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Release of Respiratory Control in *Escherichia coli* After Bacteriophage Adsorption: Process Independent of DNA Injection

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Adsorption of phages T4, T5, and BF23 to previously starved *Escherichia coli* cells triggered the immediate release of respiratory control. A similar stimulation of respiration was induced after T4 ghost attachment, showing that this process was independent of the mechanism of DNA injection. Rather, this change in the respiratory rate was related to the transient depolarization of the cytoplasmic membrane also induced after phage and ghost adsorption. Both processes were suppressed by addition of ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid in the case of T4 (phage and ghosts) but not of T5 and BF23. The increase in respiratory rate observed after phage adsorption was of a magnitude similar to that induced by protonophores. Since other treatments that depolarize the membrane without a massive proton influx did not increase the rate of respiration of starved bacteria with the same efficiency, these results suggest that phage adsorption induced an entry of protons into the cell cytoplasm.

Attachment of a coliphage to its receptor in the host cell outer membrane induces two processes. On the one hand, phages T4 and T5 trigger (after irreversible adsorption) the same immediate depolarization of the cytoplasmic membrane (11), suggesting that a signal must be transmitted between the outer and inner membranes. This signal transmission is dependent on envelope-bound calcium in the case of T4 but not of T5 (12). Indeed, addition of ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) specifically prevented the membrane potential changes induced by T4. On the other hand, phage adsorption induces a set of conformational changes of the baseplate, leading to DNA ejection (5). Then, by a mechanism still not understood, this ejected DNA is rapidly taken up by the cell. This process of DNA injection has recently been studied from an energetic point of view. Filali Maltouf and Labedan (2) have demonstrated that host cell metabolic energy is not necessary for T5 DNA penetration, suggesting that DNA enters the cytoplasm by simple diffusion. Kalasauskaite and Grinius (6) and Labedan and Goldberg (8) found that injection of T4 DNA needed the presence of the proton motive force ($\Delta\tilde{\mu}_{H^+}$). Furthermore, Labedan and Goldberg (8) and Labedan *et al.* (9) demonstrated that T4 DNA injection was possible only above a membrane potential ($\Delta\psi$) threshold of about −90 mV, suggesting that $\Delta\psi$ has only a regulatory role and would not be the motive force for DNA transport. This last point was recently demonstrated by Furukawa et al. (3), who showed that artificially contracted T4 phage normally inject their DNA in the cytoplasm of *Escherichia coli* spheroplasts in the absence of proton motive force. However, there is still controversy, since the Grinius group (7) recently claimed that phage T4 DNA enters via a proton symport. They have demonstrated (7) that T4 can induce a release of respiratory control of starved bacteria.

In the present paper, we show that this change in respiration rate was not due to the process of DNA injection since it occurred with T4 ghosts as well as with T5. Furthermore, we demonstrate that this release of respiratory control was correlated to the transient depolarization induced after adsorption of T4, T5, and BF23 since it was inhibited by EGTA in the case of T4 but not of T5 and BF23. We suggest that both the stimulation of respiration of starved cells and the membrane potential changes may be due to the entry of protons in the cell cytoplasm.

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MATERIALS AND METHODS

Strains. *E. coli* B was used to adsorb phages T4 and BF23. *E. coli* F was the host strain for phage T5. B and T4 were obtained from E. Goldberg. F and T5 originated from the Lanni laboratory. BF23 was from A. Pugsley. Phage stocks were prepared and purified as described previously (8, 10). T4 ghosts were prepared by addition of 3 M sodium acetate and rapid dilution into 100 volumes of cold distilled water (17). The titer of the ghost suspension was determined as described before (17), and the absence of viable particles was checked by plating.

Chemicals. Cyanine dye (3,3'-dimethylindodicarbocyanine iodide) (13) was a gift from A. S. Waggoner. Valinomycin, 2,4-dinitrophenol (DNP), and carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP) were obtained from Sigma Chemical Co., St. Louis, Mo. [^{14}C]TPP$^+$ (tetraphenyl phosphonium) was from the CEA, Saclay, France.

