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Role of *S* gene product of bacteriophage lambda in host cell lysis

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Abstract. Studies with the induced lysogens of λ S^+R^+, λS^-R^+, λS^+R^- and λS^-R^- phages have shown that while the *S* gene product is essential for the action of intracellular *R* gene product to release the periplasmic alkaline phosphatase in the presence of EDTA, the latter gene product can bring about this effect while acting on *Escherichia coli* cells from outside, in the absence of functional *S* gene product; chloroform, could help the intracellular *R* gene product in effecting bacterial lysis in the absence of *S* gene product. These result support the premise that the *S* gene product facilitates the *R* gene product in crossing the cytoplasmic membrane into the periplasmic space such that the latter can act on the peptidoglycan layer of the host cell thus causing both the release of alkaline phosphatase and cell lysis.

Keywords. Bacteriophage λ; λ*S* and *R* genes; periplasmic leakage; host cell lysis.

Introduction

The lytic growth of λ in *Escherichia coli* is terminated by lysis of the bacterial cell brought about by joint action of the products of three genes, *R* (Campbell, 1961), *S* (Harris *et al*., 1967) and *Rz*$^-$ (Young *et al*., 1979), of the phage. While the induced *R*$^-$ and *S*$^-$ lysogens are not lysed (Reader and Siminovitch, 1971b), induced *Rz*$^-$ lysogen is lysed if Mg^{2+} is absent in the medium (Young *et al*., 1979). The *R* gene product is a transglycosylase which degrades the peptidoglycan layer by attacking glycosidic linkages (Bienkowska-Szewczyk and Taylor, 1980), and the *Rz*$^-$ gene product has been suspected to act on the outer membrane to bring about lysis of the host cell (Young *et al*., 1979). Comparative studies made with *S*$^+$ and *S*$^-$ lysogens (Reader and Siminovitch, 1971a,b; Adhya *et al*., 1971; Mukherjee and Mandal, 1976) have shown that the respiratory uptake of oxygen, total DNA synthesis, transport function, β-galactosidase induction are all reduced after induction in the former lysogen relative to those in the latter; the induced *S*$^-$ lysogen also continues to accumulate intracellular phage beyond 60 min while the former does not. In the induced *S*$^+$ lysogen, there is evidence for phospholipid

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hydrolysis (Reader and Siminovitch, 1971b) which is considered to be secondary to the action of *S* gene product. Based on these observations, it has been suggested that the target of *S* gene product action is the cytoplasmic membrane, and that by this action, the membrane is altered in such a way that the *R* protein is now able to act on its substrate *viz.* the peptidoglycan layer in the cell wall (Reader and Siminovitch, 197lb). In this paper, we present evidence to show that in the absence of the *S* gene product, the *R* gene product does not reach the peptidoglycan layer to exhibit its lytic action.

Materials and methods

Media and solutions

Tryptone broth contained 1% bactotryptone and 0.5% NaCl. Normal saline contained 0.85% NaCl. ST buffer contained 0.5 M sucrose and 0.033 M Tris-HCl, pH 8.0.

Bacteria and bacteriophage strains

Escherichia coli 594 (Sm^r, su^-, lac^-, gal^-, F^-) was obtained from Dr M. Lieb, University of Southern California, School of Medicine, Los Angeles, USA. *E. Coli* 594-APC-12, an alkaline phosphatase constitutive strain, was prepared by mutagenizing 594 with ethyl methane sulphonate according to the procedure of Lin *et al*. (1960) and selecting the mutant by the procedure of Torriani *et al.* (1961). Phage strains λ*c*I857, λ*c*I857*Ssus*7, λ*c*I857*Rsus*5 and λ*c*I857*Ssus7Rsus*5 were obtained from Dr S. Adhya, National Institute of Health, Bethesda USA. These phage strains will be referred to in the text as λ*cItsS*$^+$*R*$^+$, λ*cItsS*$^-$*R*$^+$, λ*cIts*S$^+$*R*$^-$, and λ*cItsS*$^-$*R*$^-$ respectively.

