J Microbiol Immunol Infect
2005;38:399-408

The receptor of an oyster juice-borne coliphage OJ367 in the outer membrane of *Salmonella derby*

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Received: July 15, 2005 Revised: August 9, 2005 Accepted: August 25, 2005

The objective of this study was to identify the receptor of OJ367, an oyster juice-borne bacteriophage, in *Salmonella derby* ATCC 6960. The crude receptor outer membrane (OM) fraction was prepared and examined from the total cell envelope (TCE) by differential extraction with N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES)-$MgCl_2$ and then with Triton-HEPES-ethylenediamine tetra-acetic acid buffers. The OM proteins (Omps) were isolated by diethylaminoethyl column chromatography to screen for receptor activity. A 45-kDa protein belonging to a minor Omp species, with phage neutralization ability, was eluted in a homogeneous form. It was a non-peptidoglycan-associated protein which was digestible by trypsin. Lipopolysaccharide had no influence on its receptor activity when coexistent in the diethylaminoethyl column fractions. An *S. derby* mutant resistant to lysis by phage OJ367 was isolated. The mutant not only showed decreased receptor activity in vitro when its TCE was tested but had an altered Omp profile. This implied that the 45-kDa Omp is involved as a receptor in coliphage binding; however, this role is affected by the expression of other Omps.

Key words: Bacterial outer membrane, coliphages, outer membrane protein, *Salmonella*, virus binding sites

Salmonella spp., Gram-negative rods in the family *Enterobacteriaceae*, are major food-associated pathogens and are related to outbreaks of human salmonellosis. They have been the most serious food-borne organisms of disease worldwide for decades [1].

The Gram-negative bacterial cell envelope is 3 layers thick, composed of a unique outer membrane (OM) surface structure as an outside envelope, an intermediate peptidoglycan (PG; murein), and an inner membrane (IM). The OM contains a highly asymmetrical lipid layer with lipopolysaccharide (LPS) located in the outer leaflet and phospholipid (PL) in the inner leaflet [2]. *Salmonella typhimurium* is known to contain 1.5×10^6 molecules of PL and 5.8×10^6 molecules of LPS per nm^2 section of OM, with the remaining area covered with OM proteins (Omps). The OM is involved in the interaction of the cell with exocellular macromolecular entities such as antibacterial agents, antibodies, complements, and phagocytes [3]. Most of the interactions are receptor-mediated events. Cell surface receptors play roles in the nonspecific diffusion of small molecules and are involved in the specific uptake of macromolecules as nutrients for growth and survival. Receptors on the OM are also required for infection by antibacterial agents such as bacteriophages and bacteriocins. The receptors responsible for bacteriophages adhering to the host bacterial cells introduce lysogenic or lytic effects [4].

Bacteriophages have been commonly used as an aid to diagnostic tools for the identification and taxonomy of bacteria since the 1970s [5]. The determination of the unique receptor function on the OM is thus critical for phage-bacteria interactions. *Escherichia coli* phage receptors have been extensively investigated in both in vitro and in vivo studies. In particular, advancement of knowledge concerning receptors for bacteriophage was due to the availability of bacterial mutants resistant to bacteriophages and host-range mutants of phages. Among the common major Omps of *E. coli*, OmpF, OmpC, OmpA, and PhoE proteins are known to be membrane receptor proteins for bacteriophages TuIa, TuIb, TuII*, and TC45, respectively [6,7]. They are classified into classes of either heat-modifiable (such as OmpA) or PG-associated proteins (porins such as OmpF, OmpC, and PhoE) [8]. Some phages (e.g., bacteriophage P1), which have broad host ranges among different genera of bacteria, have been used for lysogenic transduction in genetic studies. The common receptors

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on the cell membrane of this kind of bacteriophage are less-specific cell envelope components.

The OM protein profiles have shown similarities between individual groups of *E. coli* and *S. typhimurium*. In *S. typhimurium*, the major OM proteins are the OmpF (35 kDa), OmpC (36 kDa), OmpD (34 kDa), and OmpA (33 kDa) proteins which can also be classified into the same classes of heat-modifiable and PG-associated protein as found in *E. coli*. However, a major difference is that the 33 kDa *Salmonella* protein has not been found to act as a receptor for bacteriophages [8]. *S. typhimurium* OmpD, OmpF, and OmpC are also porins. Mapping data indicated that the 35 kDa (OmpF) porin of *S. typhimurium* is homologous to that of *E. coli* [9].

A coliphage, OJ367, was isolated from oyster juice by screening batches of oysters from a collection contaminated with *E. coli* and *Salmonella* strains at Hornpoint Laboratory, Cambridge, MD, USA. In addition to its specific *E. coli* host strain, MD367, it was also infected with a *Salmonella derby* strain (American Type Culture Collection [ATCC] 6960) [10]. Common phage receptors are proteins reported to be located on the OM as mentioned earlier; however, receptor-specific phages are seldom found to be capable of interacting with different genera of host bacterial cells. Thus, this particular kind of coliphage infection led to the objectives of this study of identifying and characterizing the phage receptor on this novel host. This work was conducted using the well-developed in vitro model system of phage receptor neutralization in conjunction with a phage-resistant mutant as a comparison, to purify, determine, and partially characterize the phage OJ367 receptor from the cell envelope fractions of *S. derby*.

Materials and Methods

Materials

All microbial media were purchased from Difco (Detroit, MI, USA). All chemicals were reagent grade and were purchased from Sigma Chemicals Co. (St. Louis, MO, USA).

Microorganisms, culture media, and growth conditions

The *Salmonella* strain *S. derby* was obtained from the ATCC 6960. *E. coli* JF568, a K-12 strain from the collection in Prof. Tuu-jyi Chai's laboratory at Hornpoint Laboratory (Cambridge, MD, USA), was used as a standard strain with a known Omp profile [11].