Growth and starvation of bacteria. Bacteria were grown to 5×10^8 cells per ml in minimal medium (M9) supplemented with 1% sodium succinate. EDTA-treated cells were prepared as described previously (11). Cells were suspended in 10 mM Tris-hydrochloride (pH 7.4)–100 mM NaCl at a concentration of 3×10^9 cells per ml and starved by vigorous shaking for at least 10 min at 37°C.

Oxygen consumption. A 1.5-ml volume of starved bacteria was placed into a closed cell thermostated at 23°C. The suspension was saturated by bubbling oxygen. Oxygen consumption was then measured with a Clark electrode by using a Gilson oxygraph.

Fluorescence measurements. Cyanine dye (0.03 μM) was added to a cuvette containing 1.45 ml of 10 mM Tris-hydrochloride (pH 7.4)–100 mM NaCl supplemented with 0.4% glycerol. EDTA-treated *E. coli* B (5 μl) was added to a final concentration of 2×10^8 cells per ml. When a steady level of fluorescence was attained, phage were added with rapid mixing. Fluorescence was measured at 640 nm (excitation

wavelength, 600 nm). The sample chamber was maintained at 37°C. The fluorescence signal was calibrated in terms of $\Delta\psi$ as described previously (4).

Measurement of membrane potential. Membrane potential was determined in EDTA-treated cells from the accumulation of either $^{86}Rb^+$ in the presence of valinomycin or [^{14}C]TPP$^+$ by using the filtration technique previously described (4).

RESULTS

Release of respiratory control after phage adsorption. Growing *E. coli* K-12 cells do not exhibit respiratory control (1); their respiratory rate is not increased upon addition of uncouplers. Nevertheless, it has been shown (1) that incubation in a buffer deprived of an oxidizable carbon source leads to a decrease of the rate of respiration, which is sufficient to establish respiratory control. Indeed, under these conditions of starvation, the rate of respiration is regulated in such a manner that the rate of proton extrusion by the respiratory chain balances the rate of proton leak back across the cytoplasmic membrane. Addition of protonophores (DNP and CCCP), which increase the internal proton concentration, leads immediately to an increase in respiration (1). We found that the respiratory control ratio, i.e., the ratios of the rate of respiration in the presence of protonophores and endogenous respiratory rate were 3.5 and 4.4 for *E. coli* B and F, respectively. When these cells were treated by EDTA before the starvation procedure, they displayed a lower respiratory control ratio (1.8 for *E. coli* B).

