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Changes in Host Cell Energetics in Response to Bacteriophage PRD1 DNA Entry

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Double-stranded DNA bacteriophage PRD1 infects a variety of gram-negative bacteria harboring an IncP-type conjugative plasmid. The plasmid codes for the DNA transfer phage receptor complex in the cell envelope. Our goal was, by using a collection of mutant phage particles for which the variables are the DNA content and/or the presence of the receptor-binding protein, to obtain information on the energy requirements for DNA entry as well as on alterations in the cellular energetics taking place during the first stages of infection. We studied the fluxes of tetraphenylphosphonium (TPP^+), phenyldicarbaundecaborane (PCB^-), and K^+ ions as well as ATP through the envelope of *Salmonella typhimurium* cells. The final level of the membrane voltage ($\Delta\Psi$) indicator TPP^+ accumulated by the infected cells exceeds the initial level before the infection. Besides the effects on TPP^+ accumulation, PRD1 induces the leakage of ATP and K^+ from the cytosol. All these events were induced only by DNA-containing infectious particles and were cellular ATP and $\Delta\Psi$ dependent. PRD1-caused changes in $\Delta\Psi$ and in PCB^- binding differ considerably from those observed in other bacteriophage infections studied. These results are in accordance with the presence of a specific channel engaged in phage PRD1 DNA transport.

PRD1 is a double-stranded DNA bacterial virus. The virion is composed of an outer protein coat which surrounds the phage membrane. The linear double-stranded DNA is located inside this membrane vesicle (13). Interest has been directed to this virus system due to its internal membrane and the protein-primed replication mechanism of the genome (for a recent review see reference 4). PRD1 is a broad-host-range virus infecting gram-negative bacteria harboring a conjugative P, N, or W incompatibility type plasmid (44). Plasmid transfer by bacterial conjugation requires DNA transfer and replication (Dtr) functions and those required for mating pair formation (Mpf). The Mpf system is located in the cellular envelope and is responsible for DNA transport from the donor cell to the recipient (25). Functions responsible for conjugative transfer are encoded by two distinct regions in the plasmid known as Tra1 and Tra2 (15, 36, 54). During conjugation the DNA-TraC complex is thought to be transported through a pore at the mating bridge between the donor and the recipient cells (47, 55). The proposed formation of a channel or a pore depends on gene products of the Mpf system. The plasmid functions in the PRD1 life cycle are restricted to receptor formation and DNA entry (18, 33, 36, 40). The phage receptor complex and part of the DNA transport machinery consist of the same Mpf components (25, 33). The most commonly used hosts for PRD1 are *Escherichia coli* and *Salmonella typhimurium* cells carrying IncP-type plasmids or their derivatives (3, 44).

Very little is known about the mechanisms by which DNA crosses the cell envelope in transformation, conjugation, or virus infection. It has been shown that the penetration of DNA into the cell during phage infections depends on the presence of proton motive force (Δp) on the plasma membrane (PM) (26, 29, 30, 35). For example, phage T4 DNA injection into the bacterial cytosol is possible only if the membrane voltage ($\Delta\Psi$) exceeds a threshold of about 90 mV (30, 35). Phage DNA transport through the bacterial envelope is a rapid and highly efficient process, and DNA translocation-associated changes in PM permeability suggest that the DNA penetrates the bacterial envelope through a channel. It has been shown (1, 9–11, 21, 31) that ion-permeable channels are formed in the PM during the infection of *E. coli* cells by bacteriophages T1, T4, T5, and λ. However, an open nonselective channel would cause the dissipation of $\Delta\Psi$ and Δp very effectively. This again would hamper many energy-transducing and energy-dependent processes, such as ATP synthesis and active transport of ions and essential substrates. It is known (53) that the addition of substances which dissipate Δp abolishes the development of viruses.

Due to the stringent coupling between H^+ and other ion gradients and $\Delta\Psi$, the movement of ions becomes an indicator of the energy state of the PM. The accumulation of lipophilic cations and anions is used to assay $\Delta\Psi$ in different biological systems. Thus, tetraphenylphosphonium (TPP^+) or phenyldicarbaundecaborane (PCB^-) can be used for the estimation of $\Delta\Psi$ in systems that have a negative or a positive membrane voltage, respectively (38). The outer membranes (OMs) of gram-negative bacteria, such as *S. typhimurium*, are not permeable to lipophilic compounds. If the phage infection increases the permeability of the OM, the uptake of lipophilic cations and the effects of ionophoric antibiotics on K^+ accumulation should be observed. It has also been shown that infection with channel-forming phage T4 very effectively induces PCB^- binding to cellular membranes (16).

We have studied the effects of bacteriophage PRD1 adsorption and DNA entry on the magnitudes of host cell $\Delta\Psi$ and K^+ gradients by using ion-selective electrodes and a potentiometric monitoring system. It is shown that the postinfection level of TPP^+ accumulation exceeds the initial one both in the case of Tris-EDTA-treated and nontreated cells. Besides the effects on TPP^+ accumulation, PRD1 induces a considerable leakage

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TABLE 1. Bacterial strains, phage, and plasmids

Strain, phage, or plasmid	Genotype and/or phenotype	Reference or origin
S. typhimurium LT2 strains		
SL5676	ΔH2 H1-i::Tn*10* (Tc^s) *non rev*	B. A. D. Stocker[a]
DS88	SL5676 Sm^r (pLM2)	6
PSA	Suppressor for *sus1*	42
DB7154	Suppressor for *sus170*	42
Phage PRD1	wt	44
	sus1 (DNA packaging defect)	42
	sus170 (adsorption defect)	42
Plasmid pLM2 (RP1 derivative)	Ap^r (Am) Km^r Tc^r(Am)	41

[a] Stanford University, Palo Alto, Calif.

of ATP and K^+ from the cytosol. All these events are induced only by DNA-containing infectious particles.