Bacterial strains were maintained at 4°C on protease peptone-beef extract (PPBE) agar which contained 1.5% agar in PPBE broth (1% proteose peptone no. 3, 0.2% beef extract, and 0.5% NaCl, w/v). A loop of strain was grown overnight in 100 mL PPBE broth at 37°C. Then 0.8 mL of overnight culture was inoculated into 800 mL of PPBE broth per flask on a rotary shaker at 250 rpm to an optical density (OD) at 650 nm (OD_{650}) of 0.5-0.6. Cells were centrifuged at 5000 × *g* for 10 min at 4°C to recover the cell pellet.

Preparation of total cell envelopes

The cell pellet was resuspended in 20 volumes of 0.85% saline and centrifuged at 5000 *g* for 10 min to remove the residual medium. The pellet was then resuspended in 20 volumes of Tris-HCl buffer (50 mM Tris-HCl; pH 7.8) and centrifuged again to remove any residual NaCl. The final pellet was then suspended in 20 volumes of Tris-HCl buffer and disrupted by passing the suspension through a precooled French Press cell twice at 18,000 psi. The clear mixture of disrupted cells was ultracentrifuged at 200,000 *g* for 90 min. The supernatant was the Cyt (cytoplasmic) fraction, and the pellet was resuspended in a small amount of Tris-HCl buffer as total cell envelope (TCE) [11,12].

Preparation of OM extracts from the cell envelope

A 2-stage extraction procedure was used to prepare the OM extract from TCE with a N-(2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid (HEPES)-Triton-based buffer system (HT buffer, 10 mM HEPES, and 2% Triton X-100; pH 7.2) [13]. Each extraction was done by extruding the membrane suspension successively through hydrodermic needles with decreasing pore size (no. 18, no. 23, then no. 25 gauge) at room temperature (RT), followed by centrifugation at 200,000 *g* for 45 min at either 4°C (first stage) or RT (second stage).

The first stage was to remove the inner IM while keeping the OM intact. A protein concentration of 20 mg/mL membrane was used to perform the extractions with HTM buffer (HT buffer containing 10 mM $MgCl_2$) twice. Then, the extraction used only HT buffer to pull the residual IM into the supernatant. The HT-insoluble pellet fraction was resuspended in HTE buffer (containing extra 5 mM ethylenediamine tetra-acetic acid [EDTA] in HT) and was subjected to the 2nd stage of extraction to solubilize the Omps. The extraction was performed 4 times with HTE buffer

to extract the Omps into the supernatant. The resulting solubilized Omps (OM-1-4) and the above HT-solubilized IM supernatant (IM-1-3) were precipitated by adding 2 volumes of ice-cold 95% ethanol, allowed to stand at –20°C overnight, and collected by low-speed centrifugation. The final HTE-insoluble portion was the residue.

Purification and isolation of Omps by chromatography

Swollen Whatman DE-52 resin (460 mL) was pre-equilibrated with column buffer, TTE (Tris-N-tris [hydroxymethyl]methyl-3-aminopropanesulfonic acid-EDTA) buffer (0.05 M Tris-HCl buffer, 2% Triton X-100, and 5 mM EDTA; pH 7.2), and packed into an open column system (100 cm height × 2.5 cm; outer diameter) [14]. The column flow rate was controlled to <1 mL/min at RT. The ethanol-precipitated OM extracts were pooled and resuspended in TTE buffer at a final concentration of <20 mg protein/mL by passing them through a no. 25 gauge needle, and these were used as the samples. After sample loading, the column was first washed with TTE buffer to remove any non-charged proteins. Then, the bound Omps were eluted with 2 salt gradients, 0-0.1 M NaCl and 0.1-0.5 M NaCl in TTE buffer. Each 5-mL fraction was collected by a fraction collector. A small amount (50 μL) from 1 in 5 eluted fractions was assayed for protein concentration. The protein-containing fractions were further collected by concentration with Amicon-30 and then by precipitation with 2 volumes of cold 95% ethanol. The ethanol-precipitated Omps were suspended in 30 mM phosphate buffer (pH 7.0) for LPS and protein determinations, and phage neutralization analysis. The protein purity was analyzed by gel electrophoresis.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Proteins in the TCE, IM, OM, and diethylaminoethyl (DEAE) fractions were analyzed by a discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) system of 16 or 32 cm in height with a 0.75- or 1.5-mm thickness according to standard procedures [15]. The separation gel contained 11.5% acrylamide, 0.21% N,N'-methylene-(bis) acrylamide (BIS), 0.1% SDS, 3.0% urea, 0.04% N,N, N,N'-tetramethylethylenediamine (TEMED), and 0.015% ammonium persulfate in separation gel buffer (0.375 M Tris-HCl; pH 8.8). The stacking gel contained 0.0005% riboflavin, 2.5% acrylamide, 0.625% BIS, and 0.1% SDS in stacking gel buffer (0.125 M Tris-HC1; pH 6.8).

Protein samples were solubilized by heating for 5 min at l00°C in sample buffer which was composed of 12.5% glycerol, 2.5% SDS, 1.25% 2-mercaptoethanol, and 0.001% bromophenol blue in stacking gel buffer (pH 6.8). The harvested gel was stained in 0.05% coomassie brilliant blue G in 50% methanol and a 10% acetic acid solution and then destained in 10% acetic acid. A molecular weight standard was run for comparison containing the 66.2-kDa bovine serum albumin, 45-kDa egg albumin, 36-kDa glyceraldehyde-3-phosphate, 29-kDa carbonic anhydrase, 20.1-kDa trypsin inhibitor, and l4.2-kDa lactalbumin.