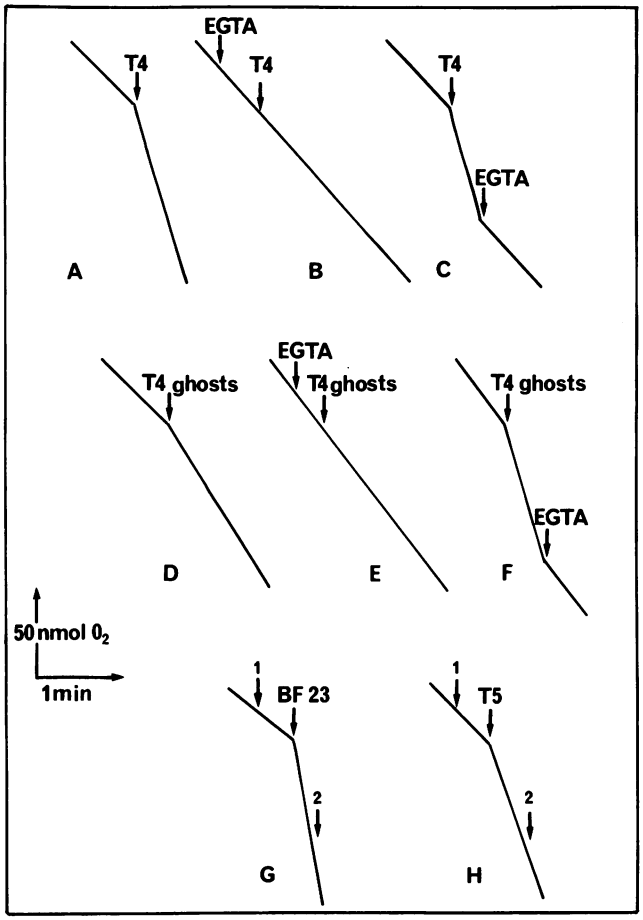

FIG. 1. Stimulation of the respiration of starved *E. coli* cells after phage adsorption. Bacteria were starved as described in the text. (A to F) *E. coli* B was infected either by T4 phage (A to C) at a multiplicity of infection of 20 or T4 ghosts (D to F) at a multiplicity of killing of 6. EGTA (400 μM) was added either before (B and E) or after (C and F) phage adsorption. (G to H) Phage BF23 (multiplicity of infection, 10) and T5 (multiplicity of infection, 13) were respectively added to *E. coli* B and F. EGTA (400 μM) was added either before (1) or after (2) phage adsorption.

TABLE 1. Effect of the multiplicity of infection on T4-induced increase of respiration rate

Multiplicity	Respiratory control ratio[a]	$\Delta\psi$ (mV)
0	1	150
3	1.5	140
4	1.7	130
5	2.6	120
20	3.5	110

[a] The respiratory control ratio is the ratio of the rate of respiration in the presence of phage and endogenous respiratory rate in starved bacteria.

Addition of different phages to previously starved host cells induced an increase of the rate of respiration (Fig. 1). Addition of phage to unstarved bacteria had no effect on the respiration rate (data not shown). The increase in respiration rate was dependent on the multiplicity of infection. Table 1 shows that the respiratory control ratio increased with increasing MOI. Moreover, this increase of respiratory control ratio correlated with the previously observed (11) decrease of $\Delta\psi$ obtained in unstarved but EDTA-treated cells (Table 1).

Upon osmotic shock, T4 phage lose their DNA and internal proteins, but these ghosts are still able to adsorb to and kill bacteria (17). T4 ghosts induced the same release of respiratory control (Fig. 1D) as do T4 phage, with the same dependence of the respiratory control ratio on the MOI (data not shown). This result showed that this stimulation of respiration observed after phage adsorption was independent of the presence of phage DNA and internal proteins and was thus not related to the DNA injection process.

We previously showed (12) that incubation of EDTA-treated cells in the presence of EGTA prevents the T4-induced transient depolarization of the host cell membrane. Moreover, this T4-induced depolarization could be stopped by addition of EGTA at any moment during the depolarization (12). Figure 2A shows that the same results were obtained using T4 ghosts. Figure 1B and E shows that addition of 400 μM EGTA (which by itself has no effect on the rate of respiration) also prevented the increase of respiration of starved cells normally observed in the presence of either phage T4 or T4 ghosts. Moreover, addition of EGTA after that of phages or ghosts induced a decrease of the stimulated respiratory rate which returned to its initial value (Fig. 1C and F).

Addition of EGTA before or after phage T5 adsorption had no effect on the increase of the respiratory rate induced by the phage (Fig. 1H). This result was expected since we previously observed that, contrary to T4, T5-induced transient depolarization normally occurs in the presence of EGTA (12).