MATERIALS AND METHODS

Bacteria, phage, plasmids, and growth conditions. The bacteria, phage, and plasmids used in this study are listed in Table 1. The strains were grown in Luria-Bertani medium (48) at 37°C with aeration. The final cell batch was grown from a diluted (2×10^8 cells/ml) overnight culture. Cells were harvested at 1.1×10^9 cells/ml. Strain DS88 was used for propagation of PRD1 wild-type (wt) virus and suppressor-containing strain PSA(pLM2) or DB7154(pLM2) for nonsense mutants *sus1*, defective in DNA packaging, and *sus170*, defective in receptor-binding protein, respectively. The titers of the nonsense mutant phage lysates were determined both on DS88 cells and cells containing the suppressor mutation. With both of the mutants the titer on the suppressor strain was at least six orders of magnitude higher than that on the DS88 strain. The wt and mutant virus particles were produced on DS88 cells, concentrated, and purified in 5 to 20% sucrose gradients (20 mM Tris-HCl, pH 7.4) as previously described (5, 7). Both wt and *sus170* infections lead to the production of DNA-filled and empty particles, whereas *sus1* infection leads to the production of empty particles only. Filled and empty wt particles but only DNA-filled *sus170* particles were used in our experiments. The empty and filled particles differ in their sedimentation velocities and can be collected separately from rate zonal sucrose gradients (7). The virus-containing zones were removed from the gradients, and the particles were collected by differential centrifugation (Sorval T647.5 rotor, 32,000 rpm, 2.5 h). The pellets were resuspended in 20 mM Tris-HCl, pH 7.4, stored on ice, and used within 10 days. No detectable loss in the infectivity of the wt particles was detected during this time period. Phage adsorption experiments were performed as previously described (17, 33). Protein concentration measurements were carried out by the Bradford assay (12), with bovine serum albumin as a standard.

Measurements of ion fluxes and determination of membrane voltage. For the ion flux experiments the exponentially growing cells were concentrated 100-fold by centrifugation and resuspension in 100 mM Tris-HCl, pH 8.0. The usage of TPP^+ for membrane voltage measurements requires OM permeabilization, which was achieved by 100 mM Tris-HCl–10 mM EDTA treatment at 37°C for 10 min (30). This cell suspension was kept on ice until used (maximally 4 h). Fifty to seventy microliters of the suspension was added to an appropriate buffer in a 5-ml reaction vessel. The vessel was thermostated, and the cell suspension was aerated by magnetic stirring. The concentrations of K^+, TPP^+, and PCB^- ions in the medium were monitored by ion-selective electrodes, connected to Orion 520A pH/ISE meters. The K^+-selective electrode was from Orion Research, Inc. (model 93-19). Ag-AgCl reference electrodes (Orion Research Inc.; model 9001 or 9002) were indirectly connected to the measuring vessels through an agar salt bridge. The construction and characteristics of TPP^+- and PCB^--selective electrodes have been previously described (22, 23). Tetraphenylphosphonium chloride was purchased from Aldrich, and potassium phenyldicarbaundecaborane was a generous gift from L. I. Zakharkin (A. N. Nesmeyanov Organoelement Compounds Institute, Moscow, Russia). Polymyxin B (PMB) sulfate (7730 U of PMB base/mg), gramicidin D (GD), and nigericin (NG) were purchased from Sigma.

The K^+ content of the cells and the nonspecific binding of TPP^+ were measured by adding PMB (150 μg/ml) and GD (4 μg/ml) to the reaction vessel. The internal K^+ concentration was calculated from the external one, assuming that 200 Klett units (A_{540}) correspond to 10^9 cells/ml, 2×10^9 cells correspond to 1 mg of dry mass, and the intracellular water volume of *S. typhimurium* is equal to that of *E. coli* (1.1 ml/g of dry mass [10]). The $\Delta\Psi$ values were calculated from a modified Nernst equation, as described previously (30):

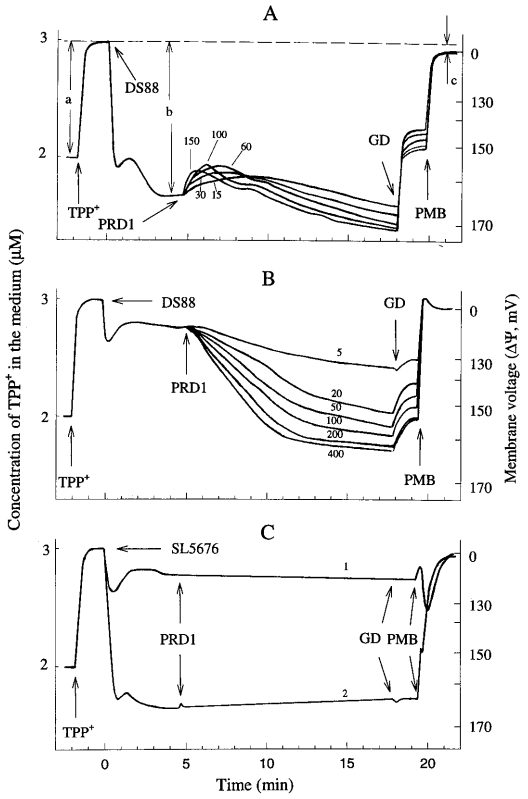

FIG. 1. Effects of phage PRD1 on TPP^+ uptake by *S. typhimurium* cells. The experiments were carried out at 37°C in 50 mM Tris-HCl, pH 8.0, initially containing 2 μM TPP^+. For the calibration TPP^+ was added to obtain a 3 μM concentration. Tris-EDTA-treated (A; curve 2 in panel C) or nontreated (B; curve 1 in panel C) *S. typhimurium* cells (3×10^9 cells/ml). Phage PRD1 was added to obtain an MOI of 150 (C) or as indicated by the numbers on the figure (A and B). GD was added to the final concentration of 4 μg/ml, and PMB was added to a final concentration of 120 μg/ml. (A and B) Experiments with PRD1-sensitive DS88 cells; (C) experiment with PRD1-resistant SL5676 cells. For the definition of *a*, *b*, and *c* see Materials and Methods (Measurements of ion fluxes and determination of membrane voltage).