Preparation of bacteriophages

Bacteriophage OJ367 was isolated by J. A. Oriani (Food Science Department, University of Maryland, MD, USA) from oyster liquor [10]. The phage stock was prepared by mixing 0.2 mL of phage lysate (10^8-10^{10}/mL) with 10 mL early logarithmic-phase culture of the host cells ($2 \times l0^8$ cells/mL). The phage-host mixture was incubated with vigorous aeration for 2 h at 37°C or until visible lysis occurred. The lysate was sterilized with chloroform and stored over chloroform at 4°C. Titers were determined by the overlaid soft agar technique [16]. The soft agar was comprised of 0.75% agar in PPBE. Bacteriophage TuIb, TuIa, and TUII* from Prof. Tuu-jyi Chai's laboratory were used to test the receptor activity of *S. derby*.

Receptor activity-phage neutralization

Phage neutralization of various extracted membrane preparations or Omp fractions was determined by mixing 0.05 mL of a bacteriophage suspension containing 3×10^3 phage particles in PPBE broth and 0.05 mL of a test sample in 30 mM phosphate buffer (pH 7.0). The 0.1 mL mixture was incubated at 37°C with slow shaking for 2 h. One drop of mid-logarithmic-phase host culture and 2.5 mL of soft agar was added. The entire mixture was overlaid on fresh PPBE agar to determine the viable bacteriophages. The receptor activity in this study was defined as the percentage of the number of viable phage particles inactivated by a certain amount of phage receptor protein.

Isolation of phage-resistant mutants

To isolate *S. derby* mutants resistant to phage infection, 0.3 mL phage lysate containing $l0^5$-10^8 phage particles was mixed with 0.1 mL of early-phase host cells

($l0^5$-10^8 cells) at 35°C for 20 min. The mixture was spread on a PPBE agar plate and allowed to dry before an overnight incubation. Then the phage-resistant cells were chosen by picking small non-mucoid single colonies and transferring them into PPBE broth for overnight growth. The isolated phage-resistant mutants were confirmed by a cross-test in which the overnight resistant cell cultures were cross-streaked with phage lysate ($l0^8$ phages/mL) on a plate. The phage OJ367-resistant single colonies were again selected for their complete resistance ability and were then grown overnight. The overnight culture was overlayed with $l0^{10}$ phage particles again to confirm the phage resistance. The TCE of the final resistant cells was analyzed by SDS-PAGE to compare protein profiles and phage neutralization.

Preparation of LPS- and PG-associated membrane proteins

LPS was prepared by the hot-phenol-water method [17]. Briefly, cell pellets suspended in hot water (68°C) at 1: 3.5 (w/w) were extracted with 17.5 volumes of 68°C-preheated 90% phenol for 15 min in a 68°C water bath. The extract was cooled in an ice bath until the appearance of the phenol/water phase, followed by centrifugation at 10,000 *g* for 45 min. The LPS-containing aqueous phase was saved, and the phenol phase was re-extracted. The final aqueous solution was dialyzed at 4°C for 4 days against deionized water (DIW) with the DIW being changed every 6 h and then lyophilized. The lyophilysate was dissolved in water (3% w/v) and centrifuged at 105,000 *g* for 7 h. The pellet was washed 3 times by repeatedly redissolving it in water and centrifuging it at 105,000 *g* for 3 h. The final LPS pellet was dissolved in a small amount of water and lyophilyzed.

The TCE was suspended in extraction buffer [2% SDS, 10 mM Tris-HCl (pH 7.3), 0.7 M 2-mercaptoethanol, and 10% glycerol] at a ratio of 20:1 (w/v) for incubation at 60°C for 30 min. The mixture was centrifuged at 45,000 *g* at RT. The insoluble pellet was the PG-associated membrane protein fraction [18].

Chemical analysis and treatment

Total protein

Total protein was determined by the Lowry method [19]. A 1-mL portion of mixed reagent containing 3% Na_2CO_3 in 0.1 N NaOH: 4% sodium potassium tartrate: 2% copper sulfate (98:1:1) was added to 0.1 mL of the test sample. Then the 1.1-mL mixture was incubated for 10 min at RT, followed by the addition of 0.1 mL of 1.0 N phenol reagent. After a 20-min reaction at RT, the OD was read at 660 nm. A calibration curve was constructed using bovine serum albumin as the standard. Any sample precipitated with the Lowry reagent (e.g., by HEPES-Triton) was centrifuged before testing the absorbance.

LPS content

The LPS content was determined by the KDO (2-keto-3-deoxy-octonate) method [20]. KDO is a conserved sugar in the R-core region of LPS. A 50-μL sample was suspended in 0.05 mL of 0.5 N H_2S0_4 and hydrolyzed at 100°C for 12 min to release the KDO. After cooling to RT and adding 0.05 mL of 0.1N HIO_4, the mixture was heated to 55°C for 10 min again and then cooled to RT. 200 μL of 4% $NaAsO_2$ in 0.5 N HCl was added and shaken intermittently for 2 min. After the mixture appeared colorless, 0.8 mL of 0.6% thiobarbituric acid was added and heated for 10 min at 100°C, and then cooled to RT. During cooling, 1 mL dimethyl sulfoxide was added to the hot sample. The OD at 548 nm was determined. A standard curve was prepared within a range of 0-60 nmol KDO solutions each time the assay was run.