Phage BF23, a phage very closely related to T5 but which adsorbs to another outer membrane protein, the BtuB protein (14), induced the same transient depolarization of the host cytoplasmic membrane as T4 and T5 (Fig. 2B). Figure 2B further shows that this depolarization was not prevented by EGTA. Figure 1G shows that a release of the respiratory

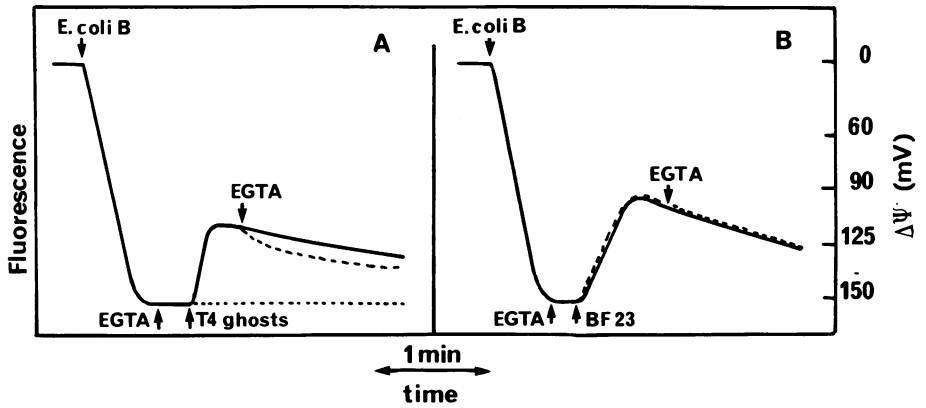

FIG. 2. Membrane potential changes induced after addition of (A) T4 ghosts and (B) BF23 phages to EDTA-treated *E. coli* B. The fluorescence of cyanine dye was measured as described in the text. The fluorescence intensity was calibrated in terms of $\Delta\psi$ as described in the text. EGTA (1 mM) was added (– – – –) either before or after phage adsorption. T4 ghosts and BF23 phages were added at the respective multiplicities of 8 and 20.

control was also observed upon infection of starved *E. coli* B with BF23 and that this change of respiratory rate normally occurred in the presence of EGTA.

Relation between release of respiratory control and decrease of the electrochemical gradient of protons ($\Delta\tilde{\mu}_{H^+}$). Since phage adsorption induces both a transient depolarization and a release of respiratory control, it is tempting to attribute both effects to an entry of protons. Nevertheless, ions other than protons may be implicated. We incubated EDTA-treated cells, which were previously starved, in buffer (pH 7.8) containing increasing concentrations of potassium in the presence of its ionophore valinomycin. Under these conditions, the membrane potential $\Delta\psi$, the only component of $\Delta\tilde{\mu}_{H^+}$ present at this external pH (18), is gradually decreased. Table 2 shows that a release of respiratory control was observed when $\Delta\psi$ was decreased, but only when its value was lowered below −110 mV, i.e., under the maximum level of the phage-induced depolarization we previously observed. However, this release of respiratory control was not maximum since addition of 6 μM CCCP after valinomycin further increased the respiratory rate. Moreover, a complete depolarization of the EDTA-treated cells obtained by adding either 100 mM potassium in the presence of valinomycin (Table 2) or 2 mM TPP^+ (data not shown) induced only a partial increase of the respiration rate which could be amplified again by a further addition of CCCP, showing that the entry of protons by protonophores was far more efficient.

TABLE 2. Respiratory control ratios for different levels of $\Delta\psi$ in EDTA-treated *E. coli* B cells

K^+ (mM)[a]	Respiratory control ratio with:		$\Delta\psi$ (mV)	
	Valinomycin[b]	Valinomycin and CCCP[c]	Rb^+	TPP^+
0	1	1.8	150	150
2	1	1.7	110	120
10	1.35	2.0	70	100
20	1.57	1.7	35	70
50	1.33	1.6	20	50
100	1.35	2.05	0	15

[a] $\Delta\psi$ was varied by changing the external concentration of K^+ in the presence of valinomycin.

[b] The respiratory control ratio with valinomycin is defined as the ratio of respiration rate in the presence of valinomycin and the endogenous rate of respiration.

