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Membrane fusion in prokaryotes: bacteriophage ϕ6 membrane fuses with the *Pseudomonas syringae* outer membrane

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Protein-triggered membrane fusion in the prokaryotic system is described using the lipid-containing enveloped bacterial virus ϕ6 and its host, the Gram-negative bacterium *Pseudomonas syringae*. Bacteriophage particles can be fused to form multiple particles where two or more nucleocapsids are surrounded by a single membrane vesicle with a volume proportional to the number of fused particles. For fusion to occur, a fusogenic protein is required in the membrane of the participating phage particles. Upon infection of the host cell, fusion of the viral membrane with the bacterial membrane takes place without leakage of the periplasmic enzyme alkaline phosphatase to the extracellular supernatant. There is a time-dependent mixing of fluorescent phage phospholipids with the bacterial membrane lipids between 5 and 20 min post-infection. The phage membrane proteins and phospholipids co-purify with the bacterial outer membrane of infected cells. The fusion is independent of divalent cations and pH, resembling Sendai virus fusion with the plasma membrane. This is the first targeted, protein-dependent fusion event described in prokaryotes.

Key words: bacteriophage/membrane fusion/outer membrane/virus entry

Introduction

The transport of macromolecules into and out of the eukaryotic cell as well as between different cellular compartments is dependent on targeted specific membrane fusion events. Enveloped animal viruses are the best understood biological fusion systems. In these organisms membrane fusion is the mechanism by which the viral nucleoprotein particle enters the cell. Paramyxoviruses fuse with the plasma membrane of the cell in a pH-independent way. Toga and orthomyxoviruses enter the cell via an endocytotic pathway and their fusion proteins are activated by the slightly acidic pH of the endosomes (for a review see White *et al.*, 1983).

The best understood fusion activation system is that of influenza virus hemagglutinin (HA). This surface glycoprotein undergoes a conformational change at mildly acidic pH exposing a hydrophobic portion of the HA close to the virus surface. The hydrophobic portion is obviously inserted into the target membrane leading to membrane fusion (Doms *et al.*, 1985). Semliki Forest virus spike glycoproteins E1 and E2 also undergo conformational changes which trigger the fusogenic activity. No hydrophobic domain has been detected in the activated glycoproteins, suggesting a different fusion triggering mechanism from that of influenza virus (Kielian and Helenius, 1985).

Prokaryotes have no intracellular membrane vesicle transport system, and it is not known whether specific protein-controlled fusion events are necessary for such functions as cell division, adhesion site formation, secretion and conjugation.

Bacteriophage ϕ6 is a dsRNA virus of *Pseudomonas syringae* pv. *phaseolicola* (Vidaver *et al.*, 1973). The virus nucleocapsid is surrounded by a lipid–protein envelope. The envelope consists of phospholipids and virus-specific proteins, P3, P6, P9 and P10 (Mindich *et al.*, 1976b; Bamford and Palva, 1980). Two of the proteins (P3 and P6) are exposed on the surface of the virion (van Etten *et al.*, 1976; Stitt and Mindich, 1983). Protein P3 is associated with protein P6 which is hydrophobic and inserted in the membrane. Nonsense mutations in gene 3 coding for protein P3 are polar on gene 6 coding for protein P6, and gene 6 nonsense mutants lacking P6 do not assemble P3 on the virion (Sinclair *et al.*, 1976, Mindich *et al.*, 1979). Particles lacking the surface proteins do not adsorb to the phage receptor, the bacterial pilus (Mindich *et al.*, 1976b).

Electron microscopic studies have indicated that the ϕ6 membrane fuses with the host outer membrane introducing the nucleocapsid into the periplasmic space (Bamford *et al.*, 1976, Bamford and Lounatmaa, 1978). A virus-encoded lytic enzyme (protein P5; Kakitani *et al.*, 1978; Mindich and Lehman, 1979) is located between the phage membrane and the nucleocapsid (Bamford and Palva, 1980). This enzyme is obviously needed to digest locally the host cell wall peptidoglycan during the virus entry, allowing the nucleocapsid to reach the cytoplasmic membrane, the following step being the penetration of the nucleocapsid into the cytoplasm.

Rather detailed knowledge of the virus structure is available (Bamford and Palva, 1980; Berger and Kennedy, 1980; Day and Mindich, 1980; V.M.Olkkonen and D.H.Bamford, in preparation), the infective particle ratio approaches 1 (Day and Mindich, 1980), and the complete nucleotide sequence, as well as the location of the genes, are known (Mindich *et al.*, 1985; McGraw *et al.*, 1986; L.Mindich, personal communication). This makes ϕ6 a useful system for membrane fusion and penetration studies in a relatively simple prokaryotic system.

Results

To study the protein complexes needed for ϕ6 adsorption and fusion, several *ϕ6h1s sus* mutants with mutations in genes 3 or 6 were used. Particles lacking protein P3 were prepared using butylated hydroxytoluene (BHT; Bamford, 1981).

Three *ϕ6h1s* derivatives that were resistant to the effect of BHT were isolated. They were designated *ϕ6h1s bht1*, *bht2* and *bht3*. The electrophoretic mobility of protein P6 in protein gels was changed equally in all three independently isolated mutants. All other proteins migrated as in *ϕ6h1s*. Figure 1 shows the protein pattern of purified *bht1* mutant particles as well as *ϕ6h1s*, *sus277* ($P3^-$, $P6^-$), and BHT-treated *ϕ6h1s* ($P3^-$) particles — the types of particles used in this study. Additional *sus* mutants with mutations in the P3 gene (*sus45*, *sus120*) and the P6 gene (*sus453*, *sus507*) were used to confirm the *in vitro* fusion results. All phages used are listed in Table I.

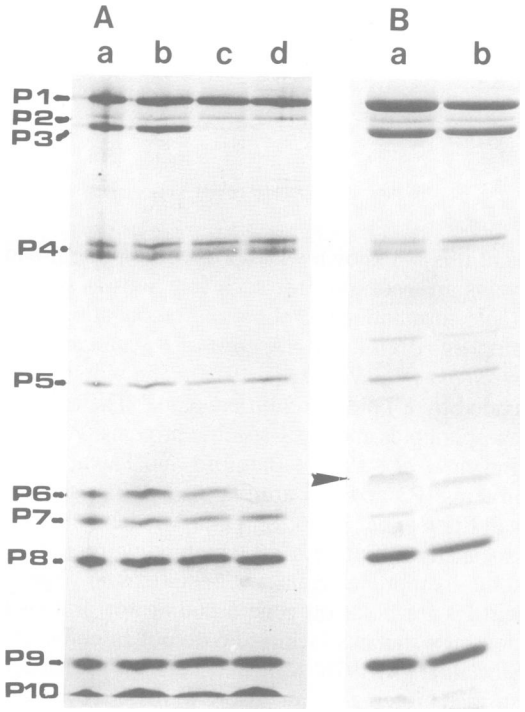

Fig. 1. Protein pattern in SDS–polyacrylamide gels of phage types used in this study. **A**: *ϕ6hls* (**lane a**); *ϕ6hls bht1*, BHT-resistant mutant with altered P6 mobility (**lane b**); *ϕ6hls* with P3 removed from the particles by BHT treatment (**lane c**); *ϕ6hls sus277* lacking the surface proteins P3 and P6, (**lane d**). **B**: *ϕ6hls bht1* (**lane a**); *ϕ6hls* (**lane b**). The arrow indicates the position of the *bht1* P6. **A** is an autoradiogram of phage labeled with a [^{14}C]amino acid mixture. In **B** the gel is stained with Coomassie brilliant blue. **B** contains ~10 times more material than **A**.