Trypsin treatment

An adequate quantity (1 mg/mL) of receptor proteins in 5 μL was incubated with 0.25 mL of bovine trypsin (1 mg/mL) for 30 min at 37°C. Trypsin degradation was terminated by the addition of 10 times the amount of a soybean trypsin inhibitor. After incubation for 5 min, the samples were heated to 100°C for 5 min. Then, the same volume of the double-strength sample buffer was added. The samples were boiled again for 5 min, and used for SDS-PAGE.

Results and Discussion

Effect of culture age on the protein profile of the TCE in *S. derby*

Cell envelope protein compositions vary depending on the bacterial strain, growth medium, temperature, and culture age [13,21]. Before the IM and OM preparations, the influence of the growth phase on the TCE protein profile of *E. coli* MD367 was examined to compare it with that of the JF568 standard strain of *E. coli*. In *S. derby* (Fig. 1), no visible difference was found in the major proteins with sizes between 35 and 40 kDa, except that the 24- and 18.5-kDa proteins became denser

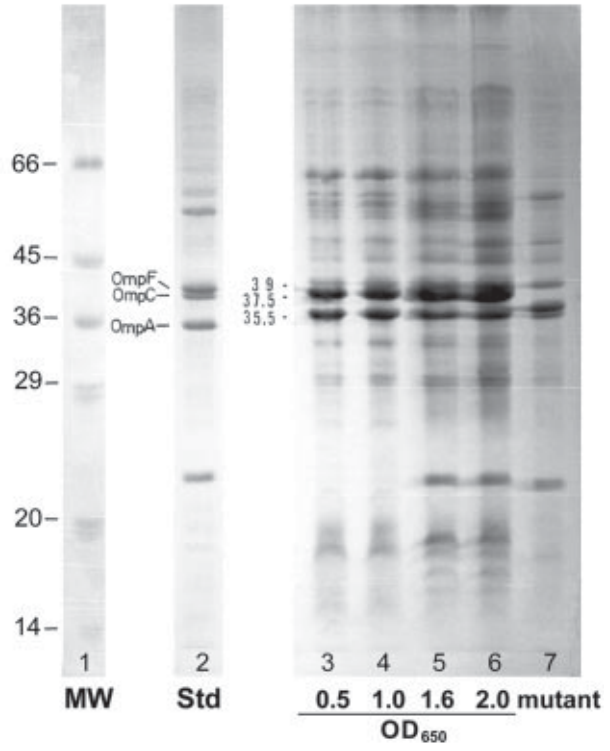

Fig. 1. Effect of culture age on the protein profile of the total cell envelope (TCE) in *Salmonella derby*. Lane 1, molecular weight (MW) standard (Std); lane 2, TCE of *Escherichia coli* JF568 as the outer membrane protein standard; lanes 3-6, TCE profile of *S. derby* harvested at optical density at 650 nm (OD_{650}) values of 0.5, 1.0, 1.6, and 2.0; lane 7, *S. derby* mutant (OD_{650} 1.6) which was resistant to phage OJ367. Lane 1 was loaded with 100 μg standard proteins, and lanes 2-7 were loaded with 250 μg of TCE proteins in a 30-cm-long, 1.5-mm-thick sodium dodecyl sulfate polyacrylamide gel electrophoresis.

after the early stationary phase (Fig. 1, lane 5, OD 1.6). The mid-logarithmic phase was chosen for use in receptor isolation and identification. This decision was made to avoid unexpected changes in the level of the receptors and difficulty in its purification when cells aged. Therefore, *S. derby* was grown in 9.6 L PPBE broth at 37°C until an OD_{650} of 0.5-0.6. About 9.4 g of wet cells was collected. After applying the French Press and ultracentrifugation, 4.6 g total wet weight of TCE was obtained.

Isolation of OM proteins

In the cell envelope fractions, Omps were isolated by extracting the IM twice with HTM buffer (IM-1 and IM-2), and once with HT buffer (IM-3) from TCE. The OM was then collected by 4 extractions with HTE buffer (OM-1-4) from the remaining TCE. This procedure provided differential solubilization of the Omps into the OM extract [22]. Analysis of the protein mass balance showed about 33% of the proteins of the starting TCE were extracted into the IM fractions, while the 4 HTE-soluble OM fractions contained about 51% proteins. About 11% of the HTE-insoluble protein was left in the final residue. The entire procedures resulted in the loss of about 4.9% of the membrane proteins. Thus, *S. derby* contained a higher quantity of protein in the OM than in the IM.

LPS analysis showed that there was an average of about 13.2% LPS in the Cyt fraction and 14% LPS in the IM fractions. This result showed that some LPS might not have been tightly bound with the OM proteins, or it might have been due to the effect of Tris which caused the loss of the exterior LPS into the IM fractions even though magnesium cations were used to neutralize the negative charges of LPS and reduce the electrostatic repulsion to protect the integrity of the OM [23]. Nikaido and Vaara [4] indicated that Tris is a bulky primary amine which can destabilize the membrane architecture by partially replacing other cations bound with LPS. By examining a pore-deficient mutant, Nogami and Mizushima [24] also reported that LPS was easily removable by 0.12 M Tris buffer.

Protein profiles of different cell envelope fractions

Protein profiles in Fig. 2 show that the 3 IM fractions comprised both high- and low-molecular-weight proteins of similar-sized polypeptides. Some proteins appeared unique to IM, while some proteins had the same mobility in both the IM and OM on the SDS gel. However, the same molecular size might not be the same protein species [25], and there may have been cross-contamination during the extraction and separation processes. Although the IM had a smaller quantity of protein, it had more protein species than the OM fractions in the TCE of *S. derby*.

