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Display of a PorA Peptide from *Neisseria meningitidis* on the Bacteriophage T4 Capsid Surface

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The exterior of bacteriophage T4 capsid is coated with two outer capsid proteins, Hoc (highly antigenic outer capsid protein; molecular mass, 40 kDa) and Soc (small outer capsid protein; molecular mass, 9 kDa), at symmetrical positions on the icosahedron (160 copies of Hoc and 960 copies of Soc per capsid particle). Both these proteins are nonessential for phage infectivity and viability and assemble onto the capsid surface after completion of capsid assembly. We developed a phage display system which allowed in-frame fusions of foreign DNA at a unique cloning site in the 5′ end of *hoc* or *soc*. A DNA fragment corresponding to the 36-amino-acid PorA peptide from *Neisseria meningitidis* was cloned into the display vectors to generate fusions at the N terminus of Hoc or Soc. The PorA-Hoc and PorA-Soc fusion proteins retained the ability to bind to the capsid surface, and the bound peptide was displayed in an accessible form as shown by its reactivity with specific monoclonal antibodies in an enzyme-linked immunosorbent assay. By employing T4 genetic strategies, we show that more than one subtype-specific PorA peptide can be displayed on the capsid surface and that the peptide can also be displayed on a DNA-free empty capsid. Both the PorA-Hoc and PorA-Soc recombinant phages are highly immunogenic in mice and elicit strong antipeptide antibody titers even with a weak adjuvant such as Alhydrogel or no adjuvant at all. The data suggest that the phage T4 *hoc-soc* system is an attractive system for display of peptides on an icosahedral capsid surface and may emerge as a powerful system for construction of the next generation multicomponent vaccines.

Since the report by Smith (30), the ability to display a foreign peptide on the surface of a viral capsid has emerged as a powerful strategy for a variety of biological investigations (21). The filamentous bacteriophages M13 and fd have been extensively used to display short random peptides (23) as fusion products of the minor capsid protein pIII (4, 22, 29) and, in some cases, as fusion products of the major capsid protein pVIII (13). The use of phage λ for peptide display has also been reported recently. The outer capsid protein gpD (20, 31) and the tail protein gpV (5, 18) were used in these systems. In all these phage display systems, the major focus has been to construct and screen either random short peptide libraries or cDNA expression libraries. Application of these systems for construction of recombinant vaccines has been limited (7, 19). Phage T4 offers some unique features that can be exploited for display of peptides on the capsid surface and for their potential use as multicomponent vaccines. The phage T4 capsid is composed of three essential capsid proteins: the major capsid protein gp23* (960 copies per phage particle) and the two minor capsid proteins gp24 (vertex protein; 55 copies per particle) and gp20 (portal vertex protein; 12 copies per particle). In addition, the outer surface of the capsid is coated with two nonessential outer capsid proteins Hoc (molecular mass, 40 kDa) and Soc (molecular mass, 9 kDa) (1). These proteins are located at symmetrical positions on the icosahedral lattice (1, 34). The important characteristics of these proteins, which are highly suitable for phage display, are that (i) Hoc and Soc are displayed at a high copy number, a combined total of about 1,120 copies per capsid particle (160 copies of Hoc and 960 copies of Soc [8–10]); (ii) these proteins are nonessential for capsid assembly and are added to the capsid surface after completion of capsid assembly but prior to DNA packaging; and (iii) elimination by mutation of one or both proteins does not affect phage productivity, viability, or infectivity. Apparently, these proteins provide additional stability to T4 phage under adverse conditions such as extreme pH or osmotic shock (3, 9).

The primary focus of this investigation is to address some basic questions about the development of a phage T4 display system which can then be used in a novel way for construction of multicomponent recombinant vaccines. Therefore, unlike the other display systems reported, in this study the following questions are analyzed from the context of using phage T4 as a recombinant vaccine. (i) Can foreign sequences be cloned into *hoc* and *soc* and are the fusion products assembled on the capsid surface? (ii) Would the assembled peptides be displayed in a form that is accessible for biological interactions? (iii) Can the peptide be displayed on an empty capsid rather than on phage? (iv) Can more than one peptide be brought together from independent clones and displayed on the same capsid surface? (v) Are the displayed peptides immunogenic and do they elicit peptide-specific antibody responses? A 36-amino-acid peptide corresponding to the loop 4 of PorA from *Neisseria meningitidis* is used as a model peptide to address these questions.

MATERIALS AND METHODS

Bacteria. *Escherichia coli* B40 (sup_I^+) (14) from our laboratory collection was used as a suppressor strain for all the phage amber mutants used in this study. *E. coli* P301 (*sup* minus) was used as the isogenic nonsuppressor strain. The nonexpression strain *E. coli* BL21 was used for the initial transformation of recombinant DNA constructs. The expression strains *E. coli* BL21(DE3) and BL21(DE3)pLYS-S (Novagen) were used for expression of DNA cloned in the pET vectors (32). *N. meningitidis* 8529 (subtype P1.3) and 99M (subtype P1.2) were kindly provided by W. Zollinger (Department of Bacterial Diseases, Walter Reed Army Institute of Research, Washington, D.C.).

Phage. Wild-type T4$^+$D and *hoc* mutant phages were obtained from stocks prepared in this laboratory. The phage mutants *24*amNG433, *24*amN65, *soc* mutant, and *17*tsL2 were kindly provided by Lindsay Black (Department of

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Biological Chemistry, University of Maryland at Baltimore Medical School, Baltimore).

Antibodies. Monoclonal antibodies against PorA subtypes P1.2 and P1.3 of *N. meningitidis* were kindly provided by W. Zollinger. Affinity-purified goat anti-mouse immunoglobulin G-alkaline phosphatase conjugate (Kirkegaard & Perry) was used as the second antibody in enzyme-linked immunosorbent assays (ELISA) and Western blotting. An affinity-purified anti-mouse immunoglobulin G–gold conjugate (beads, 15-nm diameter; EM Sciences) was used in the immunogold experiments.