$$\Delta\Psi = -\frac{RT}{F}\ln\frac{V_{out}}{v}(1.5^{(b-c)/a} - 1)$$

where V_{out} is the volume of bacterial suspension studied (5.1 ml); v is the summarized intracellular water volume (8.4 μl in these experiments); 1.5 is the increase in TPP^+ concentration as a result of calibration; a, b, and c are differences in the potential of the TPP^+-selective electrode between different time points, as shown in Fig. 1A; R is the gas constant; T is the absolute temperature; and F is the Faraday constant. The measurements of ion fluxes were carried out simultaneously in three reaction vessels, and the typical registration course of ion movements is presented in the figures.

Determination of ATP content. The ATP content of the cells was determined by the luciferin-luciferase method (BioOrbit). The cells were incubated as described for the ion flux and membrane voltage measurements. A 50-μl sample of the suspension was withdrawn and mixed with 750 μl of 50 mM Tris-HCl, pH 8.0, and 200 μl of the ATP monitoring reagent (BioOrbit). The amount of light produced was measured with a 1250 Luminometer (LKB-Wallac). The amount of ATP present in the incubation buffer was calculated from a calibration curve. The total ATP content of the cells was measured by adding 500 μg of PMB per ml to the bacterial suspension.

RESULTS

PRD1 does not depolarize the PM. When infectious PRD1 particles interact with *S. typhimurium* DS88 cells permeabilized by Tris-EDTA treatment, two stages in TPP^+ accumulation can be observed (Fig. 1A): (i) an initial release of TPP^+ accumulated before infection, followed by (ii) the uptake of an

additional amount of TPP^+. The release of TPP^+ begins immediately after the addition of phage particles. At 37°C the accumulation phase becomes detectable at the end of the first minute of infection and continues for 10 to 12 min. The final level of TPP^+ in infected cells exceeds the initial one before the infection. Both effects on TPP^+ accumulation that were caused by PRD1 infection were multiplicity of infection (MOI) dependent (Fig. 1A). The amplitude of the initial TPP^+ release, as well as the amplitude of the subsequent phage-induced uptake, increased when MOI increased. However, the amount of released TPP^+ from infected cells became lower when MOI exceeded 100 (about 40 particles bound/cell [17]). Usage of SL5676 control cells which do not contain the Mpf complex proteins in the envelope and which, therefore, cannot bind phage particles, confirmed that both types of TPP^+ fluxes are caused by the phage-cell interactions (Fig. 1C).

PRD1 increases OM permeability to lipophilic compounds. Tris-EDTA-treated *S. typhimurium* cells were highly permeable to TPP^+ but not to the rather bulky molecules of the channel-forming antibiotic GD. The addition of GD induced the depolarization of the PM and, because of that, the release of accumulated TPP^+ only in the case of infected cells (compare Fig. 1A and curve 2 of Fig. 1C). The polycationic antibiotic PMB, which does not require the increased permeability of the OM for its depolarizing action, induces an efflux of accumulated TPP^+ from the infected and noninfected cells.

When the OM was intact, the cells bound a rather small amount of TPP^+ before the infection (Fig. 1B and curve 1 of Fig. 1C). The addition of PRD1 induced a considerable TPP^+ uptake by DS88 cells starting 15 to 20 s after the phage addition. The phase of intensive TPP^+ uptake continued for 4 to 6 min and was followed by a slow one for the next 6 to 8 min. The rate of TPP^+ uptake and the final level of the accumulation were MOI dependent. This phage-induced TPP^+ uptake also indicates that PRD1 increases OM permeability to lipophilic compounds.

PRD1 induces K^+ efflux from the infected cells. In addition to the effects on accumulation of TPP^+, phage PRD1 induced a leakage of potassium ions from the cytosol (Fig. 2). The K^+ efflux started 60 s after the addition of phage particles at 25°C or after 30 to 35 s at 37°C. The rate of the leakage and the amount of K^+ left in the infected cells were MOI dependent (Fig. 2A). PMB addition finally dissipated the K^+ gradient in the case of for both treated and untreated cells. The Tris-EDTA treatment used had no considerable effect on the efficiency of the phage adsorption (Table 2), but it led to the decrease of K^+ concentration in the cytosol (compare Fig. 2A and B). However, a higher K^+ gradient on the PMs of untreated cells did not considerably increase the rate of phage-induced leakage, suggesting that a more complex system than an unspecific channel is engaged in this process. Lowering the temperature from 37 to 25°C affected the rate of K^+ leakage only weakly (Fig. 2B).