It is known that the OM of the *Enterobacteriaceae* contains a set of unique proteins that differ from those of the IM and Cyt fractions in the molecular weight range of 33,000 to 40,000, and also have relatively simple patterns [26]. In *S. derby*, the OM protein also contained the 33-40-kDa proteins which included the 39- (OmpF-like), 37.5- (OmpC-like), and 35.5-kDa (OmpA-like) proteins. Thus, those major proteins of TCE were also the major proteins in the OM and amounted to approximately 60% of the TCE proteins.

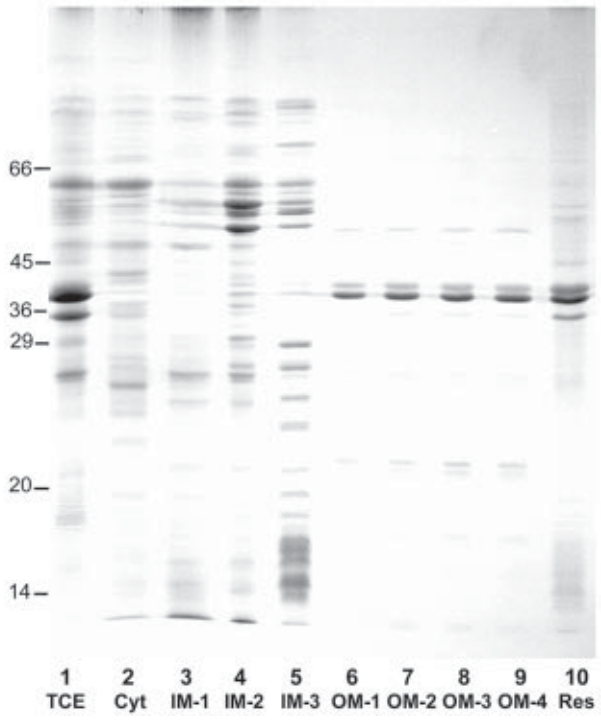

Fig. 2. Protein profiles of different cell envelope fractions (loaded with appropriate protein concentration and run in a 16-cm-long, 0.75-mm-thick sodium dodecyl sulfate polyacrylamide gel electrophoresis). Lane 1, total cell envelope (TCE); lane 2, cytoplasmic (Cyt) fraction (cell envelope Tris-supernatant), Cyt membrane; lanes 3-5, first inner membrane fraction (IM-1), second inner membrane fraction (IM-2), and third inner membrane fraction (IM-3); lane 6-9, first outer membrane fraction (OM-1), second outer membrane fraction (OM-2), third outer membrane fraction (OM-3), and fourth outer membrane fraction (OM-4); lane 10, final HTE-insoluble residue (Res).

In addition, some unextractable proteins remained in the residual pellet.

Receptor activity in cell envelope fractions

Before purification of the phage receptors from the membrane, receptor activity was confirmed by phage inactivation with 40 μg protein from the major cell envelope fractions. Results showed that there was no receptor activity in the Cyt fraction. The IM fraction was contaminated with receptors, but the activity was only up to 36%. The TCE contained about 95.3% of the receptor activity, and the OM contained 98.3% of the activity. Therefore, the location of receptor activity was distributed mainly in the OM fraction.

Since *S. derby* TCE contains the OmpF-, OmpC-, and OmpA-like major proteins when compared with those in the standard JF568 strain of *E. coli* (Fig. 1), the previously recognized Omp-specific phages of *E. coli* were tested for receptor activity with the TCE fraction. These phages were TuIb, TuIa, and TUII,* whose respective receptor proteins are OmpF, OmpC, and OmpA. Results showed that there was no phage receptor activity detected in the *S. derby* TCE fraction or in its intact cells, indicating that there are no phage receptors for TuIb, TuIa, and TUII* in *S. derby* (data not shown). If one excludes the masking effect of these phage-receptor proteins, the OmpF-, OmpC-, and OmpA-like major proteins on *S. derby* are probably not the same as those required by TuIb, TuIa, and TUII*. The receptor requirement for bacteriophages should be at the amino acid level instead of protein species as reported earlier [27]. Nevertheless, the OJ367 phage receptor of *S. derby* should be exposed on the OM and belong to the OM components; the OM extracts were pooled to purify Omps for receptor identification.

Omp purification by DEAE-cellulose chromatography

The column separated the Omps into 12 protein peak areas (Fig. 3). The protein profile of the pooled peak fractions was determined by SDS-PAGE (Fig. 4). Peaks 1 and 7 (Fig. 4, lanes 1 and 7) showed relatively pure proteins of 45 and 37.5 kDa, respectively. The OmpF-like proteins (39 kDa) were eluted at fraction no. 320 (0.05 M NaCl, area 9). The OmpC-like proteins (37.5 kDa) were eluted at fraction no. 290 (0.02 M NaCl, area 7). And the OmpA-like proteins (35.5 kDa) came down the column before the salt gradient began (area 4).

Screening receptor activity in the column fractions

Receptor activity was found in the beginning of the anion exchange column and extended to fraction no. 110 by the volume scale test or to fraction no. 130 by the protein weight test (Fig. 5, upper panel). This active fraction range appeared to share a common 45-kDa protein as shown in pooled fraction peaks 1 and 2 (Fig. 4, unbound lanes 1 and 2) although its amount in peak 2 was very faint. The 45-kDa protein in peak 1 appeared to be a homogeneous population. The 45-kDa protein was, however, very minor in the OM fractions (Fig. 2, lanes 6-9) compared to the same molecular weight protein species in TCE (Fig. 2, lane 1). Various concentrations of the pooled peak 1 fractions containing the 45-kDa protein were tested for phage neutralization ability which showed dose-dependency (data not shown). Thus, the 45-kDa protein was further characterized.