Plasmids. The phage T7 vector pET-9D (Kan) (Novagen), which has an *Nco*I site and a *Bam*HI site for cloning downstream from the T7 promoter, was used for construction of the Hoc and Soc display vectors.

Recombinant constructions. The primer sequences used for PCR amplification of T4 or *Neisseria* DNA fragments are shown below. The positions of primer sequences on the T4 genome or *porA* can be obtained from previously published reports (15, 28). All the primers were designed with a 5′ tag (indicated in italics) for efficient digestion of the adjacent restriction site (note the presence of either the *Bam*HI site, the *Nde*I site, or the *Kpn*I site as shown in bold letters adjacent to the 5′ tag sequence). The recombinant constructs were first transformed into *E. coli* BL21. For expression of the cloned DNA, miniprep DNAs were prepared from the BL21 strain and were transformed into the expression strain *E. coli* BL21(DE3) or *E. coli* BL21(DE3)pLys-S (32).

Hoc display vector, pR*hoc*. The 5-kb DNA between the T4 map units 107 to 112 kb, consisting of three open reading frames (ORFs) including *hoc*, 38 kd, and *24*, was amplified by PCR with primers 5′-*CGGG***GGATCC**AGAGTAGCATG AGCTCCGATG-3′ and 5′-*CGGG***GGATCC**ATCATTCAAGTCATCAGTA G-3′ (12, 15). The amplified DNA, after digestion with *Bam*HI was cloned into the *Bam*HI-linearized and dephosphorylated pET-9D (32). The resulting construct and the orientation of the insert were established by digestion with appropriate restriction enzymes, marker rescue with *24*amNG465, and expression of Hoc (see Fig. 1).

Soc display vector, pR*soc*. The 1.7-kb DNA containing *soc* (17) was amplified by PCR with primers 5′-*CATG***CCATGGTACC**TGGTGGTGGAGCTAGTACT CGCGGTTATGTTAAT-3′ and 5′-*CGC***GGATCC**TTGCCTACTAATGGACC GTCAGGA-3′. The former primer included a *Kpn*I site (in bold letters) and a sequence corresponding to the polyglycine spacer (underlined) immediately after the ATG initiation codon of *soc* ORF. Digestion of the amplified DNA with *Nco*I and *Bam*HI gave rise to two fragments, a 0.45-kb fragment and a 1.25-kb fragment. The 0.45-kb fragment, which contained the *soc* ORF, was then cloned into the dephosphorylated *Nco*I-*Bam*HI fragment of pET9D.

pR*porA.hoc*. pR*porA.hoc* was constructed by cloning the PCR-amplified *porA* fragment into the *Nde*I site of pR*hoc* display vector. *PorA* fragment was directly amplified from a single colony of *N. meningitidis* 8529 (subtype P1.3) or 99M (P1.2) (both strains were kindly provided by W. Zollinger) with primers 5′-*GG AATTC***CATATG**TCACATCCGATCCGGGCTTGCC-3′ and 5′-*GGAATTC***CA TATG**CTCCGGCCCAAAACAGCAAGTCC-3′ (28). The DNA was cleaved with *Nde*I prior to cloning. This construction resulted in the addition of an extra TYA sequence at the N-terminal end of the PorA sequence. For expression of PorA-Hoc, this construct was transformed into *E. coli* BL21(DE3)pLys-S.

pR*porA.soc*. pR*porA.soc* was constructed by cloning the PCR-amplified *porA* fragment into the *Kpn*I site of the pR*soc* display vector. Primers 5′-*CGG***GGT ACC**TGCCCAAAACAGCAAGTCCGCCT-3′ and 5′-*CGG***GGTACC**CACATCC GATCCGGGCTTGCCGAC-3′ were used to amplify the *porA* fragment from a single colony of *N. meningitidis* 8529 (subtype P1.3). This construction resulted in the addition of an extra V at the N-terminal end of the PorA sequence. For expression of PorA-Soc, this construct was transformed into *E. coli* BL21(DE3).

Immunoreactivity of the displayed peptide. For immunoreactivity analysis (see Table 1), the recombinant phage carrying the displayed peptide were purified by differential centrifugation followed by CsCl step-gradient centrifugation. The empty capsids were purified by differential centrifugation followed by DEAE-Sephacel column chromatography (25). Fractions 2 to 5 in Table 1 represent the DEAE column fractions of the empty capsid peak as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The immunoreactivity was determined by ELISA (6). Each well was coated with about 10^9 phage particles. The P1.3 monoclonal antibody was used as the first antibody.

Immunogenicity of the displayed peptide. Male BALB/c mice (five per group) were injected with the CsCl gradient-purified PorA-Hoc or PorA-Soc fusion phages (5 to 10 μg per injection) at 2 (experiment 2)- or 3 (experiment 1)-week intervals (see Table 2). The fusion phages were generated by the second strategy described in Results. The T4*.porA*(P1.3).Soc phage preparation used in experiment 2 had roughly 10-fold-lower copy number of PorA-Soc compared to that in experiment 1. Each injection (100 μl) contained equal volumes of phage (in 50 mM Tris-Cl [pH 7.4]) and phosphate-buffered saline (no adjuvant), or complete Freund's adjuvant (CFA) or Alhydrogel as an adjuvant. All the samples except the ones with CFA were injected intramuscularly. The CFA samples were injected subcutaneously. Mice were bled through the tail vein on day 0 (preimmune serum) and at 2- or 3-week intervals (immune serum). No booster injections were given. Only the data for the sera obtained on day 42 after the primary immunization are shown. The peptide-specific antibody titers were determined by ELISA (6) by using 1 μg of loop 4 synthetic peptide per well as the coating antigen. The amino acid sequence of the synthetic peptide is identical to that of the displayed peptide except that it has an additional GC at the N terminus and a GCC at the C terminus.