Table I. Bacteriophage strains used

Strain	Virion phenotype	Origin
ϕ6hls	Host range mutant of *ϕ6* infecting strains HB, S4 and Ro	Mindich *et al.* (1976)
ϕ6hls sus45 (3)[a]	P3$^-$, P6$^-$	L.Mindich
ϕ6hls sus120 (3)	P3$^-$, P6$^-$	L.Mindich
ϕ6hls sus277 (6)	P3$^-$, P6$^-$	L.Mindich
ϕ6hls sus453 (6)	P3$^-$, P6$^-$	L.Mindich
ϕ6hls sus507 (6)	P3$^-$, P6$^-$	L.Mindich
ϕ6hls bht1 (6)	BHT-resistant[b]	This study
ϕ6hls bht2 (6)	BHT-resistant	This study
ϕ6hls bht3 (6)	BHT-resistant	This study

[a]Number in parenthesis indicates the gene affected.
[b]Resistant to removal of P3 by BHT.

To measure the degree of hydrophobicity of the *ϕ6* membrane proteins (P3, P6, P9 and P10) we used the two-phase Triton X-114 method (Bordier, 1981). Proteins P6, P9 and P10 were always found only in the Triton phase. Protein P3, which is located on the virus surface, was found in about equal amounts in the detergent and water phases. P3 partition had to be done with isolated P3, since it showed high affinity for the nucleocapsid.

In vitro fusion of viral membranes

Since adsorption of the virus particle is totally dependent on protein P3 (see Table II), the membrane fusion of the particles lacking P3 could not be studied *in vivo*. To be able to study the influence of the *ϕ6* surface proteins on the fusion step we developed a virion-to-virion fusion assay. Radioactively labeled virus particles were brought into contact with each other by centrifugation. The formation of multiple particles was then assayed by rate zonal centrifugation (Figure 2).

Table II. Percentage of phage-specific radioactivity adsorbed to bacteria 20 min post-infection using a multiplicity of 0.1

	ϕ6hls (P3$^+$, P6$^+$)[a]	*ϕ6hls sus277* (P3$^-$, P6$^-$)	*ϕ6hls* BHT-treated (P3$^-$)	*ϕ6hls bht1*[b] (P3$^+$, P6$^+$)
HB	82.0	4.3	7.6	77.2
E. coli	3.2	3.5	6.0	3.3

The phage preparation was labeled with a [^{14}C]amino acid mixture as described in Materials and methods. The specific activity was 2.5×10^{-5} c.p.m. per phage particle.
[a]Surface protein status.
[b]Resistant to removal of P3 by BHT.

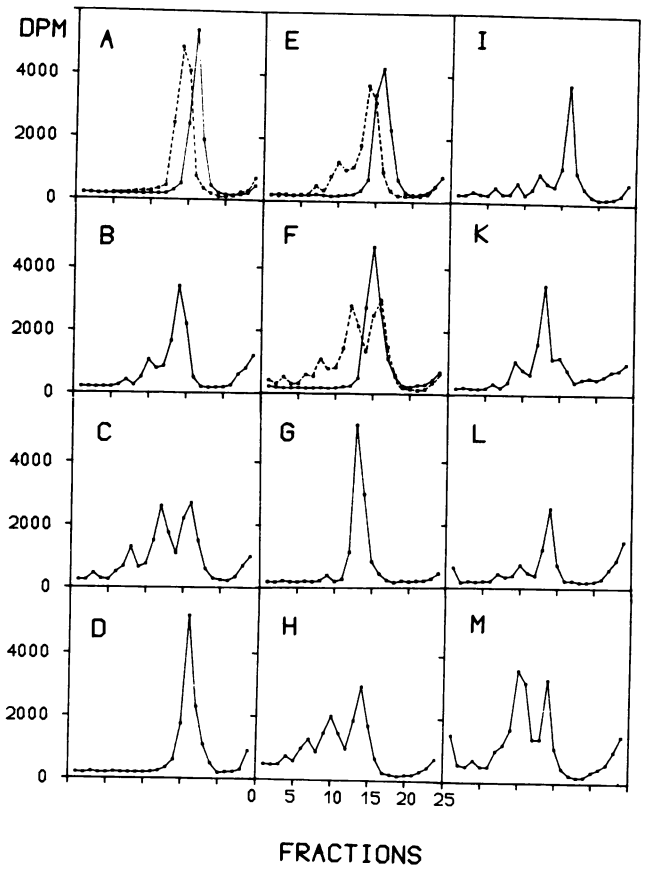

Fig. 2. The rate zonal sucrose gradient profiles of radioactively labeled phages after *in vitro* fusion assays. The sedimentation is from right to left. Monomers of *ϕ6hls* (dotted line) and *sus277* (P3$^-$, P6$^-$; solid line) (**A**); *ϕ6hls*, standard fusion assay (**B**); BHT-treated *ϕ6hls* (P3$^-$), standard fusion assay (**C**); P3$^-$, P6$^-$ particles, standard fusion assay (**D**); standard fusion assay of 10:1 mixture of *ϕ6hls* (^{3}H-labeled, dotted line) and P3$^-$, P6$^-$ particles (^{14}C-labeled, solid line) (**E**); standard fusion assay of 10:1 mixture of P3$^-$ particles (^{3}H-labeled, dotted line) and P3$^-$, P6$^-$ particles (^{14}C-labeled, solid line) (**F**); *bht1*, standard fusion assay (**G**); *ϕ6hls*, fusion assay in the presence of 10% PEG 6000 (**H**); P3$^-$, P6$^-$ particles, fusion assay in the presence of 10% PEG (**I**); *ϕ6hls*, fusion assay in the presence of 10 mM EDTA (**K**); *ϕ6hls*, fusion assay at pH 5.5 (**L**); P3$^-$ particles, fusion assay at pH 5.5 (**M**).