PRD1 induces ATP efflux but does not inhibit ATP production. The measurements of ATP-dependent light emission with the luciferin-luciferase system showed that the concentration of extracellular ATP is very low in the suspension of intact cells. However, a considerable increase in extracellular ATP concentration was induced starting 3.0 to 3.5 min postinfection (Fig. 3). This PRD1-induced leakage of ATP stopped after 18 to 20 min. The rate of the leakage and the final level of the extracellular ATP were MOI dependent. PMB induced an additional leakage of ATP. The total amount of ATP released from infected cells was higher than the amount released from noninfected intact cells, regardless of the time point of PMB addition. The total amount of ATP leaked from the infected

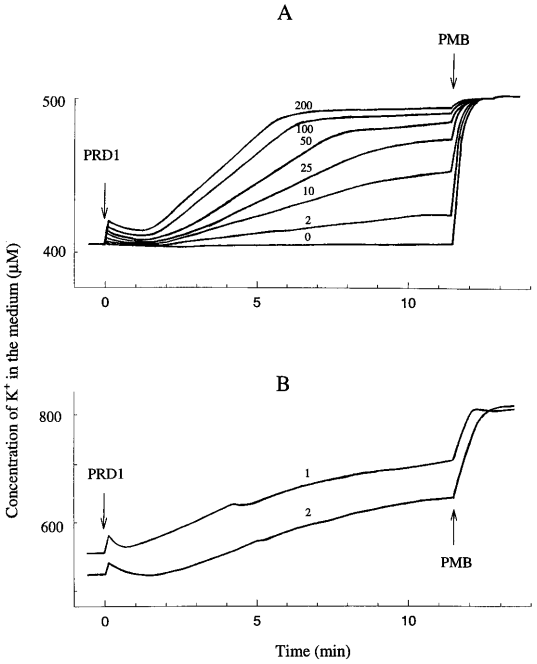

FIG. 2. Effect of the infection and PMB on K^+ fluxes across the envelope of DS88 cells. The experiments were carried out at 25°C (A; curve 2 in panel B) or 37°C (panel B, curve 1) in 50 mM Tris-HCl, pH 8.0, containing 0.1% glucose. The concentration of Tris-EDTA-treated (A) or nontreated (B) cells was 3×10^9 cells/ml. Phage PRD1 was added to obtain an MOI of 200 (B) or as indicated by the numbers on the figure (A). PMB was added to a final concentration of 120 μg/ml.

cells was maximal when an MOI as high as 1,000 was used. This indicates that a very high MOI is necessary for efficient receptor saturation in diluted cell suspensions used for ATP measurements.

PRD1 induces a small but measurable increase in PCB^- binding to cellular membranes. The phage-induced decrease of PCB^- concentration in the medium proceeded in two stages (Fig. 4): an initial fast stage, which began immediately after the addition of the phage particles, and a subsequent slow stage, which started 1.5 min postinfection and continued for 10 to 12 min. Control experiments in the absence of the cells showed that the initial fast stage of the decrease of PCB^- concentration in the medium was due to the binding of PCB^- to phage particles (16). The amount of PCB^- bound during the initial stage of the uptake was proportional to the amount of phage particles added, but the amount bound during the second stage was not considerably dependent on MOI (Fig. 4). GD induced some additional PCB^- binding, and PMB caused a high level of PCB^- binding to all infected and noninfected cells. The decrease in the amount of PCB^- bound by the infected cells at high MOIs can be explained by the lower concentration of the free anion at the moment of initiation of the second stage of the binding. Besides that, the higher $\Delta\Psi$ at higher MOIs (see Fig. 1A) also prevents the binding of PCB^- to infected cells. A strong effect of PMB on PCB^- concentration in the medium is related to the increase in the capacities of the cells as well as in those of the virions to bind this lipophilic anion (16).

Only DNA-containing particles induce the ion fluxes. Mutant PRD1 particles allowed us to distinguish between the effects caused by empty and filled particles. All the different phage particles added induced some initial release of TPP^+ from the Tris-EDTA-treated cells. For adsorbing but DNA-

TABLE 2. Effects of the medium composition on the efficiency of PRD1 adsorption

Compound (concn)	% Adsorption[g] to:				
	Untreated cells			Treated cells	
	p + c[a]	c + p[b]	c + p(−glu)[c]	p + c[d]	c + p[e]
Na phosphate (20 mM)	100	70	34	110	70
Na arsenate (20 mM)	100	2	1	98	5
NaN_3 (20 mM)	ND[f]	60	22	ND	ND
NaF (20 mM)	ND	67	29	ND	ND
NaN_3 + NaF (20 mM each)	ND	11	14	ND	ND
NG (2 μg/ml)	115	105	ND	104	79
CCCP (50 μM)	100	36	13	98	4
GD (10 μg/ml)	112	110	ND	92	73
PMB (120 μg/ml)	101	56	24	105	8
PMB (500 μg/ml)	105	13	ND	108	2

[a] The experiments were carried out in 50 mM Tris-HCl, pH 8.0, containing 0.1% glucose. The cell concentration was 3×10^9 cells/ml. After 10 min of thermal equilibration at room temperature phage (p) was added (MOI, 4). Ten minutes later the indicated compound (c) was added, and the infection mixture was incubated for an additional 10 min before the assay of adsorbed phages.
[b] The experiments were carried out as described in footnote *a*, except that the indicated compound was added before the phage particles.
[c] The experiments were carried out as described in footnote *b*, except that the medium did not contain glucose.
[d] The experiments were carried out as described in footnote *a*, except that the cells were treated with Tris-EDTA.
[e] The experiments were carried out as described in footnote *b*, except that the cells were treated with Tris-EDTA.
[f] ND, not determined.
[g] The results are normalized to PRD1 adsorption to nontreated (phosphate) cells (100%).

less particles (*sus1*) the amount of TPP^+ released was somewhat higher than for nonadsorbing particles (*sus170*) but lower than for intact infective virions (Fig. 5). Lowering the temperature reduced the amplitude and increased the duration of this fast cell reaction to phage addition (data not shown). The nature of the initial phage-induced TPP^+ release is, however, unclear.