Standard techniques. Conditions for PCR amplification of *porA* fragment from *Neisseria* or the *hoc* and *soc* DNA fragments from phage T4 have been described previously (28, 35). Restriction enzyme digestions, dephosphorylations, ligation reactions, and other recombinant DNA modifications were carried out according to the reaction conditions recommended by the manufacturer. Transformations were done by electroporation with a Bio-Rad electroporator. Cloned DNA under the control of the phage T7 promoter was expressed by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (0.4 mM) to the log-phase cultures at 37°C and shaking of the cultures for the indicated time periods (32). SDS-PAGE was performed according to the method described by Laemmli (16). Western blotting was performed according to the method of Towbin et al. (33). ELISAs were done in triplicate according to the basic procedure described by Engvall and Perlman (6). Immunogold electron microscopy (EM) was performed by the procedure described by Dunn (5).

RESULTS

Vector system. Two display vectors, a Hoc display vector and a Soc display vector, both of which allow in-frame fusion of foreign DNA to the 5′ end of either *hoc* or *soc*, were constructed (Fig. 1). The phage T7 vector system (32) was chosen for insertion of foreign DNA because it allowed the confirmation of the expression of the right fusion protein prior to the phage display experiments (Fig. 2 and 3).

The Hoc and Soc display vectors were designed to test two alternative strategies for expression and display of the fusion protein. With the Hoc display vector, the fusion would be first transferred into the T4 genome, expressed under the control of T4 promoter(s), and assembled onto the capsid during normal phage development. With the Soc-display vector, the fusion would be first expressed in *E. coli* from the T7 promoter and later assembled onto a *soc* mutant capsid during phage infection.

Target peptide. We chose a 108-bp DNA fragment corresponding to the 36 amino acids (amino acids 163 to 198 of the mature protein) of the class I porin, PorA, from *N. meningitidis* (subtype P1.3, which has been responsible for an epidemic of meningitis in Northern Chile [36]) as a target sequence for insertion into Hoc and Soc. The reasons for this choice are as follows. (i) Based on the recently developed structural model for PorA which traverses the outer membrane sixteen times, this sequence was predicted to correspond to loop 4, a loop that is most extended into the external environment (28); (ii) there is strong evidence that this loop constitutes one of the primary epitopes recognized by the human immune system since the bactericidal antibodies elicited in immune individuals were mapped to this epitope; and (iii) a battery of monoclonal antibodies specific to loop 4 from a number of subtypes of *N. meningitidis* are readily available (36).

Hoc display vector. The details of Hoc display vector (pR*hoc*) construction are described in Materials and Methods. It includes, in addition to the *hoc* coding sequence, a 884-bp flanking sequence from the 5′ end of *hoc* and a 3-kb flanking sequence from the 3′ end of *hoc*. The 3′ flanking sequence also contained the essential T4 gene *24*. These flanking sequences are included in the vector to facilitate the transfer of the *hoc-porA* fusion into T4 genome by homologous recombination (see below).

Expression of Hoc from pR*hoc* was tested by IPTG induction and SDS-PAGE (Fig. 2A). A 40-kDa protein, the expected size of Hoc, was expressed to a level of about 10% of the total cell protein (compare uninduced lane 1 with induced lanes 2 and 3). This clone also expressed a 38-kDa protein (lanes 2 and 3); this is consistent with the reported presence of an ORF of unknown function downstream from *hoc* (15). However, gp 24 was not expressed by this clone since the g24

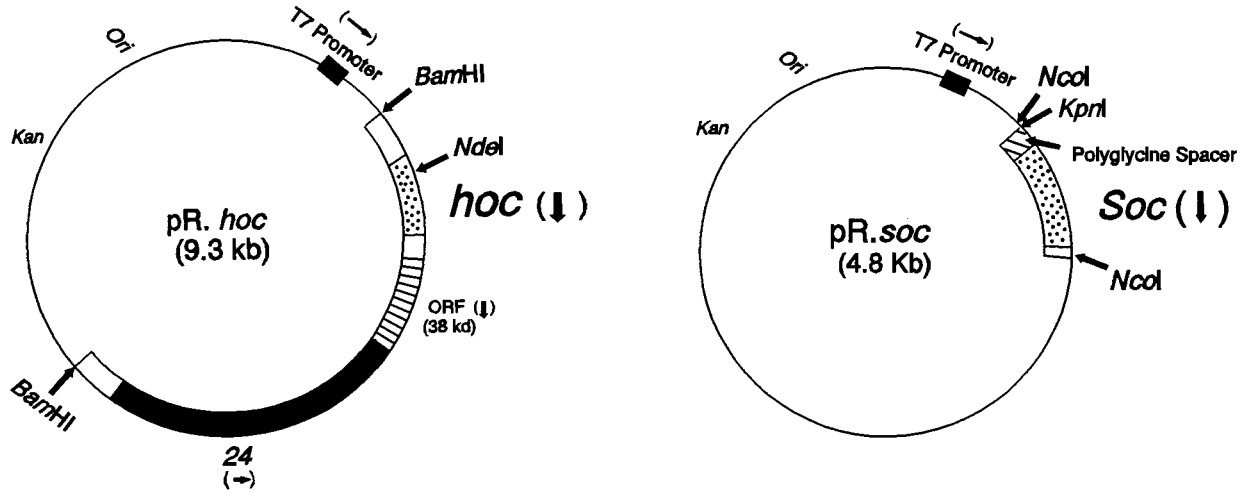

FIG. 1. Schematic diagrams of display vectors pR.*hoc*, the Hoc display vector, and pR.*soc*, the Soc display vector. See Materials and Methods for details on the construction of these vectors. Only the relevant features of the vectors are shown. The arrows in parenthesis indicate the direction of transcription from the T7 promoter. The plasmids are not drawn to scale.