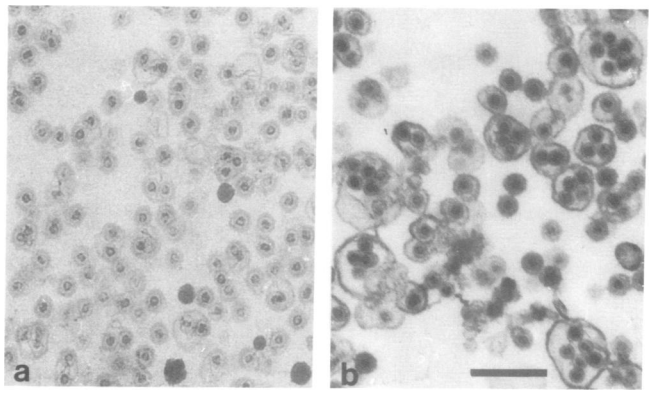

Fig. 3. Thin section of *ϕ6hIs* particles after standard fusion assay and sucrose gradient sedimentation. Pooled material from the second and third peak of a gradient like the one in Figure 2C (**a**); material sedimenting faster than the third peak (**b**). The bar represents 200 nm.

Figure 2A shows the sedimentation profiles of untreated *ϕ6hIs* and *sus277* ($P3^-$, $P6^-$) particles. The slower sedimentation of particles lacking the surface proteins has been documented previously (Mindich *et al.*, 1979).

The standard conditions were chosen so that ~25% of the *ϕ6hIs* particles formed multiple particles (Figure 2B). In the standard assay we used ~300 μg of virus in 300 μl of 50 mM K-phosphate buffer pH 7.2; 1 mM $MgCl_2$. The outcome of the assay was not sensitive to changes in virus amounts used. When particles lacking P3 (BHT-treated) were tested in this assay, they showed a much higher fusion activity than the normal particles (Figure 2C), whereas particles lacking both P3 and P6 (*sus277*) did not fuse at all (Figure 2D). Similar results were obtained with other gene 3 and gene 6 *sus* mutants (not shown). To test the ability of $P3^-$, $P6^-$ particles to fuse with normal or with $P3^-$ particles, [^{14}C]amino acid-labeled $P3^-$, $P6^-$ particles were mixed in a 1:10 ratio with either [^{3}H]leucine-labeled *ϕ6hIs* or *ϕ6hIs* $P3^-$ particles. In neither case were the $P3^-$, $P6^-$ particles able to fuse with the fusion active particles (Figure 2E and F). The *bhtI* particles showed less than half of the fusion activity of *ϕ6hIs* (Figure 2G).

Addition of polyethylene glycol (PEG) 6000 to a final concentration of 10% increased the fusion activity of *ϕ6hIs* particles (Figure 2H). In the presence of PEG the $P3^-$, $P6^-$ particles fuse at about the same rate as *ϕ6hIs* fuse without PEG (Figure 2I). Addition of 10 mM EDTA to the fusion mixture as well as to the rate zonal gradient did not affect the fusion activity (Figure 2K). This treatment, however, led to some destabilization of the virus particles in the fusion assay as shown by an additional small peak sedimenting slower than the single virus particles. Lowering the fusion assay pH to 5.5 did not alter the fusion activity of *ϕ6hIs* (Figure 2L) or the BHT-treated particles (Figure 2M).

Multiple particles of *ϕ6hIs* thus formed, were subjected to thin-section electron microscopy. Figure 3a shows fused material pelleted from the pooled double and triple particle bands from a rate zonal gradient. This fraction also contained numerous single particles, obviously as contaminants from the single particle peak. Figure 3b shows the sectioned pellet of material sedimenting faster than triple particles, revealing several nucleocapsids surrounded by a continuous membrane. Sectioned and negative stained $P3^-$ and $P3^-$, $P6^-$ single particles are indistinguishable from wild-type single particles (not shown).

The multiple particle bands were also subjected to protein gel electrophoresis. No qualitative or quantitative changes could be seen in the protein pattern as compared to single virus particles.

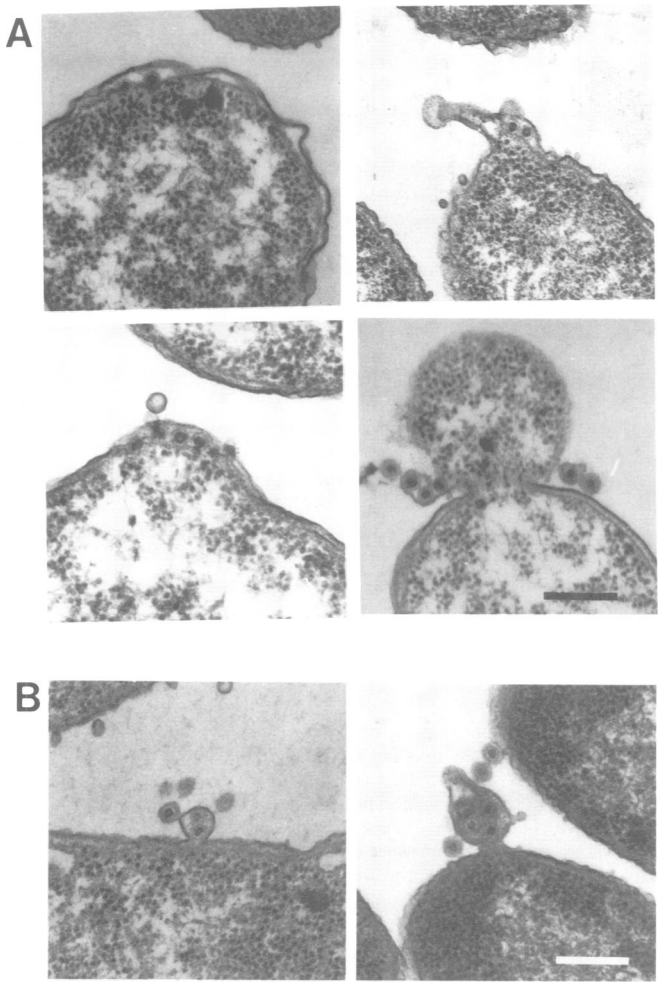

Fig. 4. (**A**) Thin-section electron microscopy of *ϕ6hIs* multiple particles infecting *Pseudomonas syringae* pv. *phaseolicola* HB10Y cells 10 min after addition of phage. The multiple particles were obtained from a sucrose gradient like the one in Figure 2B. The picture is a composite, showing nucleocapsids in the periplasmic space. The preparation also contained many bursting cells as the one in the lower right corner. The bar represents 200 nm. (**B**) Thin-section electron microscopy of cells infected as above. Before infection the phage particles were, in this case, heat treated to inactivate the virion-associated lytic enzyme. Using these particles the nucleocapsids are always seen outside the peptidoglycan layer. The bar represents 200 nm.

