INCORPORATION OF PHAGE DNA ON LYSOGENIZATION OF *SHIGELLA DYSENTERIAE**

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INTRODUCTION

Bacteria which are potentially lysogenic interact with temperate phage to produce either a lytic or lysogenic response[1]. When the bacteria become lysogenic, *i.e.* the phage reduces to prophage, it is of interest to determine the extent to which the phage material is incorporated in the cell, in particular, the extent of its participation in the formation of prophage material.

HERSHEY AND CHASE[2] have demonstrated that when the virulent phage T2 attacks *Escherichia coli* B, the DNA from the phage is injected into the cell leaving its protein coat attached to the bacterial surface. All the evidence to date indicates that the DNA of the phage is responsible for the hereditary characteristics of the phage particle. Little, however, is known concerning the transfer of temperate phage material to the host cell when the cell becomes lysogenic.

The results presented in this paper show that the temperate phage P1 transfers almost all of its DNA to the host cell when this cell becomes lysogenic and that the incorporation of DNA is specific in the sense that it is associated with prophage formation.

MATERIALS AND METHODS

The strain of *Shigella* (*Sh*) and its streptomycin resistant mutant Sh/s, and phages P1 and P1 *vir* used in these experiments were obtained from Dr. G. BERTANI and Dr. E. LENNOX. A description of these organisms and preparation of media have been given by BERTANI[3,4]. The following variations were used. Nutrient agar plates contained only 0.8% agar in place of 1% and spreading of the phage or bacteria was performed with a 2 ml 0.4% soft agar layer. The P1 plaques obtained under these conditions were 2 to 4 mm in diameter and were usually examined after 24 h at 37°C to allow sufficient time for drying to prevent the surface from running.

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EXPERIMENTAL PROCEDURES

Lysogenization at low temperatures

BERTANI AND NICE[4] have shown that *Shigella dysenteriae* sensitive to phage P1 may be lysogenized by P1 to a far greater extent by incubating infected cells at lower temperatures than by maintaining growth at 37° C. About 85 % of the infected cells are lysogenized by a two hour incubation at 20° C while only 15 % of infected cells are lysogenized by keeping them at 37° C. *Shigella* infected with P1 may be kept at 37° C for as long as 15 minutes without an appreciable decrease in the fraction of lysogenics produced upon subsequent incubation at 20° C.

Incorporation of ^{32}P into Shigella

Cells were grown in L broth plus $2 \cdot 10^{-3} M$ Ca at 37° C to a concentration of $5 \cdot 10^8$/ml from an inoculum of 10^8 actively growing cells. Phage P1 labeled with ^{32}P was added to give a multiplicity of 0.1 to 0.5 phage/bacterium, along with sufficient $CaCl_2$ to obtain a final concentration of $5 \cdot 10^{-3} M$. Adsorption was allowed to proceed at 37° C for 10 minutes and the cells were then centrifuged, resuspended in L broth containing $2 \cdot 10^{-3} M$ Ca^{++}, and placed at 20° C for two and a half hours or in the 37° control. The cells were then centrifuged and resuspended in L broth (no added Ca) at a dilution calculated to allow the cells to grow in log phase for a given number of generations. Bacterial plate counts, P1 assays, and ^{32}P determinations were made on all samples. For most experiments radioactive RNA, DNA, and protein were analyzed on both centrifuged cells and supernatants after Schmidt-Tanhauser and Schneider fractionations. The Schmidt-Tannhauser and Schneider fractions were omitted from a number of experiments when it was found that the TCA-soluble and protein fractions contained negligible amounts of ^{32}P activity, and the amount of RNA ^{32}P was of the order of 5–7 % of the DNA fraction. Since the DNA contamination of the RNA fraction in the Schmidt-Tannhauser fraction of Sh was found to be of the order of 5–7 %, the ^{32}P present in the TCA-insoluble fraction of *Shigella* infected with ^{32}P labeled phage P1 was considered to be all DNA. Therefore, in some cases it was expedient to follow phage ^{32}P DNA incorporation into Sh by simply centrifuging the cells down and following the distribution of ^{32}P in the cells and supernatant.