ORF is in the opposite orientation relative to the direction of transcription from the T7 promoter (Fig. 1).

Construction of PorA-Hoc fusion phage. The 108-bp fragment corresponding to the loop 4 of PorA was amplified from a single colony of *N. meningitidis* and was cloned into the unique *Nde*I site of pR.*hoc*. The *porA* primers were designed in such a way that insertion in the right orientation would fuse the 36-amino-acid PorA peptide after the 36th amino acid of Hoc. Consistent with this, a clone containing the recombinant plasmid (pR.*porA.hoc*) produced a new 45-kDa protein (with the corresponding disappearance of the 40-kDa Hoc protein) upon induction with IPTG (Fig. 2B, compare lanes 4 and 5 with lanes 2 and 3). That this protein was indeed the PorA-Hoc fusion protein was further established by its strong reactivity with a loop 4-specific monoclonal antibody by Western blotting followed by immunostaining (Fig. 2C, lane 7; lane 6 represents a control induced extract containing no insert) (this monoclonal antibody was previously mapped to a "linear" epitope NGANNTI within the loop 4 sequence by Geysen pin analysis [28]).

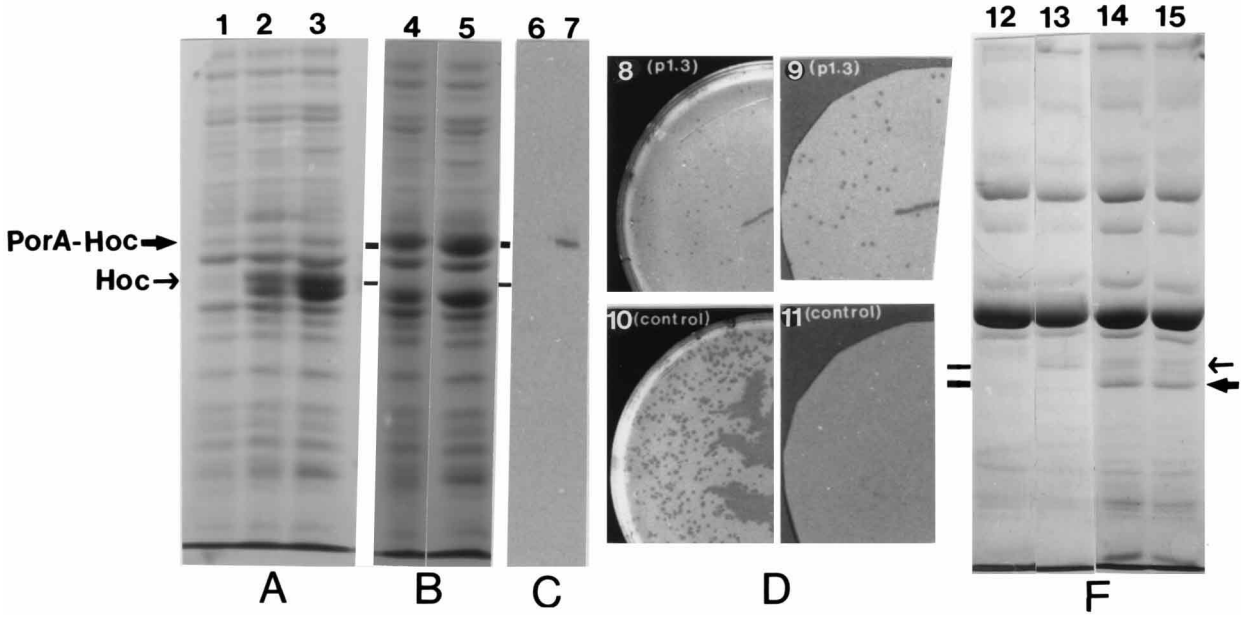

FIG. 2. Expression of PorA-Hoc fusion protein and its display on T4 phage. (A to C) Expression of Hoc and PorA-Hoc in *E. coli* BL21(DE3)pLys-S as analyzed by SDS–10% PAGE. Lanes: 1, uninduced pR.*hoc*; 2, pR.*hoc* induced for 30 min; 3, pR.*hoc* induced for 60 min; 4, pR.*porA.hoc* induced for 30 min; 5, pR.*porA.hoc* induced for 60 min; 6 and 7, Western blot of pR.*hoc* induced for 60 min and pR.*porA.hoc* induced for 60 min respectively, followed by immunostaining with the P1.3-specific monoclonal antibody. (D) Expression of PorA-Hoc in phage T4. Plates: 8 and 9, T4.*porA*(P1.3).*hoc* plaques; 10 and 11, wild-type T4 plaques; 8 and 10, plaques grown on Luria-Bertani plates; 9 and 11, the same plaques from plates 8 and 10 after transfer to a nitrocellulose membrane followed by immunostaining with the P1.3 monoclonal antibody. (E) Display of PorA-Hoc on phage T4. The phage preparations were purified by differential centrifugation followed by CsCl step gradient centrifugation. Lanes: 12, *hoc* mutant phage; 13, wild-type phage; 14, T4.*porA*(P1.3).*hoc* phage; 15, T4.*porA*(P1.2).*hoc* phage. Hoc and PorA-Hoc band positions are marked on the sides of the panels with thin and thick arrows (or lines), respectively.

