Bacteriophage φ6 nucleocapsid surface protein 8 interacts with virus-specific membrane vesicles containing the major envelope protein 9

L. Peter Sarin\textsuperscript{a,b}, Jari J. Hirvonen\textsuperscript{a,b,d}, Pasi Laurinmäki\textsuperscript{c}, Sarah J. Butcher\textsuperscript{e}, Dennis H. Bamford\textsuperscript{a,b}, Minna M. Poranen\textsuperscript{a}\textsuperscript{*}

Department of Biosciences\textsuperscript{a} and Institute of Biotechnology\textsuperscript{b}, University of Helsinki, Biocenter 2, Viikinkaari 5, P.O. Box 56, 00014 University of Helsinki, Finland
Institute of Biotechnology\textsuperscript{c}, University of Helsinki, Biocenter 3, Viikinkaari 1, P.O. Box 65, 00014 University of Helsinki, Finland

\textbf{Running title:} P8-P9 interacts with virus-specific vesicles

\textsuperscript{*Current address:} Department of Clinical Microbiology, Vaasa Central Hospital, Hietalahdenkatu 2-4 B2, 65130, Vaasa, Finland

\textsuperscript{*Corresponding author:} Minna M. Poranen, Department of Biosciences, University of Helsinki, Biocenter 2, P.O. Box 56, 00014 University of Helsinki, Finland. Phone: +358-9-19159106, Fax: +358-9-19159098, e-mail: minna.poranen@helsinki.fi

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Abstract:

Enveloped dsRNA bacterial virus pseudomonas phage φ6 has been developed into an advanced assembly system where purified virion proteins and genome segments self-assemble to infectious viral particles, inferring the assembly pathway. The most intriguing step is the membrane assembly occurring inside the bacterial cell. Here we demonstrate that the middle virion shell, made of protein 8, associates with the expanded viral core particle and the virus-specific membrane vesicle.
Pseudomonas phage φ6 is an enveloped dsRNA virus of the family Cystoviridae (22, 32). The φ6 genome, which consists of three dsRNA segments (S, M, and L), is encapsidated within a nucleocapsid (NC) surrounded by a lipid membrane envelope derived from the plasma membrane of its host, the gram-negative plant pathogenic bacterium Pseudomonas syringae (1, 3, 10, 14, 31). The NC surface consists of protein 8 (P8) arranged in a T=13 icosahedral lattice with P4 at the vertices (3, 5, 10). The internal polymerase complex (PC) is composed of four protein species (P1, P2, P4, and P7) encoded by the L-segment (1, 13, 16, 17, 23). These proteins self-assemble into empty PCs that sequentially package the genomic precursor RNAs, which are converted into dsRNA by the internally-located RNA polymerase (1, 6-8, 24, 25, 28), causing a dramatic expansion of the particle (3, 19). The dsRNA-filled PC particles transcribe genomic dsRNA templates for synthesis of late proteins encoded by the S- and M-segments (26). According to the established model of the φ6 assembly pathway, P8 is subsequently assembled onto dsRNA-filled PCs (1, 20, 21, 24) and the newly formed NC acquires a virus-specific lipid envelope from the plasma membrane in a process assisted by the viral nonstructural protein 12 (1). P9, the major viral envelope-associated membrane protein, and the assembly factor P12 are the minimal components required to induce the formation of virus-specific vesicles (1, 11, 30). In this study we have set up a recombinant expression system (Fig. 1) to further characterize the interactions between P8, the PC, and the viral envelope.

Production of P8 requires additional φ6 proteins. Previously, P8 has only been obtained through sequential disassembly of purified virions (2, 21). We set out to acquire P8 by co-expression of its gene with other φ6 genes. Constructs derived from cDNA copies of the φ6 S- and L-segments (Fig. 1) were expressed in Escherichia coli JM109 cells for 16h at room temperature in L-broth supplemented with the relevant antibiotics following induction with 1mM...
isopropyl β-D-1-thiogalactopyranoside. The pelleted cells were disrupted with a French Pressure cell and the protein content was analyzed by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) (27). Recombinant P8 was recovered only when synthesized in concert with other φ6 proteins (Fig. 2); The recovery was most prominent when the gene was co-expressed with those encoding PC proteins, P9 and P12 (Fig. 2, lane 5), as determined by Western blotting using a P8-specific polyclonal antibody and chemiluminescent detection (27).