Infection with multiple particles

The sucrose gradient fractions containing multiple particles were also used to infect HB cells. These infections were subjected to thin-section electron microscopy. Preparations fixed 10 min post-infection display many adjacent nucleocapsid particles in the periplasmic space (Figure 4A) where the infecting nucleocapsids are also normally seen early in infection (Bamford *et al.*, 1976). When multiple particles were used in the infection, cells were often lysed at the site of infection (Figure 4A). This seems to be due to the large amount of phage lytic enzyme released into the periplasm, rendering the bacterial cells sensitive to intracellular osmotic pressure.

The phage lytic enzyme is sensitive to heat (Mindich and Lehman, 1979). Multiple phage particles were incubated at 45°C for 30 min. This treatment inactivates the lytic enzyme associated

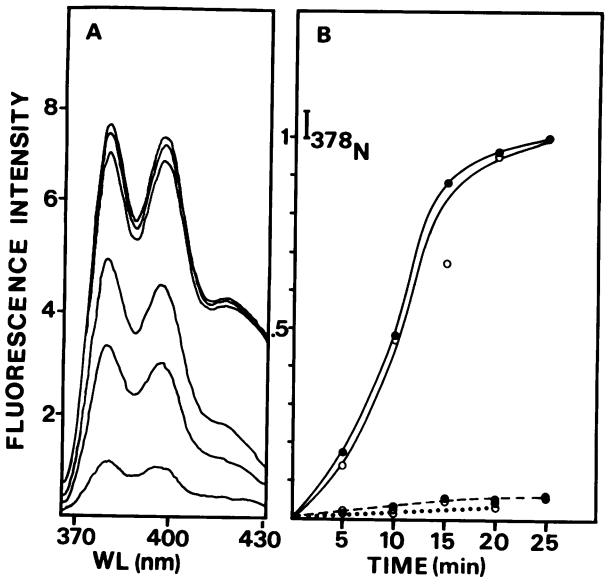

Fig. 5. A fluorescence assay of mixing of PryPG- and PyrPC-labeled and TNBS-quenched *ϕ6hls* phospholipids with bacterial membrane during phage infection at 23°C using a multiplicity of 4. The spectrum obtained immediately after infection was used as a blank, and was subtracted from spectra recorded at 5, 10, 15, 20 and 25 min after infection. **Panel A** shows a time-dependent increase of the typical pyrene fluorescence profile when phage-sensitive HB cells are used. **Panel B** is a time course of increase of pyrene monomer intensity (I_{378}) for PyrPG (○), and PyrPC (●) labeled *ϕ6hls* mixed with either HB cells or phage-resistant non-adsorbing MPO.16 (dashed), or adsorbing MP22.14 (dotted) cells. As the monomer intensities are somewhat different for PyrPC- and PyrPG-labeled phage, the data have been normalized to obtain an equal value at 25 min.

with the particle, but does not affect the structural stability or the adsorption behaviour of the virion. Infection of HB cells with these particles does not affect the growth of the host, and no viruses are produced. Thin sections of infected cells show multiple viruses fused with the outer membrane, but the nucleocapsids stay outside the rigid peptidoglycan layer (Figure 4B). In this case no lysing cells can be detected even when highly multimeric particles fuse with the outer membrane (OM).

Adsorption of ϕ6 particles to bacterial cells

The different types of ϕ6 particles were tested for their adsorption efficiency using radioactively labeled phages (Table II). The particles lacking either protein P3 or both proteins P3 and P6 did not adsorb above the background level (*Escherichia coli*), whereas the BHT-resistant derivative of *ϕ6hls* (*ϕ6hls bht1*) adsorbed at the same efficiency as *ϕ6hls*.

Strains *ϕ6hls* and *ϕ6hls bht1* behave differently in the early steps of infection. Their ability to escape removal from the cells by Triton X-100 treatment was tested. The assay measures the portion of phage-associated radioactivity that becomes insensitive to the detergent. This portion represents the particles that have penetrated at least the OM (Romantschuk and Bamford, 1985). The detergent insensitivity was compared by infecting HB cultures with radioactively labeled or unlabeled phages with a multiplicity of between 0.2 and 1 (in different tests). At 10 min post-infection the portion of adsorbed radioactivity, or the portion of infective centers that was detergent resistant, was 1.40 ± 0.19 times higher (eight separate tests) using *ϕ6hls* than with *ϕ6hls bht1*. The difference between the particle types is small but reproducible. The adsorption of the phage types to the HB cells was in all cases indistinguishable (see also Table II). When the pilitated phage-resistant strain MP22.14 is used the phage-specific radioactivity retained by the cells after detergent treatment is >5-fold lower than with the sensitive strains.

The adsorption efficiency and the detergent escape were unaffected by omission of divalent cations from the M9-medium or by addition of EDTA (5 mM final concentration).

Adsorption, detergent escape and phage growth were also tested at different pHs. HB cells were able to grow in buffered L-medium between pH 5 and pH 8.5. The phage adsorbed, became associated with the cells (as measured by the detergent escape assay), and was able to infect and lyse the cells within these pH limits).

Mixing of the virus and host membrane lipids

To investigate if mixing of viral and host membrane lipids occurs, as expected for a true membrane fusion, a pyrene phospholipid (PyrPG or PyrPC)-labeled, 95% TNB quenched *ϕ6hls* preparation was used. The infectivity of this phage preparation was decreased by 65%, but the adsorption and detergent escape properties remained normal. The phage was incubated with either sensitive (HB) cells, or resistant, non-adsorbing (MPO.16; no pili), or adsorbing (MP22.14; deficient in pilus retraction) mutant cells in a fluorometer cuvette. As shown in Figure 5, marked, time-dependent increase of the typical pyrene monomer fluorescence was observed with HB cells, while only a slight increase was observed with the adsorbing and non-adsorbing ϕ6-resistant mutants.

Especially with the mutant cells we observed considerable increase of the background fluorescence after 15 min of incubation with or without phage addition. Although the background interfered with the quantitation of pyrene fluorescence intensity with the mutants, this quantitation could be done with reasonable accuracy, since the emission spectrum of the interfering fluorophore(s) consisted of a broad, featureless peak readily resolvable from the sharp pyrene monomer peaks. Excitation spectra (not shown) recorded 25 min after mixing of the phage with the cells confirmed that only a minor increase of pyrene fluorescence had occurred with the mutants as compared to the wild-type cells.