The second stage, TPP^+ uptake, was observed only in experiments with the intact infective wt particles. Also, only the infectious wt particles were able to increase OM permeability to GD (Fig. 5). In addition, the efflux of ATP and K^+ and the second (slow) stage of PCB^- uptake were observed only when DNA-containing particles were used (data not shown).

Studies of the ion and ATP fluxes showed that subsequent additions of the virus greatly diminished the phage-induced effects. The second addition of wt particles had no effect on TPP^+ uptake by intact (Fig. 6A) or by Tris-EDTA-permeabilized (Fig. 6B) cells. Large, receptor saturating amounts of adsorbing empty *sus1* particles but not nonadsorbing *sus170* particles blocked the effect caused by subsequently added wt particles (Fig. 5). However, the infectious wt particles blocked the effects of the superinfecting ones even at low initial MOIs when not all the receptors on the cell surface were occupied (Fig. 6). These results show that the superinfection exclusion effect is expressed in PRD1 infection. When the adsorption in the superinfection experiments was measured, the superinfecting phage particles adsorbed normally (data not shown). These results rule out the possibility that the long durations of PRD1-induced TPP^+ and PCB^- uptakes as well as K^+ and ATP

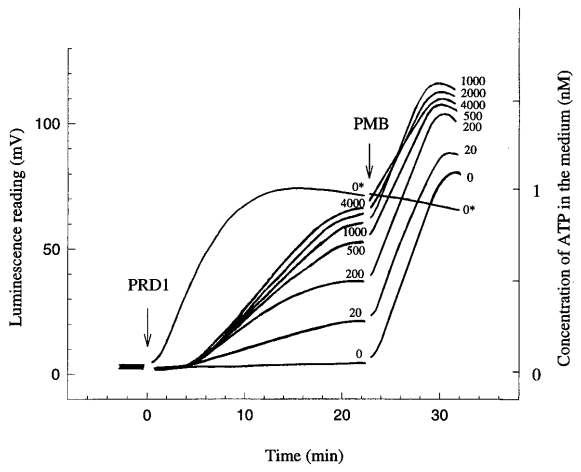

FIG. 3. Effects of the MOI on ATP release from Tris-EDTA-treated DS88 cells. The experiments were carried out at 25°C in 50 mM Tris-HCl, pH 8.0, containing 0.1% glucose and 1/4 (vol/vol) ATP monitoring reagent. The final cell concentration was 1.5×10^8 cells/ml. Phage PRD1 was added to obtain the MOIs indicated in the figure, and PMB was added to a final concentration of 500 μg/ml. 0*, instead of PRD1, PMB was added at the time of phage addition.

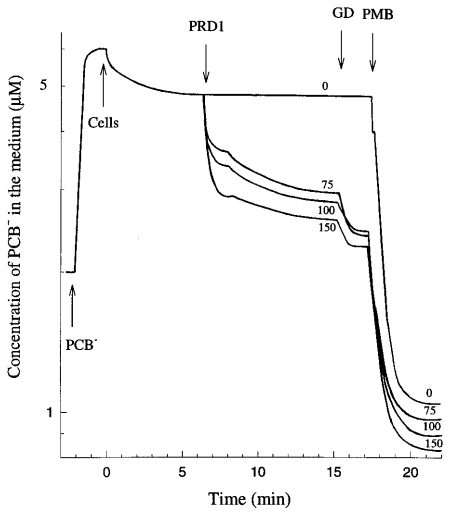

FIG. 4. Effects of the MOI on PCB^- uptake by Tris-EDTA-treated DS88 cells. The experiments were carried out at 37°C in 50 mM Tris-HCl, pH 8.0, initially containing 2 μM PCB^- and 0.1% glucose. PCB^- was added to obtain a 6 μM concentration; cells were added to a final concentration of 3×10^9 cells/ml. Phage PRD1 was added to obtain the MOIs shown in the figure; GD and PMB were added to final concentrations of 4 and 120 μg/ml, respectively.

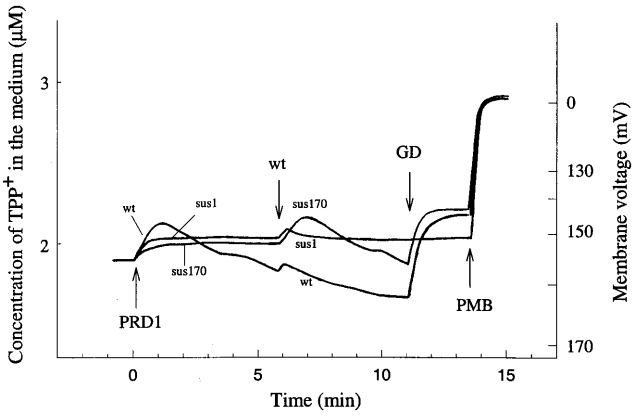

FIG. 5. TPP^+ binding dependence on the type of phage particles added. The experiments were carried out at 37°C in 50 mM Tris-HCl, pH 8.0, containing 3 μM TPP^+ and 0.1% glucose. The concentration of DS88 cells was 3×10^9 cells/ml. PRD1 was added to obtain an MOI of 200 after the first addition of different types of particles and 400 after the second addition of wt particles. GD and PMB were added to final concentrations of 4 and 120 μg/ml, respectively.

leakages are a result of slow and nonsynchronous adsorption of the phage particles to the cells.