**P8 associates with P9-specific lipid vesicles.** Previous *in vitro* assembly studies have shown that purified P8 readily reassembles onto both *in vitro* constituted and virion-derived PCs (20, 21, 24). In the absence of other viral components, virion-derived P8 forms aberrant shell-like structures (4, 21). To further analyze P8 interactions the supernatant derived from the lysate of *E. coli* BL21(DE3) synthesizing P1-P2-P4-P7-P8-P9-P12-P14 proteins (lysate centrifuged at 9300 x g for 4 min) was subjected to rate zonal centrifugation (5-20% sucrose gradient in 20mM Tris-HCl pH 8.0, 150mM NaCl, 10mM MgCl₂; Sorvall TH641 rotor, 27k rpm, corresponding approximately 90 000 x g, 110min, 15°C). After centrifugation, the gradient was fractionated and the protein content was analyzed by SDS-PAGE. Interestingly, it appeared that some P8 co-sedimented with P9 (Fig. 3A). This slowly sedimenting P8-P9 particle and a faster P8-containing particle (Fig. 3A) were collected and subjected to equilibrium flotation centrifugation [Sorvall TH641 rotor, 40k rpm, 62h, 4°C; as described in (14)] to separate lipid-protein vesicles from proteinaceous viral particles. The gradients were fractionated and the samples were analyzed by SDS-PAGE (Fig. 3B,C). Samples for transmission electron cryo-microscopy were vitrified (3) and transferred to a Gatan 626 cryostage. Micrographs were recorded on a FEI Tecnai F20 electron microscope operating at 200 kV (nominal magnification ×50000). Equilibrium flotation centrifugation of the slowly sedimenting material showed a significant excess of P8 and P9 in the...
lipid-containing low-density fractions whereas PC proteins were less prominent (Fig. 3B). Cryo-electron microscopy confirmed the presence of membrane vesicles containing aberrant shell-like structures apparently consisting of P8 (Fig. 3D, panel I). Equilibrium flotation centrifugation of the faster sedimenting material revealed expanded PCs with a P8 shell enclosed in membrane vesicles, similar to virions (12) (Fig. 3C,D, panel II). The pellet was highly enriched with PCs in their typical unexpanded conformation (Fig. 3C,D, panel III).

**Pathway for nucleocapsid and envelope assembly.** Based on the above observations P8 can be recovered when its gene is co-expressed with those encoding the PC components (Fig. 2) and it readily associates with φ6-specific lipid vesicles (Fig. 3). Previous studies have shown that synthesis of P8 is required for envelopment of the virion, but interaction between P8 and the virus-specific vesicles during φ6 infection has not been detected (1, 29, 30). Apparently, the P8-vesicle interaction observed in *E. coli* (Fig. 3) may be suppressed in *P. syringae* host until P8 is assembled on the PC. Furthermore, assembly of the P8 shell *in vitro* has only been accomplished on dsRNA-filled expanded PCs (21). Accordingly, the empty compact form of the PC is not observed in the enveloped particles (Fig. 3). These observations suggest that assembly of the NC is regulated both by interaction with the expanded P1 shell in the PC and by interaction with the P9 containing lipid vesicle. Therefore, P8 assembly requires expansion of the metastable PC, which can occur either spontaneously or through sequential packaging and replication of the genomic precursors. The expanded PC provides the scaffold around which P8 and φ6-specific lipid vesicles assemble.

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References:


**Figure legends:**

**Figure 1:** Construction of pMH expression plasmids. The schematic figure depicts the genes expressed by (A) φ6 L-segment based vectors pMH2, pMH9, and pMH10, as well as (B) φ6 S-segment based vectors pMH4, pMH7, and pMH8. The gene numbers correspond to those of the proteins. The φ6 L-segment is derived from pLM687 (18) and the φ6 S-segment from pLM659 (9). The restriction enzyme sites indicated were used for cloning (pMH2 and pMH4) or truncation of the insert (pMH7, pMH8, pMH9, pMH10). Transcription of correct length mRNA from pMH4, pMH7, and pMH8 is ensured by a 3'-terminal transcription terminator (T7 term.). Synthesis of the lytic enzyme P5 is prevented by a 4× stop codon insertion in gene 5 in the pMH4 and pMH7 vectors. Constructs based on plasmid pGZ119EH (15) contain a chloramphenicol resistance gene, whereas pLM659-based vectors carry an ampicillin resistance gene.

**Figure 2:** Western blot detection of recombinant P8 from *E. coli* lysates. The '+' indicates that the corresponding gene was included in the DNA construct(s) expressed in *E. coli* JM109 cells. P8 obtained from purified bacteriophage φ6 virions (2) is included as a control (lane 1).

**Figure 3:** P8 associates with P9-specific lipid vesicles. Denaturing 15% polyacrylamide gel electrophoresis following rate zonal centrifugation of P1-P2-P4-P7-P8-P9-P12-P14 proteins derived from gene expression in *E. coli* BL21(DE3) (A) and equilibrium flotation centrifugation of slowly (B) and fast (C) sedimenting material. The position of specific fractions following centrifugation is depicted in the schematic drawings (top). Purified φ6 (2) is included as an
electrophoresis marker (bottom, lane φ6) and the relevant proteins are labelled. The P8-containing fractions (indicated as I–III) in (B) and (C) were further analyzed by cryo-electron microscopy (D, panels I–III). The schematic drawings depict the composition of particles representative for panels I–III. The scale-bar in panel III is equivalent to 100 nm (applies to panels I–III).