Determination of lysogenicity

Shigella lysogenic for phage P1, hereafter designated as Sh(P1), is resistant to a weakly virulent mutant P1 *vir*[4], even though P1 *vir* is adsorbed. Sh is, of course, sensitive to P1 *vir*, but does not give the typical lysogenic response to it. A similar phenomenon was shown to apply to the coli phage T7. Sh is sensitive to T7, while Sh(P1) adsorbs T7 but is resistant to it. No evidence for lysogenization with T7 has been found, however. In the present study both P1 *vir* and T7 were added to samples to eliminate the cells which were not lysogenized, thereby permitting a direct count of the lysogenics. Addition of P1 *vir* or T7 also made it possible to determine the distribution of ^{32}P from phage P1 in the lysogenic and non-lysogenic bacteria.

The preparation of ^{32}P labeled P1

Sh cells sensitive to P1 were grown at 37° C in L broth with $2 \cdot 10^{-3} M$ Ca and 2–25 uc/ml of ^{32}P. At a bacterial concentration of $1 \cdot 10^8$/ml, phage P1 was added to give 10^6/ml. Thirty minutes after first signs of lysis, about 2–2 ½ hours following the addition of P1, the cells were centrifuged off and the supernatant stored in the cold overnight with 1 γ/ml of DNAse. The phage was then given two cycles of centrifugation at 9,000 g, with clarifying centrifugations at 6,000 g, to eliminate non-phage ^{32}P and debris. The purity of the preparation was characterized by a comparison of ^{32}P uptake with the adsorption of P1. The ^{32}P remaining in the supernatant of the centrifuged cell suspension after adsorption of P1 was considered as non-adsorbed non-phage material.

EXPERIMENTAL RESULTS

Incorporation of labeled phage DNA into Shigella with lysogenisation

The incorporation of phage DNA into Sh under conditions of essentially single infection are given in Table I. The data is presented in terms of the percentage of DNA adsorbed to the cells after an initial incubation of P1 with Sh for 10 minutes at 37°C and subsequent wash with nutrient broth.

The results in Table I indicate that a major portion of the original DNA adsorbed to the cells on infection with P1 was still present after lysogenization followed by more than 8 generations of growth at 37°C. The addition of P1 *vir* to the infected system

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after 3 generations of growth resulting in the lysis of more than half the cells present produced no loss in ^{32}P. This indicated that the labeled DNA was not present in those cells sensitive to lysis by P1 *vir*.

TABLE I

PERCENTAGE INCORPORATION OF PHAGE ^{32}P DNA INTO *Shigella* AFTER INCUBATION FOR 2½ HOURS AT 20° C AND SUBSEQUENT GROWTH IN L BROTH AT 37° C

Cells	*DNA C.P.M.*	*Per cent of originally adsorbed DNA*	*Per cent lysogenics Multiplicity = 0.5*
Before 20° incubation	265	100	
After 20° incubation	257	97	
2 generations	258	97	25
3 generations	225	85	
5 generations	209	79	
6+ generations	200	75	30
7 generations	196	74	27
8+ generations lag cells	187	71	27
3+ generations with P1 *vir* added	223	84	

TABLE II

COMPARISON OF THE PHAGE DNA REMAINING IN CELLS AFTER GROWTH AT 20° C AND 37° C AND AFTER TREATMENT WITH T2 GHOSTS

Cells	*DNA by ^{32}P analyses C.P.M.*	*Per cent of originally adsorbed DNA*
Before 20° or 37° cycle	167	100
Before 20° or 37° cycle + T2 ghosts	20	12
After 40 min at 37° C	158	95
After 70 min at 37° C	96	58
After 4+ gen. 37° C	31	19
After 8+ gen. 37° C	30	18
After 2½ h at 20° C	159	95
After 2½ h at 20° C + T2 ghosts	45	27
After 2½ h at 20° C 8+ gen.	135	81