The *porA-hoc* fusion was then transferred to phage T4 genome by infecting *E. coli* containing the pR.*porA.hoc* plasmid with *24*amNG465 and selecting for wild-type recombinants on *sup*-minus *E. coli* P301. Since the wild-type phage would be generated by a recombinational exchange near the gene *24* sequence in pR.*porA.hoc*, a fraction of these recombinants would also have exchanged the adjacent *porA-hoc* fusion sequence. Direct PCR of random plaques showed that about 30% of the plaques had recovered the *porA-hoc* fusion. The *porA-hoc* fusion, although originally under the control of T7 promoter, is now in the T4 genome and is controlled by the native *hoc* promoter. One of the PorA-Hoc fusion plaques [T4.*porA*(P1.3).*hoc*] was purified, and its ability to express the PorA peptide was tested by immunostaining of the plaques with the monoclonal antibody. The data, as shown in Fig. 2D, showed that virtually all the plaques in the fusion phage stock reacted strongly with the monoclonal antibody (compare the immunostained plaques in panel D9 with the unstained plaques in panel D8). On the other hand, none of the control wild-type plaques showed any reactivity (compare the immunostained filter in panel D11 with the unstained plaques in panel D10).

Display of PorA peptide on the capsid surface. To test whether the PorA-Hoc fusion protein is coated on the capsid surface, the T4.*porA*(P1.3).*hoc* phage particles were purified by CsCl density gradient centrifugation. If the PorA peptide is displayed on the capsid surface in an accessible form, it should interact with the monoclonal antibody. If so, phage particles coated on the microtiter plates should react with the monoclonal antibody in an ELISA. In a number of assays, the T4.*porA*(P1.3).*hoc* phage indeed showed strong reactivity with the monoclonal antibody (Table 1), whereas no reactivity was observed with the control wild-type phage (Table 1). In another control, the fusion phage did not show any cross-reactivity with a monoclonal antibody that is specific to a different PorA subtype P1.2 (data not shown).

The PorA-Hoc fusion protein displayed on the phage particles appears to be about 6 kDa smaller than the expected 45-kDa size (Fig. 2E, compare lanes 14 and 15 with lane 13). The same band appeared in both the T4.*porA*(P1.3).*hoc* and T4.*porA*(P1.2).*hoc* (see below) recombinant phages. However, the identity of this band as the PorA-Hoc is yet to be established. If it is indeed PorA-Hoc, it most likely represents a cleaved form of the fusion protein. But the fact that the phage particles showed strong immunoreactivity with the subtype-specific monoclonal antibody suggested that the displayed Hoc fusion protein (or a portion of it) retained the P1.3 epitope. It should however be noted that the full-length PorA-Hoc was displayed on the capsid by an alternative strategy in which the fusion protein was first expressed in *E. coli* and was then transferred to *hoc* mutant capsids (data not shown; see below for details of this strategy).

Multicomponent phage display. An attractive feature of the T4 system is that the high copy number of Hoc and Soc would allow the transfer and display of more than one fusion protein from independent constructions onto the same capsid by a mixed infection strategy. The same can also be done to manipulate the copy number of the displayed peptide by using wild-type phage in the mixed infection. To test this, a second PorA fusion phage [T4.*porA*(P1.2).*hoc*] displaying the loop 4 peptide from subtype P1.2 (36) was constructed by using the scheme described above. Display of the P1.2 peptide on the capsid surface was first confirmed by the reactivity of CsCl gradient-purified T4.*porA*(P1.2).*hoc* phage particles with the P1.2 monoclonal antibody (Table 1) but not with the P1.3-specific monoclonal antibody (data not shown). To test the mixed infection strategy, *E. coli* P301 was infected with a mixture of both T4.*porA*(P1.3).*hoc* and T4.*porA*(P1.2).*hoc* phages at a multiplicity of 2.5 each, and the progeny phage were purified by CsCl gradient centrifugation. The purified preparation was then tested for the presence of both epitopes with specific monoclonal antibodies. The data showed that the progeny phage reacted with both monoclonal antibodies (Table 1).

TABLE 1. Immunoreactivity of the displayed PorA peptide with the subtype-specific monoclonal antibodies[a]

T4 recombinant phage	Subtype-specific monoclonal antibody used	A_{495}
T4.*porA*(P1.3).*hoc*	P1.3	0.79
T4.*porA*(P1.2).*hoc*	P1.2	0.51
T4.*porA*(P1.3, P1.2).*hoc*	P1.2	0.25
T4.*porA*(P1.3, P1.2).*hoc*	P1.3	0.70
T4.*17*ts.*porA*(P1.3).*hoc* empty capsids		
Fraction 2	P1.3	0.03
Fraction 3	P1.3	0.79
Fraction 4	P1.3	0.86
Fraction 5	P1.3	0.69
T4.*porA*(P1.3).Soc	P1.3	0.91

[a] Immunoreactivity of the displayed PorA peptide was determined by ELISA with the purified recombinant phage as the coating antigen. The data were derived from a number of independent experiments, each of which was performed with a positive control (outer membrane capsule displaying the native PorA) and a negative control (wild-type phage T4). The negative control showed no reactivity with the monoclonal antibodies (the background absorbance was in the range of 0.02 to 0.05). The values (except the DEAE-fractions) represent means for triplicate assays. See Materials and Methods for more details.

Display on empty capsids. A unique feature of the T4 system is that both Hoc and Soc assemble onto the capsid only after capsid expansion but prior to DNA packaging. In fact, phage T4 is the only double-stranded DNA phage which produces expanded capsids in vivo (with assembled Hoc and Soc) when DNA packaging is arrested (2, 25). For the use of T4 recombinants as vaccines, it may be desirable to display epitopes on an empty capsid rather than on finished phage since the empty capsids will be devoid of DNA and numerous tail proteins. The absence of DNA would be highly desirable from the perspective of biosafety. The absence of tail proteins, on the other hand, may be either desirable if the tail components interfere in a negative way with the immune responses elicited towards the displayed epitope(s) or undesirable if the tail proteins offer favorable adjuvant effects.

