation could occur within the phage promoter described by Shimada et al. (8) or in another phage promoter. Second, a

TABLE 4. *HDH-I and HK activities of spontaneously cured derivatives of strain L79-20*[a]

Strain	Growth media	Sp act ($\times 10^3$)		% Threonine inhibition	Sp act ($\times 10^3$)	
		HDH	HDH + Thr		HK	*trpB*
2	Minimal	0.82	0.16	80	1.50	0.64
	Thr + Ile	0.68	0.17	76	1.12	
4	Minimal	0.54	0.10	81	0.97	0.81
	Thr + Ile	0.64	0.17	73	1.43	
6	Minimal	8.75	2.48	72	23.64	0.67
	Thr + Ile	8.94	2.29	74	21.44	
9	Minimal	0.61	0.14	76	1.02	5.26
	Thr + Ile	0.51	0.11	78	0.76	
10	Minimal	10.18	3.19	69	27.57	0.90
	Thr + Ile	8.95	2.65	70	22.43	

[a] Conditions and abbreviations are the same as for Table 3.

mutation between the prophage P.'Δ site and *thrA* could occur. (A mutation to the left of Δ.'P, outside of the prophage, is unlikely because it would have to exert its effect through the λ genome before it could affect the *thr* genes.) Both cases would accommodate the observed effect, constitutive expression of the operon, with threonine and isoleucine unable to repress the synthesis of the enzymes.

Our data indicate that heat pulse curing of strain L79 or L79-20 resulted in correct curing of the prophage. Three lines of evidence support this view. First, heat pulse curing favors expression of the λ *int* and *xis* genes (16) whose functions normally catalyze excision at the *att* (4, 16) sites, making it likely that correct excisions occur frequently. This assumption is further substantiated by the observation of superinfection curing of another secondary site lysogen located in a gene necessary for proline biosynthesis (7, 8). In the latter case, it was found that superinfection curing resulted in virtually 100% Pro^+ derivatives, indicating that the original deoxyribonucleic acid sequence within the gene was restored. The fact that heat pulse treatment increased the curing frequency 100-fold over the spontaneous frequency also implies that *int* and *xis* are involved. Secondly, when strains L79 and L79-20 are cured by a heat pulse, greater than 99% of the cured strains are Thr^+. This is in contrast to the results obtained for spontaneous curing, where both Thr^+ and Thr^- derivatives arise in significant numbers. If heat pulse curing resulted in a significant number of incorrect excisions, one would expect a large number of Thr^- derivatives owing to the deletions generated. Third, five out of five heat pulse-cured derivatives of strain L79-20 showed identical, constitutive expression of the *thr* genes. In addition, four out of four heat pulse-cured derivatives of strain L79 regained multivalent repression, characteristic of the parent strain. Although the data are not extensive, they do support the conclusion that heat pulse curing usually restores the original deoxyribonucleic acid sequence in the chromosome.

All five of the heat pulse-cured derivatives of strain L79-20 tested express the *thr* operon enzymes constitutively and at about three- to fourfold higher levels than the parent strain. This indicates the following. (i) The mutation in strain L79-20 probably arose between the prophage site and *thrA* since, if the mutation resided within the prophage, it should disappear during heat pulse curing and the resulting strains should become normally repressible by threonine and isoleucine. (ii) When the prophage is cured, the reversion mutational event can direct even higher constitutive synthesis of the *thr* enzymes. Thus, expression of the enzymes is more efficient in the absence of the prophage. This could occur if the deoxyribonucleic sequence just to the left of Δ'P (on the left side of the prophage) contains a portion of the original *thr* operator-promoter region so that when the sequence is restored after λ excision there is even better expression of the *thr* genes. Other alternatives could be advanced to explain the high-level constitutivity in heat pulse-cured derivatives of strain L79-20. However, operator function must be affected since these strains are not repressible. The order of the operator and promoter genes is not known so it is difficult to construct a meaningful model to explain the results.

Spontaneous prophage curing of strain L79-20 revealed three classes of regulatory mutants. Class A mutants appear to be identical to the mutants isolated by heat pulse curing of strain L79-20. These mutants constitutively synthesize three- to fourfold more of the *thr* operon enzymes. Thus, the mutants probably arose from correct prophage excisions. Class B mutants synthesize the *thr* operon enzymes constitutively at low levels. The class C mutant shows similar properties, except that it also deletes at least a portion of *trpR*. It is not known whether the deletion in this strain fuses *trpR* and the *thr* genes so that the *thr* genes might be controlled by the *trpR* promoter. This possibility is currently being investigated.

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