Monitoring the appearance of alkaline phosphatase in the supernatant upon phage infection

To test whether the phage penetration induces leakage of periplasmic material into the extracellular supernatant the presence of the periplasmic enzyme, alkaline phosphatase (AP) activity was analyzed following infection with *ϕ6hls* at a multiplicity of 10 (although a maximum of two particles enter the cell). Uninfected HB cells, as well as cells of the phage-resistant strain MPO.16 with and without added phages, were used as negative controls. HB and MPO.16 cells treated with SDS and chloroform, to make the OM permeable, were used as the positive control. Although the treatment affects the viability of the cells, the cytoplasmic contents are not released. Also *ϕ6hls* alone was tested. The supernatant AP activity increased above the background level only with the SDS−chloroform-treated cells (Table III). The result was identical when a multiplicity of 50 was used (not shown). The infection of the HB cells was confirmed with the detergent escape test, and by growing the cells until lysis occurred.

Fusion-induced leakage was also tested by infecting cells in the presence of gramicidin. The ϕ6 host is resistant to this hydrophobic quasi-ionophore. If the cells are sensitized with polymyxin-B-nonapeptide (Vaara and Vaara, 1983) the ionophore eliminates further growth of the cells. When the HB cells were

Table III. Activity of alkaline phosphatase in the cell supernatant

Bacterial strain	Treatment (20 min, 23°C)	Absorbance[a]
HB	*ϕ6h1s*[b] in buffer[c]	0.019 ± 0.005
HB	Buffer[c]	0.016 ± 0.004
HB	SDS+chloroform[d]	0.234 ± 0.024
MPO.16	*ϕ6h1s*	0.019 ± 0.002
MPO.16	Buffer	0.022 ± 0.006
MPO.16	SDS+chloroform	0.167 ± 0.044

[a]Absorbance ($A_{414} - A_{540}$) of digested *p*-nitrophenyl phosphate after 19 h incubation at 22°C (six independent measurements).
[b]A multiplicity of 10 was used in the infection.
[c]10 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$.
[d]0.0025% SDS, 5% chloroform.

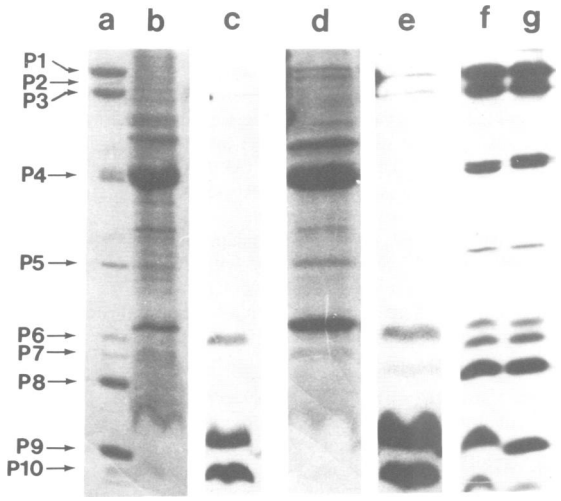

Fig. 6. *ϕ6h1s* proteins co-purified with the bacterial OM. The cells were infected with [^{14}C]amino acid-labeled *ϕ6h1s*, the outer membrane was isolated and analyzed by protein gel electrophoresis. Coomassie blue- stained *ϕ6h1s* standard (**lane a**); Coomassie blue-stained OM from infected Ro cells. The OM was partially purified by pelleting through a sucrose gradient (**lane b**); autoradiogram of lane b (**lane c**); further purified, Coomassie blue-stained OM from a CsCl density gradient (**lane d**); autoradiogram of lane d (**lane e**). [^{14}C]amino acid-labeled *ϕ6h1s* standard mixed with OM (**lane f**); and [^{14}C]amino acid-labeled virus standard. Note the effect of added OM on the mobility of lower mol. wt proteins and on the improved recovery of the P10 band.

infected with phage the growth of the cells, the onset of lysis and the phage yield were indistinguishable in the absence and presence of gramicidin (40 μg/ml), indicating that no ionophore had penetrated the OM upon infection. In the control [gramicidin (40 μg/ml) + polymyxin-B-nonapeptide (16 μg/ml)], no growth and no phage production occurred.

Localization of ϕ6 membrane proteins after infection

The phage-sensitive rough strain *P. syringae* pv. *phaseolicola* Ro49dRa1 (Ro) was used in the OM isolation experiments. The cells were infected with a multiplicity of 2. Twenty minutes post-infection ~30% of the initially adsorbed phage radioactivity was recovered in the cells after detergent wash. The cells were disrupted in a French pressure cell and the membrane fraction was collected through a sucrose gradient. The pellet obtained had the typical *P. syringae* OM protein pattern as analyzed by protein gel electrophoresis (Hurlbert and Gross, 1983; Romantschuk and Bamford, 1985; Figure 6b). This OM fraction also contained the *ϕ6* proteins P6, P9 and P10 in about the same ratios as in the phage particle. In addition, small amounts of P3 and P1 were associated with this fraction (Figure 6c).

The pelleted OM fraction was further analyzed in isopycnic sucrose and cesium chloride gradients. A single protein peak with the typical OM protein pattern was obtained in both cases. The phage membrane proteins P6, P9 and P10 were co-purified with these OM peaks. The protein composition of the CsCl peak is shown in Figure 6d, e. This fraction also contained the phage phospholipids. The presence of outer membrane material in the protein electrophoresis gel affects the mobility of some of the phage-specific proteins in a concentration-dependent way. The control lanes (Figure 6f and g) contain equal amounts of radioactive phage, but lane f also contains OM (about half as much as lanes b–e). The presence of OM is responsible for the mobility changes in fusion experiment gel, and the radioactive bands have to be compared to the OM containing standard (Figure 6f). When virus is electrophoresed alone, most of the P10 band diffuses out of the gel if the gel is stained, but the addition of OM considerably increases the P10 recovery. The protein ratio in lane f thus is close to the actual ratio in the virus (see also Figure 1).

Discussion

The evidence for the fusion between the envelope of phage *ϕ6* and the outer membrane of its host *P. syringae* are as follows. (i) The volume of the fusing compartments is additive as shown by the particle fusion assays (Figures 2 and 3), and by the thin sectioning of the cells infected with heat-inactivated particles (Figure 4). (ii) There is a time-dependent dilution of phage phospholipids during the infection, indicating the formation of a continuum between the phage and the host membrane (Figure 5). The possibility of a fusion-independent translocation of pyrene lipids between the two membranes is ruled out by the results obtained using phage-resistant adsorbing and non-adsorbing host mutants. With these only a minor increase of pyrene fluorescence was observed. (iii) The fusion event does not induce leakage of the periplasmic marker enzyme alkaline phosphatase to the medium (Table IV), or entry of gramicidin to the periplasmic space, implying that the continuity of the OM is not broken in the fusion process. (iv) The phage membrane proteins and phospholipids are found in the purified outer membrane of infected host cells (Figure 6).