ATP is necessary for the initiation of infection. In order to estimate the role of cell energetics in the adsorption process and the subsequent phage-induced events, the effects of energy

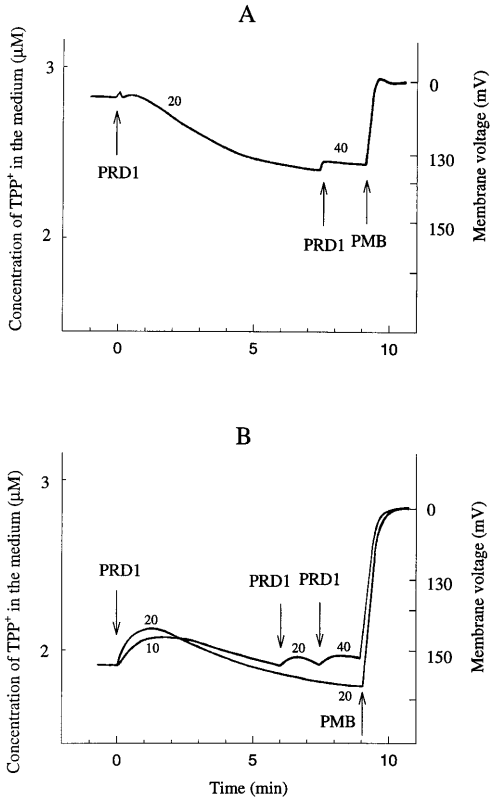

FIG. 6. Effect of repeated additions of the phage on TPP^+ uptake by DS88 cells. The experiments were carried out at 37°C in 50 mM Tris-HCl, pH 8.0, containing 3 μM TPP^+ and 0.1% glucose. The final concentration of Tris-EDTA-treated (B) or nontreated (A) cells was 3×10^9 cells/ml. Phage PRD1 was added to obtain the MOIs indicated in the figure, and PMB was added to a final concentration of 120 μg/ml.

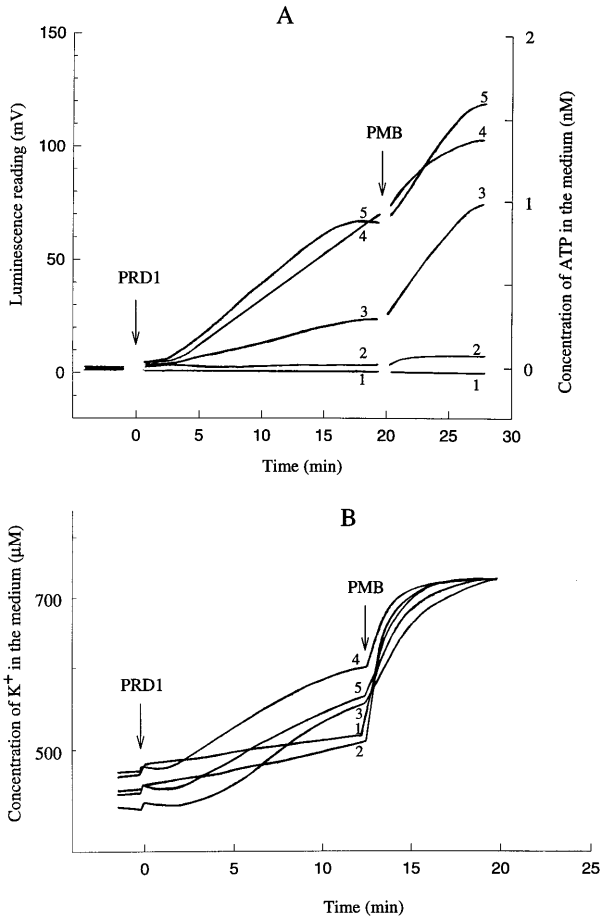

FIG. 7. Effects of metabolism inhibitors on PRD1-induced changes in the ATP and K^+ content of DS88 cells. The measurements were carried out at 25°C as described in the legend for Fig. 3 (for ATP) and in the legend for Fig. 2 (for K^+) with nontreated cells. Phage PRD1 was added to obtain an MOI of 500 or 25, and PMB was added to a final concentration of 500 or 120 μg/ml in ATP (A) and K^+ (B) measurements, respectively. Curves show the results of measurements in the presence of 20 mM sodium arsenate (1), 20 mM NaN_3 plus 20 mM NaF (2), 20 mM NaF (3), 20 mM NaN_3 (4), and no inhibitors (5).

transformation-modifying compounds were studied (Table 2; Fig. 7). Energization of the cells in buffer by the addition of glucose considerably increases the number of phage particles adsorbed. However, NaF, an inhibitor of the enolase reaction (50) and therefore of ATP formation with glycolytic substrates, did not inhibit adsorption (Table 2) and had a negligible effect on PRD1-induced leakages (Fig. 7). NaN_3 is a compound known to eliminate bacterial growth by blocking respiration and by uncoupling oxidative phosphorylation by means of the inhibition of cytochrome oxidase and membrane H^+-ATPase (27). In the presence of 20 mM NaN_3 the initial intracellular K^+ concentration was slightly lower, but this did not have a considerable effect either on PRD1 adsorption (Table 2) or on phage-induced leakages (Fig. 7). However, a preincubation of the DS88 cells in the medium containing both 20 mM NaN_3 and 20 mM NaF caused a considerable inhibition of adsorption (Table 2) and, therefore, of phage-induced K^+ leakage (Fig. 7B). The ATP concentration measurements showed that when acting together these inhibitors considerably decreased the intracellular concentration of ATP (Fig. 7A).

Arsenate is known to quickly and drastically reduce the concentration of intracellular ATP by arsenolyzation of acetyl

phosphates (28, 32). The exposure of the cells to 20 mM arsenate resulted in even more effective inhibition of phage adsorption than that resulting from the simultaneous treatment of cells with NaN_3 and NaF (Table 2). On the other hand, PMB addition showed that cells in these conditions were able to maintain a rather high K^+ gradient in the absence of ATP (Fig. 7B).