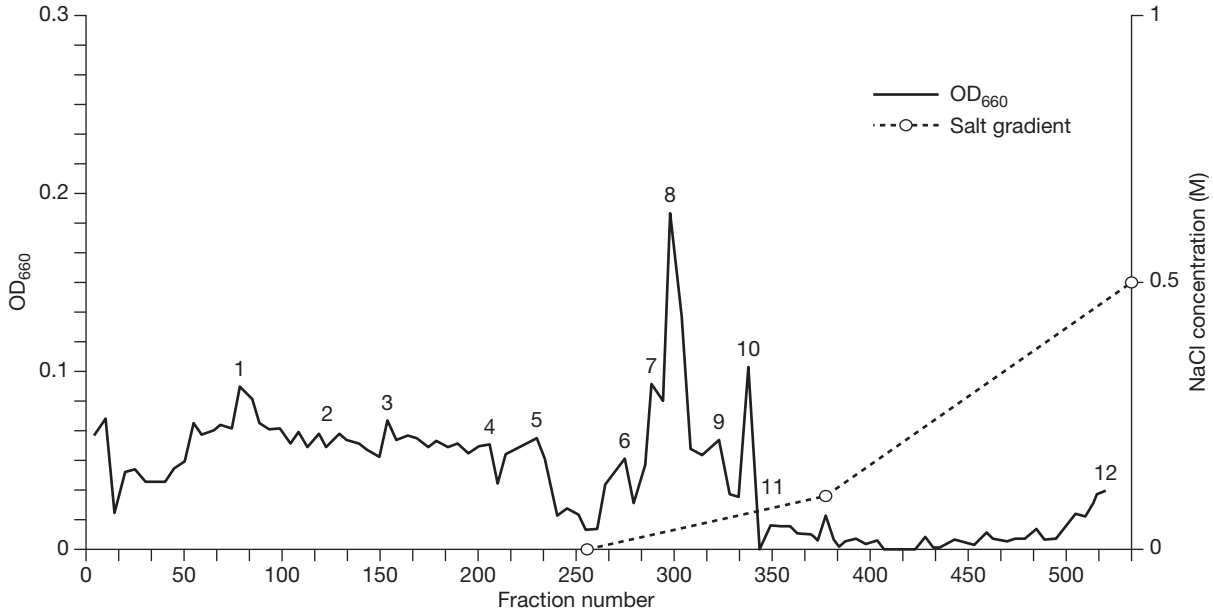

Fig. 3. Diethylaminoethyl column chromatography of *Salmonella derby* outer membrane proteins. In total, 190 mg of the outer membrane extract was loaded. The column was first washed with TTE (Tris-N-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid-ethylenediamine tetra-acetic acid [EDTA]) buffer (0.05 M Tris-HCl buffer, 2% Triton X-100, and 5 mM EDTA) to collect the unbound proteins. The first gradient was 0-0.1 M NaCl and the second gradient was 0.1-0.5 M NaCl with 350/350 mL volumes of each in TTE buffer. The column collected 520 fractions with 12 major peak areas. OD_{660} = optical density at 660 nm.

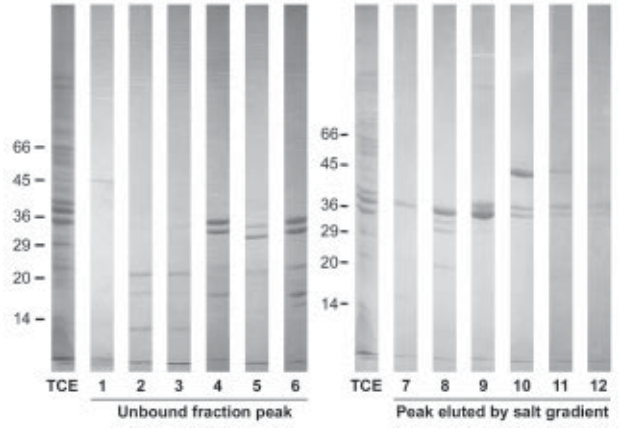

Fig. 4. Protein profile of outer membrane protein (Omp) separation in diethylaminoethyl column peak fractions. The peak samples of the unbound fraction (peak areas 1-6) and from the salt gradients (peak areas 7-12) were run in 2 separate sodium dodecyl sulfate polyacrylamide gel electrophoresis analyses. Total cell envelope (TCE) was used as the total Omp control to locate their corresponding proteins in the peak fractions. These peak fractions were pooled, dialyzed, and Amicon-concentrated. Fraction peaks 1-3, 7, 11, and 12 were loaded with 5-10 μg proteins. Fractions 4-6 and 8-10 were loaded with 15-25 μg proteins. The outer membrane (OM) extract was used as the total Omp control to locate their corresponding proteins in the peak fractions. The left OM sample was loaded with 30 μg proteins, and the right OM sample was loaded with 35 μg proteins.

Mutant resistant to phage OJ367

An *S. derby* mutant, resistant to phage OJ367 in intact cells, was isolated. The TCE protein profile of the mutant (Fig. 1, lane 7) was compared with the wild-type strain as shown in Fig. 1 (lanes 3-6). The TCE of the mutant of the same OD_{650} 1.6 contained somewhat less of the 45-kDa protein and showed very obvious alterations in the major proteins. The 37.5-kDa protein was greatly reduced and instead produced a new 36-kDa protein. When serial concentrations of TCEs of the wild type and mutant were tested for in vitro receptor activity, the wild-type strain neutralized 100% of the phage, whereas the mutant could only neutralize 64% of the phage particles with 30 μg TCE. Decreased receptor activity was indicated in the phage-resistant mutants. Combining the above results implies that the 45-kDa Omp is involved in coliphage binding as a receptor; however, such a role is affected by the expression of other Omps.