The *in vivo* fusion kinetics, measured by phospholipid mixing, thin-section electron microscopy (Bamford *et al.*, 1976; unpublished results) and detergent escape assay (Romantschuk and Bamford, 1985) are equal. According to all these parameters the majority of the fusion events take place between 5 and 20 min post-infection.

The *in vivo* fusion takes place after the protein P3-dependent adsorption to the bacterial pili. Since P3 is anchored to protein P6, and missing in the particle whenever P6 is missing, the function of the *ϕ6* proteins involved in fusion could not be studied *in vivo*. For this purpose an *in vitro* particle fusion assay was developed. In this assay the fusion activity was greatly enhanced when protein P3 was removed. If protein P6, the hydrophobic membrane anchor for P3, was also removed, no fusion activity was observed. The $P3^-$, $P6^-$ particles did not fuse, even with particles containing either protein P6, or P3 and P6. The $P3^-$, $P6^-$ particles, however, were capable of fusing in the presence of PEG. The BHT-resistant mutants, with an alteration in P6, binding P3 more firmly, showed decreased fusion activity. As measured by the detergent escape assay, the fusion *in vivo* of the BHT-resistant mutants with the host outer membrane was also somewhat slower compared to the normal phage.

The results of the *in vitro* assay were consistent with those of *in vivo* fusion when a comparison was possible (independence of divalent cations and pH; the behaviour of *φ6h1s bht1*) supporting the relevance of the *in vitro* results.

The model emerging from these experiments involves an absolute requirement of protein P6 for fusion. Further, protein P3, which is not an integral membrane protein, has to be removed or dislocated to expose P6 to activate fusion. One cannot, however, rule out the possibility that other phage membrane proteins are also involved in the fusion process. The inability of $P3^-$, $P6^-$ particles to fuse with normal or $P3^-$ particles suggests that a fusion receptor is needed in the target membrane, resembling the requirement for cholesterol in the case of Semliki Forest virus (Kielian and Helenius, 1984). The φ6 fusion independency of divalent cations and pH resembles that of Sendai virus (Hsu *et al.*, 1981; Oku *et al.*, 1982); in both cases the virus envelope fuses with the external cell membrane from the outside milieu with a mechanism that is independent of both pH and divalent cation concentration. The conformational change necessary for all known viral fusion activation events seems in the case of φ6 to take place in the interaction between proteins P3 and P6.

This investigation shows that the prokaryotic fusion system studied here is conceptually similar to the well-studied eukaryotic virus fusion systems. The fusion target, the bacterial OM, however, differs structurally and functionally from the bacterial cytoplasmic membrane and the eukaryotic plasma membrane. The latter ones act as selective barriers, whereas the OM is a Donnan-type membrane, permeable to rather large solutes and constructed largely of porin complexes (Stock *et al.*, 1977, Engel *et al.*, 1985). A protein-mediated membrane fusion in prokaryotes has also been suggested in the studies of the proteinase-sensitive fusion of *Bacillus subtilis* membrane vesicles (Driessen *et al.*, 1985). The results presented here imply that targeted, protein-controlled fusion events are also important in prokaryotes.

Materials and methods

Bacterial strains, bacteriophages and media

P. syringae pv. *phaseolicola* HB10Y (HB) (Vidaver *et al.*, 1973) and the rough strain *P. syringae* pv. *phaseolicola* Ro49dRa1 (Ro) (K.Rudolph, Georg-August-Universität, Göttingen, FRG) were used as hosts for φ6. The suppressor host for the φ6 *sus* mutants was *P. pseudoalcaligenes* ERA S4 (S4) (Mindich *et al.*, 1976a). Two phage-resistant derivatives of HB were used as control strains. MPO.16 is non-piliated and non-adsorbing, whereas MP22.14 is super-piliated and adsorbs φ6 more efficiently than HB, but the pili are non-retractable and the phage envelope does not fuse with the MP22.14 outer membrane (Romantschuk and Bamford, 1985).

The phages used in this study are listed in Table I. L-broth (Maniatis *et al.*, 1982) was used as the rich growth medium, protein labeling was done in supplemented M9-medium (Romantschuk and Bamford, 1985) and lipid labeling in TG19 medium (Sands and Lowlicht, 1976). The solid media contained 15 g and top agar 6 g Bacto agar (Difco) per liter.

Production, radioactive labeling and purification of phage

HB was grown in L-broth to 6×10^8 cells/ml at 23°C with aeration. Phage was added to obtain a multiplicity of ~10. The *sus* mutant phage stocks were prepared using the suppressor strain S4. The virus was concentrated and purified as previously described (Bamford and Palva, 1980). The purified viruses were resuspended in 10 mM potassium phosphage pH 7.2; 1 mM $MgCl_2$ (buffer A) and used fresh or stored at −75°C.

For radioactive labeling of the virus proteins, the host was grown in supplemented M9-medium. At 40 min post-infection 10 μCi/ml of a [^{14}C]amino acid mixture (CFB.104, Amersham) or 100 μCi/ml of [^{3}H]leucine (TRK.510, Amersham) was added. In lipid labeling 20 μCi/ml of ^{32}P (PBS.11, Amersham) was added at the time of infection to cells growing in TG19 medium. Purification was done as with the unlabeled virus.

BHT treatment of the virion and isolation of BHT-resistant mutants

In order to remove protein P3 from the surface of purified viruses they were treated with 4 mM BHT (100 mM stock in ethanol) at room temperature for 10 min. The BHT-treated viruses were sedimented through a 5−20% (w/v) sucrose gradient in buffer A. The virus pellet was resuspended in buffer A. The BHT treatment was repeated once.

Viruses resistant to BHT were isolated by adding BHT (5 mM final concentration) to the top agar used in virus titration. BHT-resistant strains were isolated and purified by three subsequent single plaque isolations. Three independent virus mutant strains were collected.

In vitro fusion assay

Radioactively labeled and unlabeled virus preparatons were combined to obtain the desired specific activities and suspended in 50 mM potassium phosphate buffer pH 7.2, 1 mM $MgCl_2$. In the standard treatment the particles in a 300-μl reaction mixture were centrifuged in a Beckman Ti 50 rotor (25 000 r.p.m., 15 h at 15°C). The pellets were left to resuspend in their own buffer on ice for 4 h. By varying the conditions in the 300-μl reaction mixture the effects of EDTA, PEG, temperature and pH were tested. To analyze the fusion products the resuspended mixtures were centrifuged in a 5−20% (w/v) sucrose gradient in 10 mM phosphate buffer, pH 7.2 with appropriate ions (Beckman SW41 rotor, 23 000 r.p.m., 35 min, 15°C). The gradients were fractionated and the radioactivity of the fractions was measured.