Centrifugation of cells which had incorporated labeled phage DNA or treatment of these cells with DNAse did not result in the loss of ^{32}P. The possibility still existed that the phage may have adsorbed to the cell and not injected its DNA. In order to eliminate lack of injection of DNA as a source of cell phage DNA, cells were lysed with T2 ghosts[9] both before and after lysogenization. The results in Table II indicate that the phage DNA adsorbed to Sh was in a form which could be solubilized by lysis of the cells either immediately after adsorption to the cells or after incubation of the cells for two and a half hours at 20° C. The production of phage along with the release of ^{32}P DNA which was not sedimentable with cell debris following the lytic cycle of growth at 37° C was confirmation that the phage DNA was actually injected into the cell. The latent period of P1 on Sh at 37° C in L broth is 46 minutes. Very little loss of

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phage DNA was shown to occur in the 40 minute period after the initiation of adsorption. A marked drop has occurred at 70 minutes while at 4^+ and 8^+ generations the amount of DNA incorporated had stabilized at about 18 or 19% of the original DNA adsorbed. This was close to the 15% of lysogenics expected from infection of Sh by P1 at 37°C under our experimental conditions.

Since the phage preparations used in the study of ^{32}P incorporation were not completely pure either from the point of view of ^{32}P adsorbable to the cell or in terms of DNA ^{32}P it was desirable to find some measure of phage DNA ^{32}P broken down in the adsorption period before incubation at 20°C. An upper limit to the amount of breakdown of DNA was given by the fraction of DNA adsorbed to cells compared to the fraction present in the original phage preparation. This was found to be 67% for a number of preparations, or a maximum of 33% breakdown of DNA P. However, this 33% figure would be expected to be reduced by contamination with non-infectious, non-adsorbable material in the phage preparation.

To test the release of ^{32}P from DNA following adsorption the ^{32}P activity per infectious unit was determined for various times after phage P1 adsorption. Assuming that all or a major portion of the breakdown does not occur within the first minute following adsorption, the data of Table III suggest that there was no appreciable loss of ^{32}P from DNA during the 12 minute adsorption period before submitting Sh to lysogenization at 20°C. In the case of Sh(P1) superinfected by labeled P1 there was considerable release of ^{32}P into the medium during adsorption.

TABLE III

THE EFFECT OF TIME OF ADSORPTION ON THE STABILITY OF PHAGE DNA ADSORBED BY *Shigella*

	Time of adsorption (min)	*Phage adsorption (per cent)*	*P1 adsorbed Total*	*Adsorption C.P.M.*	*C.P.M./10^7 adsorbed*
Shigella	1	35	$3.0 \cdot 10^7$	63	21
Shigella	3	73	$6.4 \cdot 10^7$	115	18
Shigella	5	89	$7.8 \cdot 10^7$	153	20
Shigella	12	95	$8.4 \cdot 10^7$	169	20
Sh(P1)	12	94	$8.5 \cdot 10^7$	99	12

Specificity of ^{32}P incorporation

Shigella cells which are already lysogenic for phage P1, Sh(P1), are immune to lysis by superinfection with P1 or weakly virulent mutants such as P1 *vir*; however, both of these phages are adsorbed by the cell and at rates which are not measurably different from the sensitive cell. In order to obtain some evidence on the question of a non-specific incorporation of DNA from the P1 phage by Sh the lysogenic Sh(P1) was infected with radioactive P1 and tested for the retention of ^{32}P-DNA after incubation at 20°C followed by growth at 37°C. The data are presented in Table IV, and indicate that the amount of DNA-P retained by the cells under conditions where the cell was not being lysogenized was greatly reduced. The value of ^{32}P retained by Sh (P1) after adsorption indicates a loss of ^{32}P after adsorption since a similar number of infectious P1 were adsorbed by both Sh and Sh(P1).

Since the number of prophage units per cell has been estimated to be small and of the order of one prophage per bacterial nucleus, it was feasible to determine the

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number of phage ^{32}P units incorporated during lysogenization using a multiplicity greater than one and, consequently, to obtain information as to whether the incorporation of phage ^{32}P involved a specific "site".