Uncouplers of oxidative phosphorylation, such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), are known to effectively deplete the energized state of the PM (43). CCCP drastically reduced the number of adsorbed phage particles when Tris-EDTA-treated cells were used. However, the adsorption-inhibiting efficiency of GD was rather low. Therefore, it is possible that the high adsorption-inhibiting efficiency of CCCP is a result of the membrane deenergization-induced decrease in the ATP content of the cells. The considerably higher adsorption-blocking efficiency of PMB at 500 μg/ml (effectively releasing ATP) than at 120 μg/ml (sufficient only for complete depolarization of the PM; Fig. 1) supports this suggestion. All these results indicate that ATP and/or related acetyl phosphates are crucial for phage adsorption. However, none of these adsorption-blocking agents reduced the number of phage particles adsorbed when added after the phage to the infection mixture (Table 2).

The increase of OM permeability is a gradual process. The channel-forming antibiotic GD only slightly affected adsorption but it had a considerable effect on the later phage-induced events. The GD effect on TPP^+ accumulation depended on the time interval between PRD1 and GD additions (Fig. 8A and B). Only the PRD1-induced TPP^+ efflux, not the uptake of this cation, was observed (Fig. 8A) if GD was added to the cellular suspension before the phage. The second addition of GD after PRD1 was introduced had no effect on TPP^+ accumulation, though the cells had rather high $\Delta\Psi$. A considerable depolarizing effect by GD was observed if this antibiotic was added 2 min or later after the phage. The amount of TPP^+ remaining in the infected cells after GD addition was less than the amount accumulated by the cells before the phage addition. This indicates that the inability of GD to induce TPP^+ efflux at the early stages of infection is associated with restricted permeability of the OM to this antibiotic, but not with low $\Delta\Psi$.

These results indicate that the increase in the OM permeability of infected cells is a rather slow and gradual process. The level of OM permeability sufficient for free electrophoretic distribution of TPP^+ between the interior and exterior of PRD1-infected cells with initially intact OMs is achieved in 0.5 min (Fig. 1B), but the penetration of rather large GD molecules starts 2 to 2.5 min postinfection and is self inhibiting. One possibility is that the TPP^+-permeable and the GD-permeable states of the OM are results of different events caused by the PRD1 infection.

Depolarization of the PM stops the ATP leakage. GD (Fig. 8C and D) as well as CCCP (data not shown) stopped ATP leakage if added 5 to 15 min postinfection and prevented it if added before or just after the infection. However, if NG was added instead of GD, the rate of phage-induced ATP leakage was higher than that in untreated cells. Further addition of PMB showed that the blockage of the ATP leakage caused by GD was not due to the depletion of the intracellular ATP. None of these agents added after the phage had a considerable effect on the number of PRD1 particles adsorbed (Table 2). These results suggest that ATP leaks from the infected cells through a voltage-dependent channel which closes when $\Delta\Psi$ decreases below a threshold value. This threshold could be rather high, because GD does not completely depolarize the PM at these conditions (Fig. 8A). ATP permeability through PMB-induced pores is not sensitive to membrane voltage.

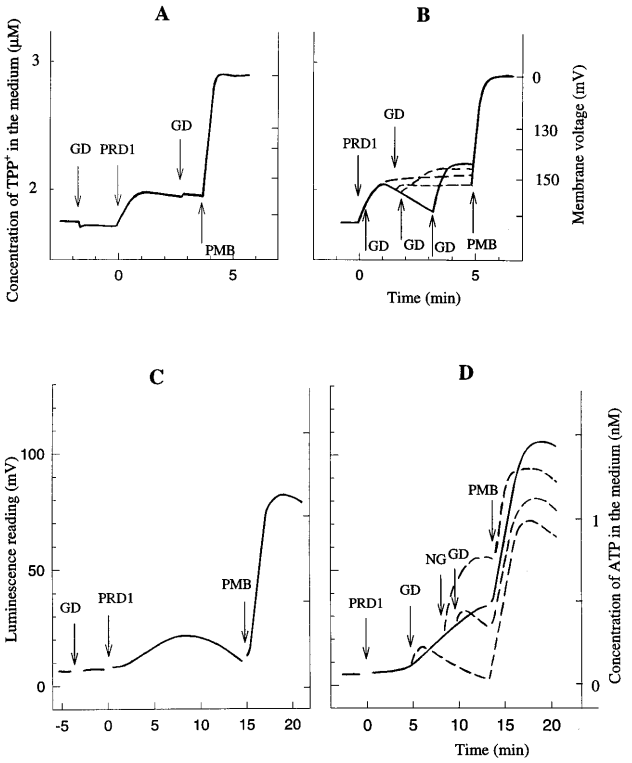

FIG. 8. Effects of GD and NG on phage-induced TPP^+ and ATP fluxes. (A and B) TPP^+ measurements carried out at 37°C in 50 mM Tris-HCl, pH 8.0, containing 3 μM TPP^+. The concentration of Tris-EDTA-treated DS88 cells was 3×10^9 cells/ml. Phage PRD1 was added to obtain an MOI of 50. GD was added to a final concentration of 4 μg/ml (after the first addition) or 8 μg/ml (after the second addition), and PMB was added to a final concentration of 120 μg/ml. (C and D) ATP measurements carried out as described in the legend for Fig. 3. Phage PRD1 was added to obtain an MOI of 500, and GD, NG, and PMB were added to final concentrations of 4, 2, and 500 μg/ml, respectively.

