Bacterial ice nucleation activity after T4 bacteriophage infection

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The changes in ice nucleation activity of transformed *Ina*⁺ *Escherichia coli* K12 after infection with T4D bacteriophage have been examined. Within 2 min after infection class A nucleation activity (measured at -4°C) fell about 100–1000-fold whilst class B (measured at -5.5°C) and class C (measured at -9°C) nucleation activities increased 50–100-fold and then rapidly decreased. These changes also occurred after interaction with T4D ghost particles or T4D 11⁻/12⁻ particles. Since ghost particles lack DNA and 11⁻/12⁻ particles lack short tail fibres, the T4D particles appear to be exerting their effect by the attachment of the phage long tail fibres to the cell. The changes were not influenced by the addition of chloramphenicol.

Introduction

The interaction of a virulent bacteriophage with an ice nucleating bacterium has been examined to learn more about both the physiology of the first steps in viral infection and the structure of the ice nucleating site. Little is known of the interaction of the naturally occurring *Pseudomonas syringae* phages with their ice nucleating host cells. The conversion of *Ina*⁻ (ice nucleation activity) *Escherichia coli* K12 to *Ina*⁺ cells by transformation with a plasmid containing the *inaZ* gene of *Pseudomonas syringae* placed a highly sensitive and easily assayed reporting structure in the membrane of a bacterium sensitive to *E. coli* T4 bacteriophage (Kozloff *et al.*, 1984). In addition, whilst new information has recently been gained about the ice nucleating structures and their attachment to the cell outer membrane (Govindarajan & Lindow, 1988; Kozloff *et al.*, 1991*a, b*; Turner *et al.*, 1990, 1991; Wolber *et al.*, 1986), the structure is clearly complex and a number of membrane-bound biosynthetic intermediates complicate its isolation. Earlier it was found that infection of *Ina*⁺ *Erwinia herbicola*, a second naturally occurring ice nucleating bacterium, by a small double-stranded DNA *E. herbicola* phage with a short tail structure and no long or short tail fibres resulted in the marked loss of ice nucleating activity only in the middle of the phage latent period (Kozloff *et al.*, 1984).

Methods

The main bacterial strain used was derived from *E. coli* K12 HB101. This strain was transformed with a pUC8 plasmid which contained an ampicillin resistance gene and about 15 kb of DNA which included the *inaZ* gene from *P. syringae* C9 (Kozloff *et al.*, 1984) and is referred to as *Ina*⁺ *E. coli* C91a. Wild-type *E. coli* T4D bacteriophage was found to form plaques with equal efficiency on the normal host, *E. coli* Be, and on *E. coli* C91a. For experiments on ice nucleation the bacteria were grown with aeration at 30°C in L-broth, supplemented with 1% (w/v) yeast extract, and with 5×10^{-4} M-inositol, 2×10^{-6} M-Mn²⁺ and 25 µg ampicillin ml⁻¹, and infected with five T4D particles per bacterium. Phage growth appeared normal; the latent period, determined after a dilution of 1:200, lasted about 35 min and the burst size was 17 phage particles per infected cell (Fig. 1). The culture was sampled at various times before and after infection by dilution into cold PBS (0.01 M-phosphate, pH 7.4, in 0.9% NaCl) plus 10^{-3} M-MgSO₄ in an ice bath. Further dilutions were carried out in an ice bath and ice nucleation activity was measured immediately using a standard precooled cold plate (Vali, 1971). Since the measurement of nucleation activity takes 15–20 min, the times are listed for when the cultures were sampled and chilled. The freezing nucleus units ml⁻¹ (FNU ml⁻¹) or per cell were calculated by standard methods (Turner *et al.*, 1991; Vali, 1971).

T4D ghosts were prepared by osmotic shock and purified in a D₂O gradient. The purified T4D 11⁻/12⁻ particles were prepared after growth of a T4D double amber mutant, N63/N69 (Kozloff *et al.*, 1977) on non-permissive host cells. The latter particles contain DNA but lack two important tail baseplate components, gP11 and gP12, the short tail fibres, (Kozloff *et al.*, 1977; Zorzopulos & Kozloff, 1978). Both preparations of defective particles were assayed by measuring their protein content.

Results and Discussion

There were immediate and marked changes in the ice nucleating activity upon the addition of T4 bacteriophage (Fig. 1). The changes in the actual FNU ml⁻¹ in

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Abbreviations: FNU, freezing nucleus units; PI, phosphatidylinositol.