Table V lists the results of two experiments with two different phage preparations using multiplicities of 5 and 2. These results indicate that cells undergoing lysogenization under condition of multiple infection retained ^{32}P equivalent to only one phage per cell.

TABLE IV

A COMPARISON OF THE RETENTION OF ^{32}P DNA BY Sh AND Sh(Pl) AFTER INCUBATION AT 20° C AND GROWTH AT 37° C

Cells	*C.P.M. Adsorption by cells*	*Wash*	*C.P.M. NA before 20° incubation*	*C.P.M. After 20°*	*C.P.M. 4+ gen. at 37°*
Sh	200	38	178	173	165
Sh(Pl)	145	93	79	76	52
Sh	115	19	90	93	84
Sh(Pl)	87	34	56	48	34
Sh		38	169	119	91*
Sh(Pl)		56	99	45	11

* Low value for per cent ^{32}P retained due to larger fraction of non-lysogenics.

TABLE V

EFFECT OF MULTIPLICITY OF INFECTION ON RETENTION OF PHAGE ^{32}P DNA WITH LYSOGENIZATION

Multiplicity of infection	*C.P.M. Adsorption present before 20°*	*After 20° incubation*	*1 gen.*	*2+ gen.*	*4+ gen.*	*Fraction lysogenic cells*
M = 5	427	173	119	79	80*	1.0
M = 0.2	178	173		166	165	0.1
M = 2	20	16		9	8	
M = 0.2	21	20		17	18	

* 8+ generations.

Fraction of cells lysogenized by P1

When Sh cells undergo lysogenization with P1 at 20°C, the multiplication of these cells in L broth at 37°C is reduced[4] approximately one generation time compared to the uninfected control. Under conditions of low multiplicity of infection the fraction of cells lysogenic, $P(a)$, in a population should be:

$$P(a) = \frac{a}{a + 2b} \tag{1}$$

where, a = fraction infected, b = fraction not infected if there is no segregation of non-lysogenic cells from the infected cells. If there is segregation of non-lysogenic cells, then the expression will become:

$$P(a) = \frac{na}{(a + 2b)} \tag{2}$$

where, n is the fraction of lysogenic cells derived from originally infected bacteria, *i.e.*,

$$n = \frac{\text{Lysogenic cells}}{\text{Lysogenics} + \text{non-lysogenics from infected cells}}$$

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The data presented in Table VI fits equation 1 and not equation 2 showing that there was no appreciable segregation of two types of cells.

TABLE VI

FRACTION OF CELLS LYSOGENIC AS A FUNCTION OF THE MULTIPLICITY OF INFECTION

Multiplicity	*Fraction lysogenic*	*T7 resistant colonies**
0.125	0.07	
0.20	0.10	4/32 = 0.13
0.52	0.34	14/40 = 0.35
0.52	0.30	
5	1.0	30/30 = 1.0

Cells infected with P1 were incubated at 20° C for 2 h and then returned to 37° C. One sample was plated for bacterial count and another was shaken for 5 minutes with P1 *vir* (M = 10) and then plated.

* Colonies from the bacterial count plates were treated with phage T7.

DISCUSSION

In a recent monograph JACOB[5] has presented the important arguments relating to the concept of prophages as hereditary material located on a specific bacterial structure capable of giving rise to mature phage particles. Prophage may be considered as derived from the infection of a sensitive bacterium by a temperate phage under conditions where lysis does not occur. Phage multiplication is then postponed to that event which causes a dissociation of the prophage from the bacteria's normal hereditary mechanism.

When the phage P1 attacked the sensitive *Shigella dysenteria*, its DNA was "injected" into the cell. Since the phage P1 preparation labeled with ^{32}P was not free of non-infectious ^{32}P material, it was not possible to state unequivocably that all of the DNA was "injected". However, the value was certainly 70% or greater, for one phage preparation gave a value of 70% for ^{32}P adsorbed over ^{32}P total. Since the amount of ^{32}P adsorbed per unit phage adsorbed was constant from 1 to 12 minutes (see Table III) and the loss of ^{32}P from bacteria infected for 40 minutes at 27° was only 5%, it seems unlikely that an appreciable amount of ^{32}P was lost after adsorption.