Extraction of PG-associated proteins

On TCE, some proteins are associated with PG and some are not. In order to characterize the 45-kDa receptor protein, separation of PG-associated proteins and non-PG-associated proteins (NPG-associated proteins) was performed by extracting the TCE with 2% SDS at 60°C [18].

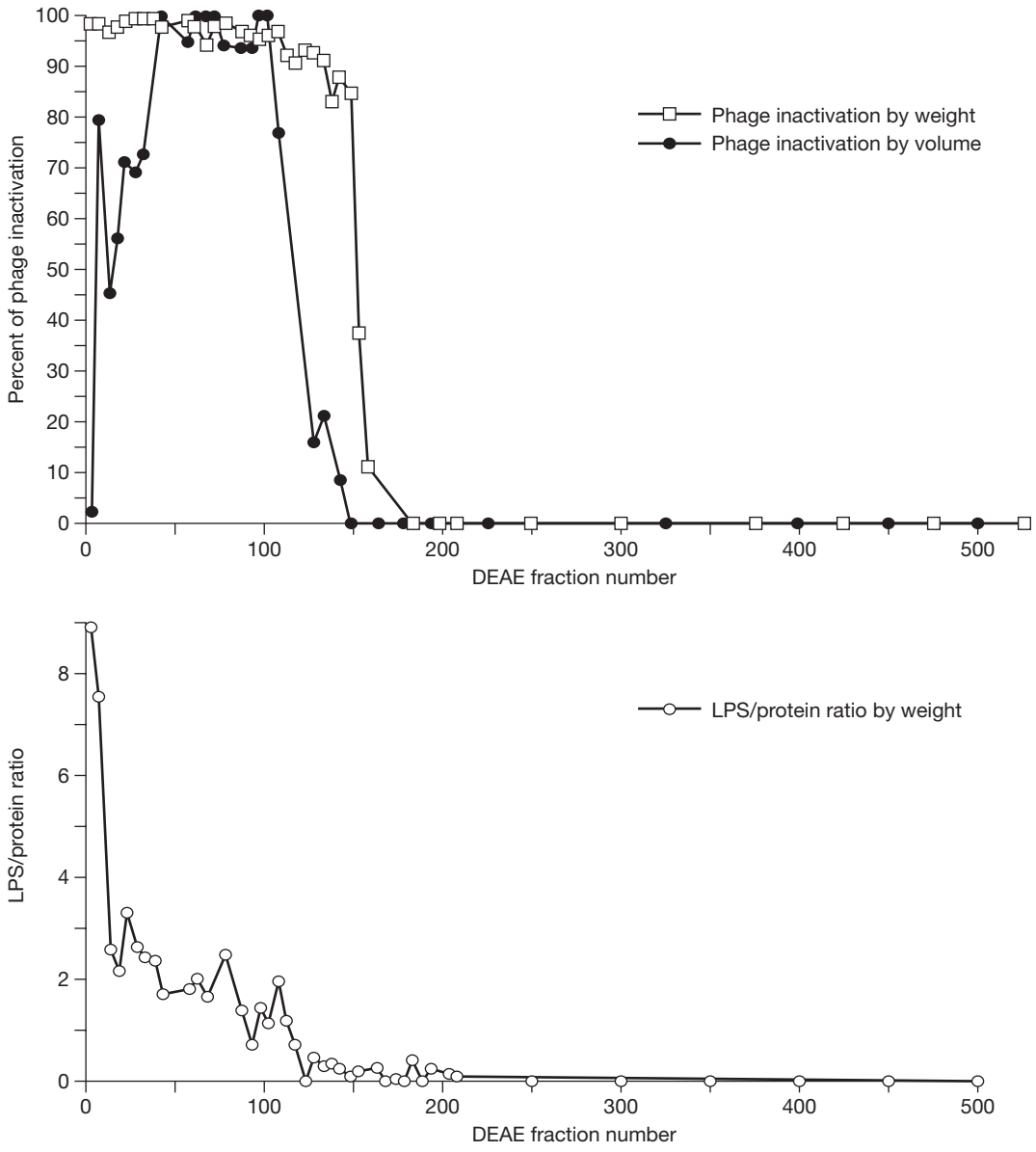

Fig. 5. Phage OJ367 receptor activity and the relative lipopolysaccharide (LPS)/protein ratio distribution in the diethylaminoethyl (DEAE) column fractions. The reactions in the upper panel used 100 μL or 10 mg protein from the individual fractions to react with 3000 phages for the 2 scales of volume and weight. This assay was performed in column buffer in which TTE (Tris-N-tris [hydroxymethyl]methyl-3-aminopropanesulfonic acid-ethylenediamine tetra-acetic acid [EDTA]) buffer (0.05 M Tris-HCl buffer, 2% Triton X-100, and 5 mM EDTA) satisfactorily dissociated protein molecules without damaging the receptor activity. The percent phage OJ367 inactivation was monitored by determining the number of viable phage particles lost = $[(N_0 - N)/N_0] \times 100$, where N is the number of viable phage particles after inactivation with receptor, and N_0 is the initial number of viable phage particles used in the inactivation experiment.