To test whether the peptide can be displayed on the empty capsid, a packaging defective *17*tsL2 mutant was first crossed into T4.*porA*(P1.3).*hoc* followed by selection of a PorA-expressing *17*ts plaque [T4.*17*ts.*porA*(P1.3).*hoc*] by immunoscreening with the monoclonal antibody. Capsids were purified from cells infected with the T4.*17*ts.*porA*(P1.3)*hoc* phage at nonpermissive temperature by differential centrifugation and DEAE-Sephacel chromatography (25, 26). EM and SDS-PAGE showed that the purified capsids were empty and expanded and that the expected five major capsid proteins constituted the empty capsid (data not shown). ELISA of the peak capsid fractions showed a corresponding peak of immunoreactivity with the P1.3 monoclonal antibody (Table 1).

Soc-display vector. The details of the Soc display vector (pR.*soc*) construction are given in Materials and Methods. In this construct, a unique *Kpn*I cloning site as well as a 15-bp sequence corresponding to the amino acid sequence VPGGG was created at the 5′ end of *soc*. This polyglycine spacer was incorporated in order to create a structureless sequence and to minimize interferences between the displayed peptide and the Soc protein. Expression of Soc from pR.*soc* was tested by IPTG induction and SDS-PAGE. A 9-kDa protein, the size expected for Soc, was expressed only from the clones containing *soc* in the right orientation (Fig. 3A, lanes 1 to 3).

Cloning and expression of PorA-Soc fusion protein. The 108-bp *porA* fragment with a *Kpn*I site at the ends was directly amplified from *N. meningitidis* and inserted into the *Kpn*I site of the Soc display vector. A clone (pR.*porA*.*soc*) that has the insert in the right orientation was selected. In-frame fusion of the *porA* fragment will add the 36-amino-acid PorA peptide immediately after the ATG initiation codon followed by the 5-amino-acid polyglycine spacer at the N terminus of Soc. This was confirmed by the expression of a 14-kDa protein, upon induction, which reacted strongly with the P1.3-specific monoclonal antibody by Western blotting (Fig. 3B, compare lanes 4 and 5 with lanes 2 and 3) (Western blotting data not shown).

Display of PorA-Soc fusion protein on the capsid surface. As discussed above, this vector was designed to test an alternative strategy to display PorA-Soc on the capsid surface. In this, the PorA-Soc was first expressed in *E. coli* and was then allowed to assemble on the capsid surface during a *soc* mutant phage infection. Progeny phage [T4.*porA*(P1.3).Soc] were purified by differential centrifugation followed by CsCl banding and were analyzed by SDS-PAGE, Western blotting, and ELISA (Fig. 3C and D and Table 1). A number of conclusions can be drawn from these analyses. (i) The SDS-PAGE data showed that, as hypothesized, the PorA-Soc that was preexpressed in *E. coli* assembled onto the *soc* mutant capsids produced later in the infection, a new band corresponding to PorA-Soc position was seen only in the T4.*porA*(P1.3).Soc lane (Fig. 3C, compare lane 6 with the wild type and *soc* mutant controls [lanes 7 and 8, respectively]. Note that, as would be expected, the Soc band is missing in the *soc* mutant and the T4.*porA*(P1.3).Soc lanes. However, a closely migrating band of unknown origin is seen just above the PorA-Soc band in all the lanes). That this band was PorA-Soc was further demonstrated by the reactivity of this band with the P1.3-specific monoclonal antibody (Fig. 3D, compare lane 9 with the wild type and *soc* mutant controls [lanes 10 and 11, respectively]). (ii) One or two lower-molecular-weight bands, which are presumably the proteolytic degradation products of PorA-Soc but which reacted with the monoclonal antibody, were also seen in a number of experiments (note the presence of bands just below the PorA-Soc band in lanes 6 and 9). (iii) IPTG induction of PorA-Soc was not essential for production of T4.*porA*(P1.3).Soc phage; in fact, phage yields were greatly diminished by prior induction, presumably because the intracellular metabolite pools, depleted as a consequence of abundant transcription, could not support phage development. (iv) The bound PorA-Soc was stable during CsCl density gradient centrifugation (step gradient or equilibrium gradient centrifugation) followed by storage for several weeks at 4°C. (v) While some preparations showed as high as, or even greater than, the expected copy number of PorA-Soc per phage particle (Fig. 3), other preparations showed as low as 1/10 the expected copy number. This variability was most likely due to variations in the expression levels of PorA-Soc and/or the intracellular solubility of the expressed PorA-Soc (greater than the expected copy number was presumably due to binding of Soc aggregates, a likely consequence of abundant protein expression). (vi) The copy number of PorA-Soc could be manipulated by infecting the PorA-Soc-