Electron microscopy

For thin sectioning of the *φ6h1s* multiple particles, the viruses were fused in K-phosphate buffer, pH 7.2, 1 mM $MgCl_2$. Two fractions of the rate zonal sucrose gradient were collected. One containing the second and the third peak, and the other containing material sedimenting faster than the third peak. The fractions were pelleted in a SW 50.1 rotor for 2 h (35 000 r.p.m. at 5°C). The pellets were fixed with 3% glutaraldehyde and processed for thin sectioning as described previously (Bamford and Mindich, 1980).

For sectioning of the cells infected with multiple particles, HB cells were grown in L-broth to $\sim 6 \times 10^8$ cells/ml, infected with *φ6h1s* multiple particles, at a multiplicity of ~50. Ten minutes post-infection the infected cells were fixed with 3% glutaraldehyde in suspension for 20 min at room temperature before processing for thin sectioning.

Protein gel electrophoresis

Proteins were analyzed in 15% SDS−polyacrylamide gels, principally according to Laemmli (1970) but modified as described by Romantschuk and Bamford (1985). The protein gels containing ^{14}C-labeled virus material were soaked in Amplify (Amersham) before drying. Autoradiography was done with pre-flashed film at −75°C.

Triton X-114 partition of φ6 membrane-associated proteins

The partition experiments were done using Triton X-114 as described by Bordier (1981). Either the whole virus or protein P3 isolated with BHT were treated with Triton-114 and the protein content of the Triton and water phases were analyzed by protein gel electrophoresis, or in the case of isolated P3, by assaying the radioactivity.

Labeling of φ6 membrane with pyrene phospholipids

sn-1-Palmitoyl,2-pyrenedecanoyl-phosphatidylcholine (PyrPC) and -glycerol (PyrPG) were synthesized as previously described (Somerharju *et al.*, 1985). Egg yolk phosphatidylcholine was purified as described by Singleton *et al.* (1965), and phosphatidic acid was prepared from this lipid by phospholipase D treatment (Davidson and Long, 1958). Phosphatidylethanolamine (PE) was prepared from egg phosphatidylcholine by phospholipase D-catalyzed transphosphatidylation in the presence of ethanolamine and was trinitrophenylated as described by Gordensky and Marinetti (1973). 2,4,6-Trinitrobenzenesulfonic acid (TNBS) was obtained from BDH Chemicals. The non-specific phospholipid transfer protein was purified from beef liver essentially as described by Crain and Zilversmit (1980).

φ6h1s was labeled with PyrPC or PyrPG as follows: donor vesicles consisting of PyrPC/phosphatidic acid/TNB−PE (30/7/6 nmol) or PyrPG/egg phosphatidylcholine/TNB−PE (20/40/6 nmol) were prepared by injecting the lipids dissolved in 18 μl ethanol into 400 μl of 10 mM Tris−HCl, pH 7.4. After a 5-min equilibration period, 250 μg of *φ6h1s* and 3.5 μg of non-specific transfer protein were added and the mixture was incubated for 2 h at room temperature. Transfer of the pyrene lipids from the TNB−PE quenched donor vesicles to the phage particles was followed by recording the increase of pyrene monomer fluorescence. The degree of labeling was not determined directly but it can be calculated that the virus contained ~20% and ~9% PyrPC and PyrPG respectively. These calculations were based on the amounts of donor and acceptor lipid present in the incubation mixture and on the assumption that 70% of the donor and 50% of the acceptor lipids are in the outer, exchangeable pool. The phages thus labeled were then incubated with TNBS (1 mM in 100 mM $NaHCO_3$, pH 8.3) for 20 min at room temperature (Gordensky and Marinetti, 1973) in order to quench the pyrene fluorescence. The labeling conditions were chosen so that ~95% of the pyrene monomer fluorescence was quenched. Finally the phage

was purified by rate zonal centrifugation in a 5–20% (w/v) sucrose gradient. The pyrene-labeled, TNB-quenched phage preparation retained ~35% of the original infectivity, but had normal adsorption and detergent escape characteristics.

Lipid mixing assay

The PyrPC- or PyrPG-labeled, TNB-quenched *ϕ6h1s* was added to a washed bacterial suspension (6×10^8 cells/ml) to obtain a multiplicity of ~4 in M9-medium supplemented with 0.01 mM $FeSO_4 \cdot 7H_2O$ in a fluorometer cuvette thermostatted to 23°C. The pyrene emission spectrum was reduced immediately after phage addition and this value was used as a blank and subtracted from the subsequent spectra that were recorded at 5-min intervals up to 25 min. If fusion takes place between the viral and bacterial membranes, dequenching of pyrene phospholipid fluorescence is expected, since upon fusion the viral lipids are diluted by the bacterial membrane lipids whereupon the average distance between the pyrene moieties and the quenching TNB groups bound to phage lipids and proteins becomes too long for efficient energy transfer, i.e. quenching.

All fluorescence measurements were carried out with a Hitachi F-3000 fluorometer equipped with crossed polarizers to reduce light scattering by the cells.

Fusion leakage assay

Exponentially growing bacteria in L-broth (6×10^8 cells/ml) were pelleted and washed twice in 10 mM Tris–HCl, and resuspended in TG19 medium, without the phosphate supplement, to obtain 7×10^9 cells/ml. The bacteria were used alone or infected with *ϕ6h1s* (in 10 mM Tris–HCl, pH 7.5), at a multiplicity of 10. Cells treated with SDS (0.0025%) and chloroform (5%), to permeabilize the OM (Michaelis *et al.*, 1986), were used as positive control. This treatment affects the viability of the cells but releases only the periplasmic proteins from the cells. After 20 min incubation at 23°C the cells were removed by centrifugation and the supernatant was assayed for presence of alkaline phosphatase activity according to Michaelis *et al.* (1986).

Influence of pH and divalent cations on adsorption, fusion and growth of ϕ6 in vivo

Phage adsorption at different pHs was measured by resuspending exponentially growing bacteria in L-broth buffered with 50 mM Na-phosphate (pH 5–7) or 50 mM Tris-HCl (pH 7–8.5) to a density of 1×10^9 cells/ml. Adsorption and detergent escape efficiency was measured at 0.5 pH intervals as previously described (Romantschuk and Bamford, 1981, 1985). The cell lysis and phage production was also assayed at the different pHs. The pHs were always monitored during and after the incubation.

The requirement for divalent cations for adsorption and detergent escape was tested by growing the bacteria in M9-medium. At a cell density of 1×10^9/ml the cells were collected, washed once with unsupplemented M9 salt solution (no divalent cations), and resuspended in the M9 salt solution or in EDTA (5 mM) supplemented M9 salt solution. Adsorption and detergent escape were tested as described above.