It was calculated that there was an average of 60% PG-associated proteins and 40% NPG-associated proteins in the TCE of *S. derby*. The major 39- (OmpF) and 37.5-kDa (OmpC) proteins were PG-associated proteins as shown in Fig. 6 (lane 7). The NPG-associated proteins, in addition to the 35.5-kDa (OmpA) protein, consisted of a number of minor proteins including the 45-kDa receptor protein (although the gel photo does not show the 45-kDa band in lane 6 very clearly). This pattern is in accordance with the findings of previous workers [11] that PhoE, OmpF, and OmpC porin proteins are PG-associated proteins and the OmpA protein is an NPG-associated protein. The result is also in parallel with the report of Rosenbusch [18]. Therefore,

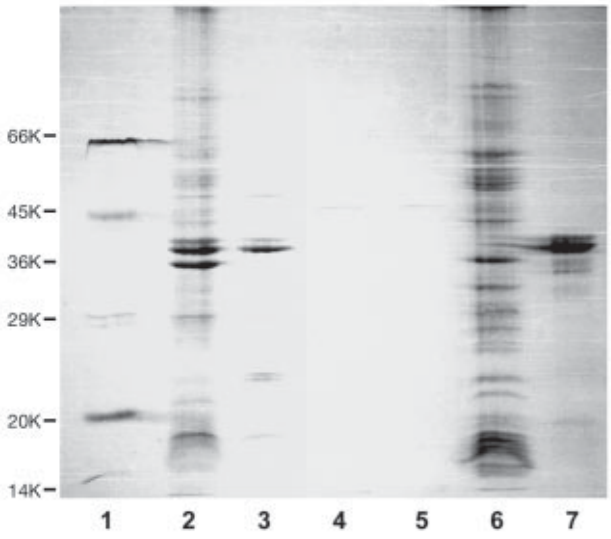

Fig. 6. Phage OJ367 receptor protein in *Salmonella derby* and peptidoglycan-associated proteins. Lane 1, molecular weight standard; lane 2, total cell envelope; lane 3, outer membrane extract; lanes 4 and 5, the 45-kDa receptor protein; lane 6, non-PG-associated proteins; lane 7, PG-associated proteins.

the 45-kDa receptor protein is an NPG-associated protein.

Effects of the LPS/protein ratio and trypsin on receptor activity

Phage receptors have been reported to have 2 component characteristics, i.e., it was reported that PhoE and OmpC require LPS in order to maximize the receptor activity of phages TC45 and T4 [28,29]. The LPS-to-protein (LPS/P) ratio by weight was measured throughout the column fractions (Fig. 5, lower panel). Most of the LPS and the PL were eluted from the DEAE column in the beginning fractions, as reported by Schnaitman [22]. In Fig. 5, the LPS/P ratio was as high as 9 in the very beginning and dropped to 2-3 before fraction no. 50, then only 0.01-0.02 was left from fraction no. 140 to the end of the column. When these values were compared to the receptor activity, no relationship between the LPS/P ratio and receptor activity was observed. Because the receptor activity extended before fraction no. 130, the LPS/P ratio in fraction no. 34 was 100-fold higher than that of fraction no. 130. Whether LPS was eluted from the column as a bound or free form with the protein is not known in this case.

Since it was reported that NPG-associated proteins were trypsin sensitive [30], the 45-kDa protein was treated with trypsin. Results showed that the 45-kDa protein was digested by trypsin and disappeared when compared with the control (Fig. 7, lane 2). This test corresponded to the result in Fig. 6 that the 45-kDa protein is an NPG-associated protein.

In the OM phage receptor studies, many receptor-mediated phage recognition patterns were reported. For example, phage K3 uses 2 Omps (PhoE and OmpA) as receptors [7], phage OX2 uses both PG-associated protein and NPG-associated protein as receptors and both OmpC and OmpA in an *E. coli* K-12 strain contain a common amino acid region [27]. In this study, a different pattern was found for an *E. coli* phage OJ367 which uses the NPG-associated 45-kDa protein, a minor protein of OM on the novel *S. derby* host, as the receptor for infection. However, such a receptor role is affected by the expression of other Omps.

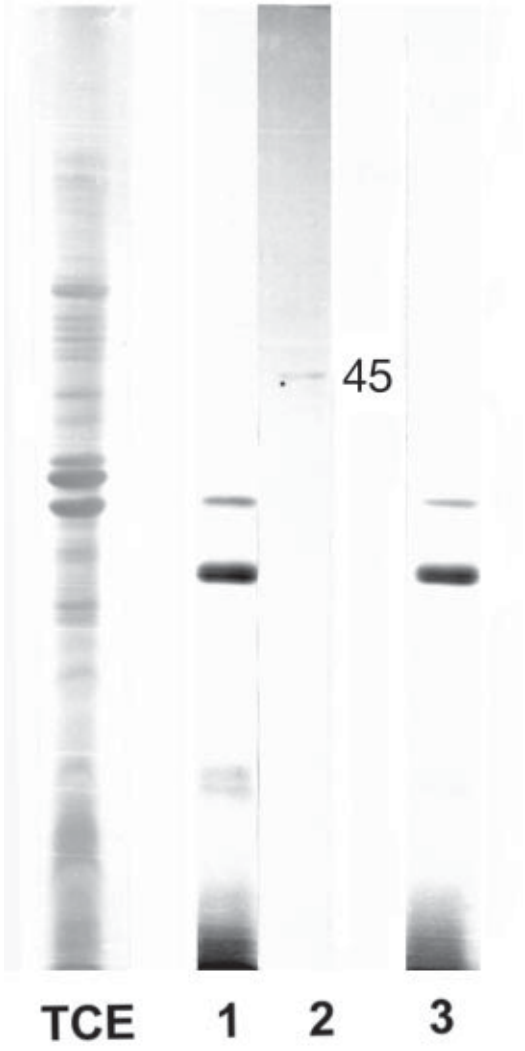

Fig. 7. Effect of trypsin treatment on receptor protein. Left lane, total cell envelope (TCE); lane 1, trypsin enzyme; lane 2, the 45-kDa receptor protein; lane 3, the 45-kDa receptor protein after trypsin treatment.

Acknowledgment

The author thanks Prof. Tuu-jyi Chai for advice in this study.

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