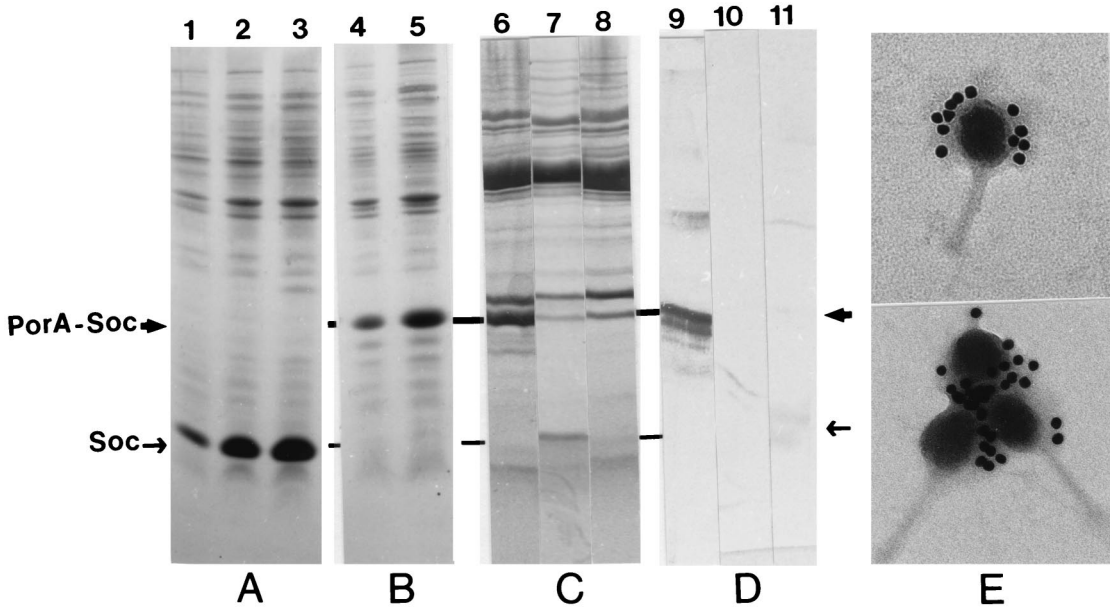

FIG. 3. Expression of PorA-Soc fusion protein and its display on T4 phage. (A and B) Expression of Soc and PorA-Soc in *E. coli* BL21(DE3) as analyzed by SDS–15% PAGE. Lanes: 1, uninduced pR.*soc*; 2, pR.*soc* induced for 30 min; 3, pR.*soc* induced for 60 min; 4, pR.*porA*.*soc* induced for 30 min; 5, pR.*porA*.*soc* induced for 60 min. (C and D) Display of PorA-Soc on phage T4. Lanes: 6, T4.*porA*(P1.3).Soc phage prepared from pR.*porA*.*soc* containing *E. coli* after infection with *soc* mutant phage, and the phage were purified by differential centrifugation followed by CsCl step-gradient centrifugation; 7, wild-type phage; 8, *soc* mutant phage; 9 to 11, same as lanes 6 to 8 but with Western blotting followed by immunostaining with the P1.3 monoclonal antibody (33). Soc and PorA-Soc band positions are marked on the sides of the panels with thin and thick arrows (or lines), respectively. (E) EM of negatively stained T4.*porA*(P13).Soc phage after treatment first with the loop 4-specific monoclonal antibody and then with the gold-conjugated anti-mouse second antibody (beads, 15-nm diameter; EM Sciences) according to the procedure described by Dunn (5).

expressing cells with both the wild-type and *soc* mutant phages. Since in these cells, both Soc and PorA-Soc are expressed (Soc from wild-type infection and PorA-Soc from the pR.*porA*.*soc* plasmid), both forms are expected to occupy the numerous sites available on the capsid surface. Indeed, SDS-PAGE analysis of the CsCl-purified phage from such a mixed infection showed the presence of both Soc and PorA-Soc bands (data not shown). (vii) ELISA analyses suggested that the PorA peptide is displayed in an accessible form since a number of independent CsCl gradient-purified phage preparations reacted strongly with the monoclonal antibody (Table 1). Immunogold-EM further showed cross-linking of phage particles in the presence of the monoclonal antibody and specific association of gold particles with the capsids but not with the tails (Fig. 3E).

Immunogenicity of the displayed peptide. The immunogenicity of the displayed peptide was tested by injecting purified PorA-Hoc or PorA-Soc fusion phage particles into mice either with no adjuvant or with CFA or Alhydrogel as an adjuvant. Induction of peptide-specific antibodies was analyzed by ELISA with the synthetic PorA 1.3 peptide as the coating antigen on microtiter plates. The data showed that high titers of peptide-specific antibodies were elicited with the recombinant fusion phages (Table 2), whereas no peptide-specific antibodies were detected either in the preimmune sera or in the sera of mice injected with the control *soc* mutant phage. The antibody titers were lower in the second experiment apparently because of the lower copy number of the PorA-Soc phage used for immunization (see Materials and Methods). It is particularly significant that Alhydrogel, a weak adjuvant approved for human use, also elicited high antibody titers. Furthermore, the PorA-Soc phage without any accompanying adjuvant also elicited comparable antibody titers. Finally, the PorA-Hoc fusion phage, which presumably have a lower copy number of the displayed peptide than the PorA-Soc phage, also elicited high antibody titers. This is consistent with the fact that Hoc is known to be a highly immunogenic component of the T4 capsid surface (9).

DISCUSSION

Icosahedral phages that assemble in the cytoplasmic milieu will have distinct advantages over the filamentous phages for display of peptides (21). Most importantly, these phages, unlike the filamentous phages, will be able to display peptides with various sizes and sequences in a much less restricted way. In phage T4, genetic and structural studies by Yanagida and coworkers (8, 9, 34) established that a combined total of 1,120 copies of Hoc and Soc are coated on the icosahedral capsid surface. Unlike the proteins used in the phage M13 display system, phage T4 Hoc and Soc are nonessential for phage infectivity and productivity and are added onto the capsid surface after the completion of capsid assembly. We reasoned, and showed in this study, that these distinctive features allowed the display of peptides not only on finished phage but also on intermediate empty capsids.

This study answered a number of basic questions. First, the data showed that fusions to the N terminus of either Hoc or Soc were tolerated and did not appear to disrupt capsid binding functions. Additional constructs are being made by incorporating features, such as the insertion of a longer spacer with multiple cloning sites and the addition of flanking cysteines to facilitate disulfide bridge formation, in order to display peptides in different structural contexts. Also, while this study was being prepared for publication, we learned that fusions to the C terminus of Soc were also tolerated and displayed on the capsid surface (27), suggesting that both termini of Soc can be used for peptide display.