Isolation of bacterial OM after ϕ6 infection

Ro cells were grown in L-medium to a density of 1×10^9 cells/ml. The cells were collected at 21°C and resuspended in fresh L-medium and incubated with agitation for 15 min. Radioactively labeled phages were added to obtain a multiplicity of 2 and the incubation was continued for 20 min. The culture was chilled on ice and the cells were collected by centrifugation at 4°C. Non-fused phages were removed with the Triton X-100 treatment. After washing the cells with L-medium they were resuspended in 1 ml of supplemented L-medium (20% sucrose, 0.5 M NaCl and 10 mM Tris–HCl, pH 7.9), and disrupted in a French pressure cell twice at 12 000 p.s.i. The suspension was layered on a 5–20% (w/v) sucrose gradient in 10 mM potassium phosphate pH 7.2, containing 0.5 M NaCl, and centrifuged in a Beckman SW41 rotor (24 000 r.p.m. for 65 min at 15°C). For isopycnic centrifugations the pellet was resuspended in 10 mM Tris pH 7.9, 20% sucrose.

The partially purified OM from the 5–20% sucrose gradient pellet was analyzed in both isopycnic sucrose (30–65% w/w) or in CsCl (3 ml 24% w/w, on top of 2 ml 48% CsCl) density gradients. The sucrose gradients were centrifuged in a Beckman SW41 rotor (35 000 r.p.m. for 24 h at 5°C), and the CsCl gradients in a Beckman SW50.1 rotor (40 000 r.p.m. for 36 h at 15°C).

Fractions were collected and their radioactivity was measured. The density was determined by measuring the refraction and/or by weighing known volumes. Lipid extraction was done according to Bligh and Dyer (1959). The material in the gradient bands was concentrated either with Centricon filter concentrators (10 000 mol.wt cutoff, Amicon) or by precipitation with 10% trichloroacetic acid and analyzed in SDS–polyacrylamide gels.

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References

Bamford,D.H. (1981) In Du Bow,M.S. (ed.), *Bacteriophage Assembly*. Alan R.Liss, New York, pp. 477–489.
Bamford,D.H. and Lounatmaa,K. (1978) *J. Gen. Virol.*, **39**, 161–170.
Bamford,D.H. and Mindich,L. (1980) *Virology*, **107**, 222–228.
Bamford,D.H. and Palva,E.T. (1980) *Biochim. Biophys. Acta*, **601**, 245–259.
Bamford,D.H., Palva,E.T. and Lounatmaa,K. (1976) *J. Gen. Virol.*, **32**, 249–259.
Berger,H. and Kennedy,K. (1980) *Biochim. Biophys. Acta*, **633**, 68–76.
Bligh,E.G. and Dyer,W.J. (1959) *Can. J. Biochem. Phys.*, **37**, 911–917.
Bordier,C. (1981) *J. Biol. Chem.*, **256**, 1604–1607.
Crain,R.C. and Zilversmit,D.B. (1980) *Biochemistry*, **19**, 1433–1439.
Davidson,F.M. and Long,C. (1958) *Biochem. J.*, **69**, 458–463.
Day,L.A. and Mindich,L. (1980) *Virology*, **103**, 376–385.
Doms,R.W., Helenius,A. and White,J. (1985) *J. Biol. Chem.*, **260**, 2973–2981.
Driessen,A.J.M., Hoekstra,D., Scherphof,G., Kalicharan,R.D. and Wilschut,J. (1985) *J. Biol. Chem.*, **260**, 10880–10887.
Engel,A., Massalski,A., Schindler,H., Dorset,D.L. and Rosenbusch,J.P. (1985) *Nature*, **317**, 643–645.
Gordensky,S.E. and Marinetti,G.V. (1973) *Biochem. Biophys. Res. Commun.*, **50**, 1027–1031.
Hsu,M., Scheid,A. and Choppin,R.W. (1981) *J. Biol. Chem.*, **256**, 3557–3563.
Hurlbert,R.E. and Gross,D.C. (1983) *J. Gen. Microbiol.*, **129**, 2241–2250.
Kakitani,H., Emori,Y., Iba,H. and Okada,Y. (1978) *Proc. Jap. Acad. Ser. A*, **54**, 337–340.
Kielian,M.C. and Helenius,A. (1984) *J. Virol.*, **52**, 281–283.
Kielian,M. and Helenius,A. (1985) *J. Cell Biol.*, **101**, 2284–2291.
Laemmli,U.K. (1970) *Nature*, **227**, 680–685.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York.
Michaelis,S., Hunt,J.F. and Beckwith,J. (1986) *J. Bacteriol.*, **167**, 160–167.
McGraw,T., Mindich,L. and Frangione,B. (1986) *J. Virol.*, **58**, 142–151.
Mindich,L. and Lehman,J. (1979) *J. Virol.* **30**, 489–496.
Mindich,L., Cohen,J. and Weisburd,M. (1976a) *J. Bacteriol.*, **126**, 177–182.
Mindich,L., Sinclair,J.F. and Cohen,J. (1976b) *Virology*, **75**, 224–231.
Mindich,L., Lehman,J. and Huang,R. (1979) *Virology*, **97**, 171–176.
Mindich,L., MacKenzie,G., Strassman,J., McGraw,T., Metzger,S., Romantschuk,M. and Bamford,D.H. (1985) *J. Bacteriol.*, **162**, 992–999.
Oku,N., Nojima,S. and Inoue,K. (1982) *Virology*, **116**, 419–427.
Romantschuk,M. and Bamford,D.H. (1981) *J. Gen. Virol.*, **56**, 287–295.
Romantschuk,M. and Bamford,D.H. (1985) *J. Gen. Virol.*, **66**, 2461–2469.
Sands,J.A. and Lowlicht,R.A. (1976) *Can. J. Microbiol.*, **22**, 154–158.
Sinclair,J.F., Cohen,J. and Mindich,L. (1976) *Virology*, **75**, 198–208.
Singleton,W.S., Gray,M.S., Brown,M.L. and White,J.L. (1965) *J. Am. Oil Chem. Soc.*, **42**, 53–56.
Somerharju,P., Virtanen,J., Eklund,K., Vainio,P. and Kinnunen,P. (1985) *Biochemistry*, **24**, 2773–2781.
Stitt,B.L. and Mindich,L. (1983) *Virology*, **127**, 459–462.
Stock,J.B., Rauch,B. and Roseman,S. (1977) *J. Biol. Chem.*, **252**, 7850–7861.
Vaara,M. and Vaara,T. (1983) *Nature*, **303**, 526–528.
van Etten,J., Lane,L., Gonzalez,C., Partridge,J. and Vidaver,A.K. (1976) *J. Virol.*, **18**, 652–658.
Vidaver,A.K., Koski,R.K. and van Etten,J.L. (1973) *J. Virol.*, **11**, 799–805.
White,J., Kielian,M. and Helenius,A. (1983) *Q. Rev. Biophys.*, **16**, 151–195.

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