[c] The respiratory control ratio with valinomycin and CCCP is defined as the ratio of respiration rate after addition of valinomycin and further addition of CCCP and the endogenous rate of respiration.

DISCUSSION

We showed in this paper that addition of three different phages, T4, T5, and BF23, induced an immediate release of respiratory control of starved *E. coli* cells. This result allowed us to draw conclusions about two different aspects related to the first steps of phage infection, i.e., the process of DNA injection (5) and the transient depolarization induced after phage adsorption (11).

Release of respiratory control is not related to DNA injection. Kalasauskaitė et al. (7) recently claimed that the release of respiratory control of starved host cells induced by T4 infection is a good indication that penetration of phage DNA is dependent on the proton motive force. Our current results showed that this conclusion is not warranted. Indeed, T4 ghosts also induced an acceleration of the respiration rate of starved *E. coli* cells similar to that triggered by intact phages. Furthermore, T5 phage, which does not use metabolic energy to inject its DNA (2), triggered a release of respiratory control. Addition of EGTA, which has no effect on T4 DNA injection (12), prevented the changes in respiratory rate induced by either T4 phage or T4 ghosts. These results reinforce the preceding ones (3, 8, 9) which showed that $\Delta\psi$ is not the driving force for T4 DNA entry into the cytoplasm.

Release of respiratory control is related to phage-induced transient depolarization. Results presented above correlated well with our previous observations about the membrane potential changes induced after phage adsorption (11). Both release of respiratory control and the transient depolarization were induced by phages which differed in the nature of their receptors and in their mode of infection. Occurrence of these two processes was independent of the presence of DNA and internal proteins and of DNA expression since they happened with T4 ghosts as well. Furthermore, the release of respiratory control was dependent on envelope-bound calcium in the case of T4 but neither T5 nor BF23. This last result confirms our previous working model (12) that phages which adsorb to lipopolysaccharide (T4) have a calcium-dependent transmission of the signal calling for membrane depolarization and that phages which adsorb to outer membrane proteins (T5 or BF23) have a direct, calcium-independent signal transmission.

Does phage adsorption trigger entry of protons in the host cell cytoplasm? The exact relationship between the rate of respiration and the proton motive force (and consequently the mechanism of respiratory control) is far from being clearly understood (16). Nevertheless, it is generally observed that increasing the proton conductance leads to a release of the respiratory control which, in most cases, is correlated with a decrease of $\Delta\tilde{\mu}_{H^+}$ (15). This dual effect, which is generally observed in the presence of protonophores, was also induced after adsorption of phages T4, T5, and BF23 to starved host cells. This led us to propose that phage adsorption would trigger an entry of protons into the cell cytoplasm. This hypothesis was supported by the following results. Firstly, addition of either high (up to 100 mM) concentrations of external K^+ in the presence of valinomycin or of high (2 mM) concentrations of TPP^+ are two established ways to strongly diminish or totally collapse $\Delta\psi$ (4). We showed that both treatments induced a release of respiratory control, but the small increase of the rate of respiration could be in each case further increased by addition of CCCP. Secondly, Burstein et al. (1) showed that the concentration of CCCP giving an optimal release of respiratory control is 6 μM. In our hands, this CCCP concentration diminished $\Delta\psi$ of EDTA-treated cells from −160 to −110 mV and increased the rate of respiration of starved cells by a factor of four. On the other hand, addition of phages at multiplicities of infection decreasing the $\Delta\psi$ of EDTA-treated cells to a value close to that obtained with CCCP gave a respiratory control ratio similar to that of CCCP (Table 1). Thirdly, this phage-induced effect occurred in a medium containing less than 1 μM potassium. Taken together, these different results argue in favor of the hypothesis that adsorption of a phage to its outer membrane receptor induces the emission of a signal which after transmission to the cytoplasmic membrane calls for an entry of protons in the cell cytoplasm leading both to a depolarization of the inner membrane and a stimulation of the respiration of starved cells.

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