Growth of bacteria

Bacteria were routinely grown in tryptone broth at 30°C on a shaker, and the growth was monitored turbidimetrically at 590 nm in a spectrophotometer.

Heat-induction of lysogens

Lysogens, grown at 30°C to about 0.3 $A_{590nm,}$ were induced at 43°C for 15 min and then incubated at 37°C for the desired period on a shaking water bath.

Assay of alkaline phosphatase

Alkaline phosphatase was assayed by the method of Garen and Levinthal (1960).

Unless otherwise stated, all the operations involved in the processing and different treatments of bacteria as described below were done at 0–4°C.

Measurement of the release of alkaline phosphatase by EDTA treatment

The bacteria were harvested, washed once with normal saline, and then suspended in ST buffer to give an $A_{590\ nm}$, of 4.0. To this suspension, EDTA was added to a final concentration of 0.5 mM, and the mixture was divided into two equal parts. One part was kept at 4°C for 15 min with occasional shaking and then centrifuged at 10,000 *g* for 10 min. The supernatant was called EDTA extract. To the other part,

lysozyme was added to a final concentration of 400 µg/ml, and the mixture was incubated at 20-25°C for 40 min and then centriruged at 10,000 *g* for 10 min. The supernatant was called lysozyme extract. The total periplasmic alkaline phosphatase was determined in the lysozyme extract and the EDTA-released enzyme, in the EDTA extract.

Measurement of alkaline phosphatase releasing activity

Preparation of substrate bacteria: E. coli 594-APC-12 was grown to about 0.6 $A_{590\ nm}$ and processed upto the EDTA-step as described above. The EDTA treated cells were then centriruged at 10,000 *g* for 10 min, and the pellet was suspended in ST buffer to give an $A_{590\ nm}$ of 4.0.

Preparation of crude extract: Lysogens were grown and induced as described. After 60 min of post-induction growth, cultures were chilled; cells were harvested and suspended in 0.1 volume of 0.01 M Tris-HCl buffer, pH 8.0. The cells were then broken by sonication in a Braunsonic 1510 Sonicator. The sonicates were centriruged at 3000 *g* for 10 min. the supernatants were dialyzed against the above buffer overnight, and the alkaline phosphatase releasing activity was then measured as follows.

To 1 ml portions of the substrate-cell suspension prepared as above were added different aliquots of the dialyzed extracts, and incubation was carried out at 4°C for 10 min. These were then immediately centriruged at 0-4°C. Alkaline phosphatase was then measured in the supematants. Alkaline phosphatase releasing activity was calculated from the linear region of the activity *vs* protein concentration curve.

Results

In gram negative bacteria like *E. coli*, the alkaline phosphatase and a few other hydrolytic enzymes are localized in the periplasm, the space in between the cytoplasmic membrane and the peptidoglycan layer (Heppel, 1971). The peptidoglycan layer is the most rigid layer which together with the outer membrane maintains cell shape and osmotic stability (Henning and Schwarz, 1973) and acts as a diffusion barrier (Leive, 1974). The periplasmic enzymes, under normal conditions, are not released into the medium unless the peptidoglycan layer is degraded by lysozyme added externally to EDTA-treated bacteria (Neu and Heppel, 1965); these enzymes are also released when EDTA-treated bacteria are given osmotic shock (Leive, 1965), and or when penicillin-induced spheroplasts of *E. coli* are treated with EDTA (Ananthaswamy, 1977).

Based on the release of periplasmic enzymes as a consequence of the structural damage of the peptidoglycan layer as mentioned above, we thought that the action of intracellular *R* gene product of λ on this layer could be monitored by measuring the release of periplasmic enzymes from the induced lysogen under suitable conditions. While working with a converted polylysogen of λ, we observed that this bacterium, unlike its nonlysogenic parent, released periplasmic enzymes on treatment with 0.1 mM EDTA in hypertonic medium (Mandal and Barik, 1979; Barik, 1981). So, to determine whether the availability of intracellular *R* gene

product for its action on the peptidoglycan layer is dependent on the functional *S* gene product, we tested the ability of different lysogens of λ collected at different times after induction to release periplasmic alkaline phosphatase on treatment with EDTA. The experiment was done as described under legend to figure 1. It can be