DISCUSSION

Events coupled to PRD1 infection. The cell into which PRD1 DNA is penetrating or has penetrated can be characterized by the following features: (i) lower intracellular K^+ concentration; (ii) increased OM permeability to lipophilic compounds; (iii) increased membrane voltage; and (iv) activated synthesis of ATP and increased envelope permeability to this compound. A transient leakage of K^+ and some other intracellular solutes has been observed after adsorption of many bacteriophages (45, 46, 51). The characteristics of PRD1-induced K^+ leakage are the same as those for most other phages studied: (i) the intensity of leakage increases when MOI increases and (ii) the leakage is irreversible. Phage-induced channels are responsible for K^+ leakage in T1, T4, T5, and λ infections (10, 11, 21, 31). However, in those cases, depolarization of the PM also takes place. PRD1-induced changes in $\Delta\Psi$ are very different than those induced by the other phages studied. An intriguing characteristic of PRD1 infection is that the final level of TPP^+ accumulated by infected cells exceeds the initial level. In addition, the amplitude of the PRD1-induced TPP^+ uptake increases with the MOI, and the maximal uptake is achieved when the receptors are saturated (MOI > 400 [17, 33]). The opposite MOI effect was

observed in the case of bacteriophage T4 (34). T4-induced depolarization increases, and the extent of repolarization decreases, when MOI increases. TPP^+ uptake by the PRD1-infected Tris-EDTA-treated cells starts at the same time as the leakage of intracellular potassium. Thus, an electrogenic K^+ efflux could be one of the possible causes of the PRD1-induced increase in $\Delta\Psi$. The PM-depolarizing agents GD and CCCP very effectively blocked ATP leakage from the infected cells. GD also inhibited the PRD1-induced increase in the OM permeability to lipophilic compounds. These results indicate that there is a $\Delta\Psi$-dependent component in the PRD1-induced effects on the permeability of the host cell membranes.

In the case of PRD1 all membrane-related events are induced only by infectious phage particles, although T4 "ghosts" induce the same initial effects as the infective virions (19). PRD1, using the Mpf complex for its adsorption, may also employ this system or part of it for its DNA translocation. The relatively small effect of phage PRD1 on PCB^- binding by infected cells indicates that PRD1 does not induce nonspecific channels in the bacterial envelope and, therefore, does not disrupt the metabolism-dependent barrier to lipophilic anions as T4 does (16). Electrochemical measurements have shown that the IncP plasmid-encoded Mpf complex consists of a structure that makes the cells permeable to lipophilic compounds, increases $\Delta\Psi$, and decreases intracellular K^+ concentration (17). If PRD1 DNA injection uses the same complex as conjugative DNA transfer, it is not surprising that the behavior of cells containing only the Mpf system resembles that of the phage PRD1-infected cells. It is likely that phage PRD1 in some way blocks the action of Dtr gene products and that the phage-induced effects on the permeability of infected cells are the effects of the "unmasked" Mpf complex (17). Electron microscopic observations of PRD1 infection (2, 39) have indicated a virus-specific tail-like structure involved in DNA penetration. The possible association of this structure with the Mpf complex will be the focus of our future studies.

Mode of the phage-induced changes. It is logical to suggest that the ions and ATP move through the same channel in the bacterial envelope through which the phage DNA enters the cell. Active DNA transport mechanisms have also been proposed (20, 52), in which protons are preferred as counterions for the DNA polyanion. In T5 infection a specific coupling of DNA transport to K^+ leakage was observed by Boulanger and Letellier (11). However, in the case of PRD1 DNA transport takes less than 5 min at 37°C (42) but K^+ leakage continues for more than 10 min (Fig. 2B). It seems that the latest measured event, the ATP leakage, starts immediately after the DNA transport is over. The possibility cannot be excluded, however, that phage adsorption activates some channels preexisting in the bacterial PM (8) and that DNA penetration and leakage of the intracellular compounds occur through different channels.

The duration of the "leaky" state of infected cells suggests that phage-induced changes to intracellular ion concentrations might have a physiological role in the later stages of the life cycle. The PRD1-induced changes in the energetic state of the cell should lead to stimulation of $\Delta\Psi$-dependent processes and inhibition of ATP-dependent processes and should lead to changes in the gradients of all actively transported compounds, irrespective of whether the channels induced and/or used by phage are permeable to them or not.

Infected cells have lower K^+ and ATP concentrations in most of the virus infections studied (for review see reference 37). The observation that the ionic compositions of the media optimal for some PRD1 enzymes in vitro differ from that found for cytosolic ones (14, 49) supports the idea that changing electrochemical conditions in the bacterial cytosol might be a part of the virus reproduction strategy. The studies on PRD1 infection showed, however, that the decrease in the intracellular K^+ concentration is not coupled to the depolarization of the PM. The decrease in intracellular ATP concentration during the initial stages of infection is surprisingly stable. It is known that intracellular ATP can be hydrolyzed by membrane H^+-ATPase (31, 52) or as a result of a shift in ATP equilibrium due to the efflux of phosphate through the phage-induced channels (24). In the case of PRD1, ATP efflux most probably takes place through a specific voltage-dependent channel which closes when the PM is depolarized. The channel also closes 20 to 25 min postinfection even though there is no depolarization of the PM. This type of closing, however, can be prevented by the addition of NaN_3 (Fig. 7A). It is worth noting that PRD1-induced ATP leakage does not lead to a drastic depletion of intracellular ATP due to the activation of ATP synthesis.

The expression of the PRD1 receptor on the cell surface was dependent on the presence of ATP, as indicated by the adsorption experiments with the drug-treated cells. The PRD1 receptor-DNA transfer complex thus seems to be a dynamic structure that is assembled and disassembled depending on the energetic conditions. However, adsorbed phages continue the infection process even if drugs leading to receptor depletion are added.

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