Two basic modes of expression and display of the 36-amino-acid PorA peptide were tested. In one case, the fusion was transferred to the T4 genome by recombination, and the fusion protein was expressed in a native context during phage infection. In the second case, the fusions were first expressed in *E. coli* and were then transferred to either *soc* mutant or *hoc* mutant phage produced during infection. By the two criteria used, (i) cosedimentation of the fusion protein with the phage particles during CsCl gradient centrifugation and (ii) immunoreactivity of purified phage particles with specific monoclonal antibodies, both strategies allowed stable display of the peptide in an accessible form. In fact, phage recovered after two CsCl gradient centrifugations, or after storage for several weeks at 4°C, did not show a significant loss of the fusion protein from the capsid surface.

Limited proteolysis was observed with both strategies. Proteolysis is a common problem in phage display systems since the fusion protein is abundantly expressed and it "sticks out"

TABLE 2. Induction of PorA-specific antibodies in mice upon immunization with PorA-Hoc or PorA-Soc fusion phages[a]

Recombinant phage used for immunization	A_{405} at the dilution indicated			
	Expt 1		Expt 2	
	Preimmune (1:50)	Immune (1:1,000)	Preimmune (1:50)	Immune (1:100)
Control *soc* mutant phage with CFA		ND	<0	<0
T4.*porA*(P1.3).Soc with				
No adjuvant		ND	<0	0.58 ± 0.47
Alhydrogel	0.02	0.59 ± 0.30	<0	0.69 ± 0.52
CFA	0.01	1.85 ± 0.12	<0	0.61 ± 0.40
T4.*porA*(P1.3).*hoc* with				
Alhydrogel	0.01	0.39 ± 0.13		ND
CFA	0.02	1.34 ± 0.33		ND

[a] Immunogenicity of the displayed PorA peptide was determined by injecting BALB/c mice in groups of five with the purified recombinant phage. The peptide-specific antibody titers were determined by using a synthetic PorA peptide as the coating antigen. Each serum was serially diluted with the cold blocking buffer, and appropriate dilutions were titrated in triplicate assays. The absorbance data represent the values after subtraction of the blank in which the first antibody was omitted from the ELISA procedure. Each value represents an average for five serum samples from mice belonging to that group ± standard deviation. No data were excluded in calculation of the values shown. See Materials and Methods for more details. ND, not done.

on the capsid surface, making it an easy target for proteolysis. However, it should be noted that unlike the phage systems developed mainly for display of peptide libraries (21), limited proteolysis will be more tolerated for vaccine development because the main objective here is to allow the host system to elicit antibodies against all the possible epitopes of the displayed peptide. In the case of Hoc, it appears that the fusion protein synthesized during the T4 infection is susceptible to proteolysis whereas the fully assembled Hoc (as in the case of the overexpressed protein) is not. Additional vectors are being constructed to clarify this phenomenon and possibly overcome it. In the case of Soc, proteolysis was very limited and was not a significant issue. There is previous evidence suggesting that a portion of Soc is cleaved at the C terminus (even when expressed from T4) to a form that is 2 kDa smaller (27). Our results are consistent with this finding since the truncated PorA-Soc is immunoreactive with the peptide-specific monoclonal antibody in the Western blotting experiments (Fig. 3D). This would also imply that the N-terminal fusion strategy for Soc that has been developed in this study is more desirable than the C-terminal fusion strategy (27).

We have shown that T4 genetics imparts enormous flexibility to the manipulation of this display system. For instance, two PorA subtype peptides, the P1.2 and P1.3 peptides, can be brought together presumably onto the same capsid surface by a mixed infection with both the P1.3 and P1.2 fusion phages. This strategy also allowed manipulation of the copy number of PorA-Soc by a mixed infection with both the PorA-Soc and wild-type phages. While Maruyama et al. (18) described a conditional chain termination strategy to alter the copy number of the fusion protein, the mixed infection strategy described here offers a simpler yet more flexible way to alter the copy number or to display multiple peptides on the same capsid surface. We have also shown that the PorA peptide can be displayed on empty capsids rather than on finished phage particles. Extension of this strategy should allow the display of peptides on either the giant empty capsids (26) (under the genetic background of *23ptg* mutations) or the abortive polyheads (27) (under the genetic background of *20*am mutations).

Immunogenicity experiments with mice established that the displayed PorA is immunogenic. This is evident by the elicitation of peptide-specific antibodies upon injection of highly purified T4*.porA*.Soc and T4*.porA.hoc* phage particles. On the other hand, in earlier experiments, no detectable antibodies were elicited upon injection of mice with the synthetic peptide either alone or with Alhydrogel as an adjuvant (35a). More interestingly, the results seem to suggest that the phage T4 particle may have a favorable adjuvant effect on the immunogenicity of displayed PorA-Soc. For example, we found that the PorA-Soc phage was immunogenic in the presence of a weak adjuvant such as Alum or no adjuvant at all as it was in the presence of a strong adjuvant such as CFA. These responses were not unique to PorA-Soc since similar high antibody responses were also obtained against the other phage structural proteins (data not shown). Similar observations were also reported recently with the potyvirus (a plant virus) display system in which chimeric capsids carrying *Plasmodium* epitopes were found to be highly immunogenic in the absence of an adjuvant (11).

In conclusion, we have shown that the loop 4 PorA peptide from *N. meningitidis*, a peptide that is known to have significance in vaccine development, can be displayed on the phage T4 capsid surface and that the displayed peptide is immunogenic in mice. We believe that the distinctive features of the T4 *hoc-soc* system would, upon further development, make it an attractive general system for construction of multicomponent vaccines against infectious diseases. In cases where the vaccine target is known, the T4 system can be used to manipulate the number of peptides displayed on the capsid surface as well as the sequence, size, structural context, and copy number, for optimal elicitation of neutralizing antibody responses. Such a flexible customization of multicomponent vaccines has not yet been realized with any currently available systems, including the multiple antigenic peptides system (24) or other phage display systems.

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