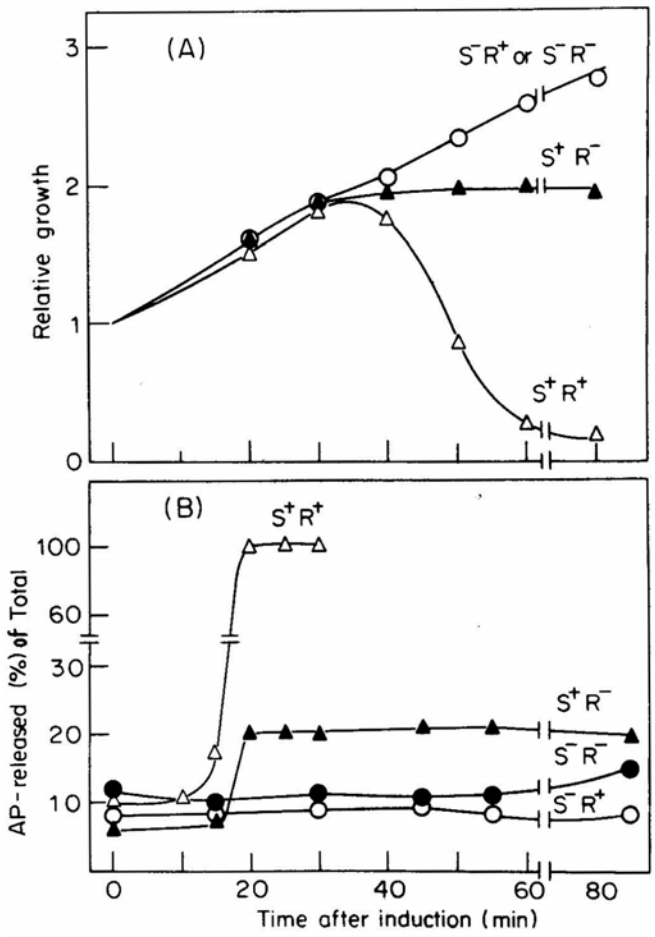

Figure 1. Growth of different lysogens after induction and the release of alkaline phosphatase by EDTA treatment of induced λ lysogens. Lysogens of λ*cItsS*$^{+}$*R*$^{+}$, λ*cItsS*$^{-}$*R*$^{+}$, λ*cItsS*$^{+}$*R*$^{-}$and λ*cItsS*$^{-}$*R*$^{-}$ in *E. Coli 594*-APC-12 were grown and induced as described in Methods. During post-induction growth, aliquots were taken out at indicated times and chilled. A portion from each aliquot was used to measure the growth and the residual portions were used to measure the release of alkaline phosphatase by EDTA treatment as described in Methods. A: Growth; the relative growth was measured by taking the ratio of $A_{590\ nm}$ of the induced culture at indicated time to that at zero time; the $A_{590\ nm}$ at zero time varied from 0.2 to 0.3 for different cultures (1 $A_{590\ nm}$ = 5×10^{8} cells/ml during log phase of growth for this strain). B: Release of alkaline phosphatase. Other details are in methods.

seen from figure 1B that the $S^{+}R^{+}$ lysogen started showing increased EDTA-induced periplasmic leakiness above the basal level at 15 min which reached 100% at 20 min from the start of induction. On the other hand, the induced $S^{-}R^{+}$

and S^-R^- lysogens did not show any such increase in this leakiness even upto 60 min of post-induction growth. In contrast with the latter two lysogens, induced S^+R^- lysogens showed a slight increase in the leakage which reached 20% at about 20 min and remained unchanged thereafter. An examination of the growth pattern (figure 1A) reveals that the induced S^+R^+ lysogen started lysing after about 35 min, while S^-R^+ and S^-R^- lysogens, under identical conditions, showed a steady increase in the turbidity beyond that period; but the induced S^+R^- lysogens neither lysed nor showed any increase in turbidity after 35 min.