In the process of lysogenization almost all of the phage ^{32}P adsorbed to bacteria destined to become lysogenic was conserved in lysogenic progeny. At 37°C where approximately 15% of the infected preparation becomes lysogenic only 18–19% of the phage ^{32}P DNA asdorbed was conserved and this for a period of more than 8 generations, whereas a population producing 85% lysogenics at 20° C conserved for a similar number of generations 81% of the originally adsorbed ^{32}P DNA. Experiments carried out for more than 11 generations have yielded similar results.

Treatment of an infected population of Sh with a weak virulent mutant, P1 *vir*, showed that phage ^{32}P DNA injected into the cell was conserved in lysogenic progeny. Phage ^{32}P could be solubilized by rupturing the infected cells remaining after lysogenezation; however, the ^{32}P of infected cells remaining after lysogenization was not subject to release by treatment with the virulent mutant P1 *vir* which lysed 60–70% of the sensitive cells present.

Although phage DNA was to a large measure incorporated into those cells destined to contain prophage, these studies gave no evidence as to whether or not the DNA was specifically involved in prophage formation. Three types of experiments were

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attempted to resolve the question of the specificity of DNA incorporation—the use of ultraviolet inactivated P1, superinfection of Sh (P1) with ^{32}P labeled P1, and using a multiplicity of infection of ^{32}P–P1 greater than 1. The use of ultraviolet inactivated P1 proved unsuccessful since most of the ^{32}P did not adsorb to the bacteria either because of non-adsorption of the phage or release of DNA from the phage during irradiation.

Superinfection of Sh(P1) by ^{32}P labeled P1 resulted in the retention of only 10 to 30% of the ^{32}P adsorbed while corresponding controls using Sh sensitives retained 55–85% of the adsorbed ^{32}P. The loss of ^{32}P seems analogous to the phenomenon of superinfection breakdown in T2[6] and may account at least in part for the immunity of the lysogenic cell to superinfecting phage. Since immunity applies primarily to homologous phage, it appears likely that the loss of superinfecting phage ^{32}P was due to a specific rejection of P1 phage DNA by the lysogenic bacterium.

Bacteria lysogenized with a multiplicity of infection greater than one retained only one ^{32}P phage equivalent per cell, which indicated that the incorporation of ^{32}P DNA was of a very specific nature, *i.e.* did not involve any large fraction of non-specific incorporation into host DNA. The high percentage of incorporation of phage DNA into lysogenizing cells, and the fact that only one phage unit of ^{32}P DNA was incorporated into a single cell when it was infected with multiplicities greater than one, does not mean that phage DNA is directly transformed into prophage. The possibility of direct transformation, however, has not been excluded. The question arises, then as to what significance can one attach to the incorporation of one phage ^{32}P DNA into Sh under multiple infection?

BERTANI[7], using *Shigella*, and JACOB[5], using *P. pyocanea*, have suggested that the number of prophages per cell was 2 to 4 or 5, or approximately one per "nucleus". This value of 2 to 5 prophages per cell was based on the ratio of superinfecting phage and carried phage from single cell burst experiments, and is dependent upon the argument that there is no selective advantage of carried phage over superinfecting phage. If one accepts these assumptions, then one is led to the conclusion that in *Shigella* the phage genetic material or its analogous prophage must replicate to provide one prophage per "nucleus" before cell division, or the phage particle itself must be multiparticulate with regard to prophage formation since there was no appreciable segregation of non-lysogenic bacteria from singly infected cells which became lysogenic.

In *Shigella* double lysogenics are relatively rare from either multiple infection or superinfection with homologous phage which indicates that there is probably only one "site" per cell from the point of view of prophage formation or cellular immunity, and when this "site" is occupied double lysogenics or replication of homologous phage are not likely to occur.