Fig. 1. The effect of TD4 infection on the ice nucleation activity of *Ina*⁺ *E. coli* K12. The top of the figure shows the change in T4 infective centres at various times. In the same experiment samples were diluted in the cold and the FNU per cell determined for the three classes of nucleation activity. The arrows indicate the time of TD4 infection (4 particles per bacterium).

the infected culture are shown for the three different classes of nucleation activity characterized previously (Turner *et al.*, 1990). Class A activity (at -4°C) represents a relatively rare structure on the outside bacterial membrane of a small number of cells in a culture and it consists of aggregates of the ice protein each substituted with mannose and glucosamine residues and anchored to the cell membrane by phosphatidylinositol (PI) (Kozloff *et al.*, 1991a; Turner *et al.*, 1991). Class B activity (at -5.5°C) is due to a structure on the bacterial membrane occurring in more cells in the culture which consist of smaller aggregates of the ice protein each substituted with mannose and glucosamine residues but not anchored with PI. Class C activity (at -9.0°C) is due to a structure in almost every cell membrane which consists of still smaller aggregates of the ice protein substituted with a few mannose residues and it does not

Fig. 2. The effect of addition of either (a) T4D ghost particles (15 per bacterium) or (b) T4D 11⁻/12⁻ particles (8 per bacterium) on the ice nucleation activity of *Ina*⁺ *E. coli* K12. The FNU per cell values for the three different classes of nucleation activity were all normalized to that at the time of infection. The arrows at the top of the figure indicate the time of T4D infection.

contain glucosamine or PI. Immediately after infection class A activity fell rapidly and irreversibly, and by 7.5 min after infection it had decreased over 100-fold. Class B activity (at -5.5°C) on the other hand increased almost 100-fold immediately after infection and then decreased rapidly. Class C activity (at -9°C) increased 30-fold and then also decreased.

The portion of the phage structure or phage gene product responsible for these changes on the host cell membrane was examined by using either phage ghosts (DNA-free particles) or T4 11⁻/12⁻ particles. T4D ghosts have both short and long tail fibres while 11⁻/12⁻ particles still have their long tail fibres but have no short tail fibres (gP12). The results of using these defective particles to infect *Ina*⁺ *E. coli* is shown in Fig. 2. The FNU ml⁻¹ of the three different classes of activity were normalized by comparing them to the activity just before infection. Ghost particles added at a high multiplicity caused a rapid decrease in class A activity, and again a marked increase in class B and C activities. Class B and C activities then dropped and finally, after about 10 min, these activities increased to the level just before

Table 1. Release and stability of class A nucleation activity after T4D infection

Samples were removed and chilled at the times indicated after infection. The total ice nucleation activity at -4°C was measured and the sample centrifuged at high speed to sediment the cells. The supernatant fluid was then again assayed for nucleating activity. The bacterial pellet was resuspended and its activity again determined.

Time after infection (min)	Total activity (FNU ml ⁻¹)	Non-cell-bound activity (FNU ml ⁻¹)	Cell-bound activity (FNU ml ⁻¹)
0	2.6×10^3	0	2.6×10^3
2.5	2×10^2	7.9×10^2	0
5.0	2×10^2	3.2×10^2	0
8.0	1.5×10^2	4.6×10^1	1.0×10^2
12.0	9.5×10^1	4.6×10^1	5×10^1
16.0	3.8×10^2	4.6×10^1	3.3×10^2

infection. The T4D 11⁻/12⁻ particles, added at a lower multiplicity, gave a similar pattern of changes although in this case by 20 min all three classes of activity were above that at the time of infection. These results indicate that binding of the long tail fibres of T4D to the cell is sufficient to cause these changes in ice nucleation activity and neither the attachment of the short tail fibres nor DNA injection are necessary. Additionally, a similar experiment with live T4D particles was carried out in which chloramphenicol was added to a final concentration of $25 \mu\text{g ml}^{-1}$ at the time of infection to inhibit all protein synthesis and results similar to those in Figs 1 and 2 were obtained.

The possibility that T4 attachment caused the release of ice nucleating structures from the cell was examined. Cells were infected, samples removed and rapidly chilled, and a portion centrifuged in a microfuge to sediment the cells. Class A ice nucleation activity in the total culture and in the cell-free supernatant medium is shown in Table 1. In this experiment, class A activity of the culture decreased markedly in the first 12 min and then recovered somewhat. However, class A activity was found in the supernatant fluid up to 5 min after infection and was actually higher than that found for the total activity in the cultures. It appears that there was no measurable cell-bound class A nucleation activity 2–5 min after infection. Our interpretation is that T4 infection triggered the release of the structure responsible for class A activity, but, separated from the cells, this activity decayed, possibly by disaggregation, at a rate slower than the structures still attached to the cells. By 16 min the total culture and the cell-bound class A activity was beginning to recover. There was no significant release of class B or C activities from the cells

and no evidence that membrane vesicles were shed (Phelps *et al.*, 1986) after phage infection.

Attachment of phage long tail fibres to the cell outer membrane is known to cause a transient depolarization of the membranes (Kalasauskaitė *et al.*, 1983; Labedon & Letellier, 1981). We suggest that this depolarization activates a bacterial phospholipase in the cell membrane possibly by allowing an influx of Ca^{2+} or other ions. The phospholipase attacks the PI anchor of the class A structure releasing the structure into the medium where its activity rapidly decays. The phospholipase cannot directly attack the class B or C structures since neither structure has PI. However, the phospholipase could increase the ability of the class B and C structures to nucleate by removing other interfering phospholipids and thus favouring aggregation. With increasing lipase destruction of the cell membrane, the B and C structures would no longer function efficiently (Govindarajan & Lindow, 1988) and these activities would also decay. The effects of initial ghost attachment (as well as of 11⁻/12⁻ particles) are known to be transient and a later recovery of cell membrane integrity would be in accord with the later recoveries of class A, B and C ice nucleation activities.

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