To ensure that the alkaline phosphatase relasing activity was associated with the *R* gene product of λ and that this was present inside the induced S^-R^+ lysogen, the above activity was measured in the crude extracts prepared from different lysogens harvested at 60 min after induction. The results presented in table 1 show that the amount of alkaline phosphatase releasing activity was maximum in the extract of induced S^-R^+ lysogen; the extracts of induced S^+R^- and S^-R^- lysogens contained respectively 1.4% and 0.1% of the activity present in the S^-R^+ extract.

Table 1. Alkaline phosphatase releasing activity at 60 min after induction of different lysogens.

Lysogen	Alkaline phosphatase releasing activity[a]	
	(units/mg protein)[b]	%
594 ($\lambda cIts S^-R^-$)	2540.0	100.0
594 ($\lambda cIts S^+R^-$)	35.00	1.4
594 ($\lambda cIts S^-R^-$)	2.5	0.1

[a] Details are given in Methods.

[b] The unit of alkaline phosphatase releasing activity was defined as the amount of protein which released one unit of the enzyme from the substrate cells during 10 min of incubation at 4°. One unit of alkaline phosphatase was defined as the amount of enzyme which could liberate 1.0 absorption unit of *p*-nitrophenol (measured at 420 nm) from *p*-nitrophenyl phosphate under the condition of assay in 10 min. The extract from a noninduced lysogen was used in blank experiment.

These results (figure 1 and table 1) suggest the following: (i) The *R* product-independent alkaline phosphatase releasing activity in these lysogens is very negligible compared to the *R* dependent one; (ii) though the *R* product dependent alkaline phosphatasee releasing activity was present inside the induced S^- R^- lysogen at a very high concentration (table 1), it could not act on the peptidoglycan layer in the absence of the functional *S* gene product (figure 1); (iii) the same *R* product present in the extract of S^- R^- lysogen could act from outside on the peptidoglycan layer of EDTA treated bacteria effecting the release of periplasmic alkaline phosphatase in the absence of *S* gene product.

The failure of intracellular *R* product to act on the peptidoglycan layer in induced S^-R^+ lysogen (figure 1) suggests, therefore that the former could not reach its substrate which is situated outside the cytoplasmic membrane. So, the cytoplsamic membrane seems responsible for the above non accessibility of the intracellular *R* product to the peptidoglycan layer in the absence of *S* product. The cytoplasmic membrane has the lipid bilayer structure (Davson and Danielli, 1943), and any lipid solvent can disrupt this bilayer structure by solubilizing the lipid components (Dewey and Barr, 1970). If the role of *S* gene product in host cell lysis is to damage the cytoplasmic membrane through which the *R* product can move out into the periplasm to become accessible to the peptidoglycan layer, then chloroform, a lipid solvent, can be expected to bring about such a phenomenon in the absence of S protein. The results of the experiment presented in figure 2 clearly

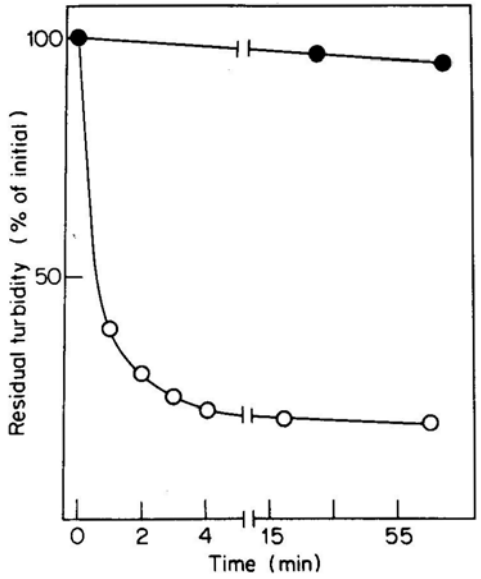

Figure 2. Effect of chloroform on nonlysogen and induced lysogens of different λ phages. Lysogens were grown and induced as described in methods. At 60 min after induction, cultures were chilled, and cells were harvested and then suspended in 0.05 M potassium phosphate buffer, pH 7.4 at a cell density of 3×10^9/ml. This suspension was then diluted 10-fold in the same buffer previously saturated with chloroform, and the decrease in turbidity with time was followed spectrophotometrically at 590 nm. O, S^-R^+ lysogen; ●, S^-R^- or S^+R^- lysogen or 594.