Since the equivalent in DNA of only one phage particle was incorporated into a single cell, one is led to postulate, therefore, from both a biochemical and genetic point of view that there is only one "site" per cell available for prophage formation. LIEB[8] has found that infected K12s gave rise to clones consisting of both lysogenic and non-lysogenic cells and suggested that this segregation was due to multiple sites interacting with prospective prophage. The possibility was also noted that phage particles had not yet reduced to prophage and were maintained as preprophage while cell division continued. A preprophage state, similar to that described by BERTANI[8] for *Shigella*, would, of course, be consistent with the idea of a single "site" for prophage formation.

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The injection of ^{32}P DNA from P1 into *Shigella* is in agreement with what is known of the injection of DNA by coli phages and suggests that DNA plays a similar role in the transfer of hereditary information from the phage to the host-phage complex. It is interesting to note that in the lytic cycle of growth Sh infected with ^{32}P labeled P1 released ^{32}P DNA over a period of 2 hours. This DNAw as non-sedimentable at 5,000 *g* for 30 minutes, was insoluble in 5% TCA, and amounted to 75% of the original ^{32}P DNA adsorbed to the cell. Since the lysogenic cells contain approximately 18% of the adsorbed ^{32}P, about 90% of the phage DNA involved in phage multiplication was released as acid insoluble DNA which was non-sedimentable at 5,000 *g*. It had not been possible to recover a large enough fraction of the phage to state that this large fraction of infecting phage DNA had been transferred to phage progeny, but the fraction of acid soluble ^{32}P released was considerably less than corresponding results with the T-even phages where ^{32}P transfers of the order of 40–50% have been reported. It seems possible, therefore, that in *Shigella dysenteriae* parent to progeny transfers of the order of 90% may occur.

SUMMARY

Shigella dysenteriae lysogenized by phage P1 under conditions of single infection was found to incorporate almost all the ^{32}P DNA of the phage into a stable bacterial DNA component. Bacteria infected with more than one phage retain DNA equivalent to only one phage per cell. Superinfection of lysogenic Sh(P1) by ^{32}P labeled phage P1 results in the retention by the cells of only 10–30% of the label. These results indicate that the DNA of the phage P1 is specifically involved in the formation of prophage material.

During the lytic cycle of growth parent to progeny transfer of ^{32}P of the order of 90% may occur.

RÉSUMÉ

Shigella dysenteriae rendu lysogène par le phage P1 dans des conditions d'infection unique incorpore la presque totalité du DNA marqué par ^{32}P du phage dans un constituant désoxyribonucléique bactérien stable. Les bactéries infectées par plusieurs phages retiennent la quantité de DNA équivalente à un seul phage par cellule. Une surinfection de Sh(P1) lysogène par le phage P1 marqué par ^{32}P s'accompagne d'une rétention par les cellules de 10 à 30% seulement de l'élément marqué. Ces résultats montrent que le DNA du phage P1 participe spécifiquement à la formation du matériel prophagique.

Au cours du cycle de croissance lytique, 90% du ^{32}P peut être transféré des parents aux descendants.

ZUSAMMENFASSUNG

Durch *Shigella dysenteriae*, welche mit Phagen P1 bei Einzelinfektionsbedingungen lysogenisiert wurde, wurde fast der gesamte DNS-^{32}P-Gehalt des Phagen in eine stabile Bakterien-DNS-Komponente einverleibt. Mit mehr als einem Phagen infizierte Bakterien halten DNS-Mengen zurück, welche nur einem Phagen pro Zelle entsprechen. Durch Superinfektion von lysogenem Sh(P1) mit ^{32}P-markiertem Phagen P1 stellt man fest, dass die Zellen nur 10–30% der markierten Substanz zurückhalten. Diese Ergebnisse deuten darauf hin, dass die DNS des Phagen P1 in der Bildung des Prophagenmaterials eine spezifische Rolle spielt.

Während des lytischen Wachstumszyklus kann ungefähr 90% des ^{32}P von einer Generation auf die andere transferiert werden.

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