show that in the presence of chloroform, induced S^-R^+ cells were lysed rapidly while S^-R^- and S^+R^- cells were not. These results indicate that in the presence of chloroform, the *R* product is accessible to the peptidoglycan layer to exert its lytic action even in the absence of the *S* gene product. It may also be noted that chloroform, by itself, cannot effect lysis unless the functional *R* gene product is present inside the cell.

Discussion

As the peptidoglycan layer is structurally the most rigid component of the bacterial envelope, its degradation is a must for lysis of the cell. In order that the *R* gene

product of λ could reach this rigid layer while approaching from inside, it has to cross the cytoplasmic membrane.

We have shown that the intracellular action of *R* gene product on the peptidoglycan layer can be monitored by measuring the EDTA-induced release of periplasmic alkaline phosphatase from intact bacterial cells. It has been shown further that the intracellular *R* product cannot effect release of any alkaline phosphatase unless functional *S* gene product is also present (figure 1), though the former can bring about such a release while acting from outside, even in the absence of the latter (table 1). This directly supports the premise that the *S* gene product helps the *R* gene product in reaching the periplasmic space to act on the peptidoglycan layer.

With regard to the mechanism by which the *S* product helps the *R* product in crossing the cytoplasmic membrane nothing is known; from the known effects of *S* gene product on the properties and functions of the cytoplasmic membrane discussed earlier (see introduction), it appears possible that this protein, would somehow derange the structure of the above layer thus allowing the cytoplasmic *R* protein to enter the periplasm; this premise is supported by the fact that chloroform, by its lipid solubilising action on the membrane, performs the function of *S* gene in facilitating *R* gene product to become accessible to the peptidoglycan layer (figure 2).

In the absence of the *R* gene product, the *S* gene product also contributes towards the EDTA-induced leakage of alkaline phosphatase (figure 1 and table 1). The possibility that this is not due to the primary effect of *S* gene product is evident from the fact that after induction of λ lysogen, the intracellular level of *S* gene product along with those of several other late proteins of the phage increases with time (Reader and Siminovitch, 1971a), and this would have caused a progressive increase in the release of alkaline phosphatase provided that this protein bear any direct relationship with the release phenomenon. But the results in figure 1 show that this release from induced $S^{+}R^{-}$ lysogen reaches 20% at 20 min and remains unchanged thereafter. It is, therefore conceivable that the *S* protein dependent small release is secondary to the action of this protein on the cytoplasmic membrane. When the *S* protein damages or deranges the structure of cytoplasmic membrane, the membrane-bound lytic enzymes of bacterial origin (Daneo-Moore and Shockman, 1977) may be released in a soluble form and these can be expected to act either from inside (figure 1) or from outside (table 1), on the peptidoglycan layer thereby causing a small relase of alkaline phosphatase.

The mechanism of *S* protein action on the cytoplasmic membrane is not yet clear. It is however known that when the multiplication of the phage having *ts* mutation in one of its replication genes is arrested by continuous growth at 44°C, the cells which are not lysed even after 2 h, are now lysed on addition of chloroform (Mandal, unpublished result). Also, it was shown by others that the initiation of lysis is not solely determined by the intracellular level of *R* gene product but must await the intraceullular accumulation of *S* gene product (del Campillo-Campbell and Campbell, 1965; Protass and Korn, 1966). These observations suggest that the action of the above gene product is not catalytic unlike that of *R* gene product. The

stoichiometric mechanism of action of the *S* protein was also suggested by Reader and Siminovitch (1971a). Further studies are necessary to unravel the exact mechanism of its action on the cytoplasmic membrane.

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