Identification of structural genes for *Clostridium botulinum* type C neurotoxin-converting phage particles

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Abstract

The structural genes for strain C-Stockholm (c-st) phage particles, a representative type C toxin-converting phage of *Clostridium botulinum*, have been determined. First, by determining the N-terminal amino acid sequences of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) bands of c-st phage particles, it became clear that four proteins, 14, 25, 32 and 42 kDa, are the products of the ORFs, *cst166*, *cst165*, *cst160* and *cst164*, respectively, of the c-st phage genome. The Western blot analyses reacting these phage bands with an antiphage serum prepared previously indicated that the products of *cst165* and *cst160* are the main proteins of the phage particles. Then, six candidates for the phage structural proteins, including *cst165* and *cst160* gene products, were prepared as recombinant proteins. Also, the protein corresponding to the *cst164* gene product was excised from SDS-PAGE gels. The antibodies against these seven proteins were prepared in rabbits, and finally, the reaction of these antibodies to the c-st phage particles was analyzed by electron microscopy. It was concluded that a sheath protein and a head protein of the c-st phage are the products of genes *cst160* and *cst165*, respectively, and that these two proteins are conserved in the other three converting phages, but not in the nonconverting phage.

Introduction

The neurotoxins produced by *Clostridium botulinum* are among the most poisonous substances. Of the seven types of neurotoxins, genes for type C (or C1) and D toxins are carried by bacteriophages (Inoue & Iida, 1970, 1971; Eklund *et al.*, 1971, 1972; Oguma *et al.*, 1973). Usually, C and D cultures are (pseudo) lysogenized by two different phages, β and γ, and only β phage controls the toxin production (Eklund *et al.*, 1972, 1971; Oguma, 1976). Oguma *et al.* (1976) reported that C- and D-converting β phages can be classified into three groups based on their antigenicity and infection spectrum. The first group consists of strain C-Stockholm (c-st) and c-468 phages, the second is c-203 and d-1873 and the third is d-sa and d-4947. They demonstrated that the antigenicity of c-st and c-468, and the antigenicity of c-203 and d-1873, are quite similar to each other. They also demonstrated that anti-c-468 phage serum cross-neutralizes the toxin-converting or plaque-forming activities of c-203 and d-1873 phages, indicating that the structural proteins of phage particles belonging to group 1 and 2 have some homology.

Recently, the complete genome sequence of the c-st phage was determined (Sakaguchi *et al.*, 2005). This phage has 198 potential protein-coding regions. Of the identified 198 ORFs, only 57 were functionally assigned based on the sequence homology to known proteins, whereas more than half (102 ORFs) shared no significant sequence homology with any proteins in databases. The remaining 39 ORFs exhibited sequence similarity to the proteins of unknown function. In this study, it was attempted to identify the structural proteins of c-st phage. This study demonstrated that the products of ORFs *cst160* and *cst165* are the structural proteins for the sheath and head, respectively, and that these two proteins are conserved in the three converting phages, c-468, c-203 and d-1873, as well as the c-st phage, but not in the nonconverting c-st γ phage.

Materials and methods

Strains, phages and media

All of the *C. botulinum* strains and phages used were from our laboratory stock (Oguma *et al.*, 1973, 1976). Type C toxin-converting phages, c-st, c-468 and c-203, were isolated from respective toxigenic strains of C-ST, C-468 and C-203. Also, a toxin-converting d-1873 phage was isolated from a toxigenic strain of D-1873. A nonconverting phage named c-st γ was isolated from a toxigenic strain of C-ST. These phages were propagated on the nontoxigenic indicator strains of (C)-AO2 or (D)-151, which were obtained from C-ST and D-1873 by the acridine orange treatment, respectively; c-st, c-468 and c-st γ phages were propagated on (C)-AO2, and d-1873 and c-203 phages were propagated on (D)-151. The phage titers were increased as reported previously (Oguma *et al.*, 1973, 1976). The young cultures of (C)-AO2 or (D)-151 in LYG medium (1% lactalbumin, 2% yeast extract, 0.5% glucose, 0.15% cysteine hydrochloride, pH 7.2) were mixed with each phage suspension. After incubation at 37 °C for 3–4 h, and the lysis of cells became clear, the cultures were clarified by centrifugation (6000 ***g*** for 20 min at 4 °C). The pellet was discarded and the supernatant fluids were filtered through a membrane filter with pore size of 450 nm. These filtrates were used for the conversion experiment. In order to observe the reaction of antisera to the phage particles, these filtrates were again centrifuged to sediment the phage particles (60 000 ***g*** for 1 h at 4 °C) in an RP45T rotor with a Beckman 80-P-7 ultracentrifuge. The final pellet was suspended in 0.5 mL of 10 mM phosphate-buffered saline (PBS; pH 7.4).

The converting phages of d-sa and d-4947 were not used this time, because the lytic activity of these phages is quite low (Oguma *et al.*, 1976), and therefore sufficient phage particles could not be prepared for the experiments.

Preparation of recombinant proteins

As described in 'Results and discussion', a total of 14 putative structural genes were cloned for phage particles from the complete genome sequence of the c-st phage, but only six clones succeeded in expressing them in *Escherichia coli* (BL21) as GST-fusion proteins. By observing the reaction of antibodies against these six recombinant proteins to phages, it was found that the products of gene *cst160* and *cst165* were sheath protein and head protein, respectively. Therefore, the procedure for preparing recombinant proteins of *cst160* and *cst165* is described below. For cloning, the coding sequences of *cst160* and *cst165* were first PCR-amplified using the following primer sequences: *cst160*-F (5′-GGAATTCATGATAAATGGAAAAAAGAAAAAATGGC AC-3′), *cst160*-R (5′-CCGCTCGAGCGGCTATTCATCGG CTAATAAAGGTTCT-3′), *cst165*-F (5′-GGAATTCCATGGA TTTAAAAAATAATAAAATTAAATTAATT-3′) and *cst165*-R (5′-CCGCTCGAGCGGTTATTGACACTCTAGTCTTACTGT TTG-3′). PCR amplification was carried out using the LA long PCR kit or the Ex TaqPCR kit (Takara Bio Inc., Otsu, Japan) as reported previously (Sakaguchi *et al.*, 2005). The PCR fragments were digested with EcoRI-XhoI and then cloned into EcoRI-XhoI sites of pGEX-6P-3 (Amersham Biosciences, Piscataway, NJ). The sizes of cloned *cst160* and *cst165* genes are 634 and 623 bp, respectively. The sequences of the constructs were verified as follows (Kageyama *et al.*, 2005): the DNA products were amplified and purified using a Centri-Sep Column (Princeton separations, Adelphia, NJ). The DNA sequences were determined by an ABI PRISM 3100 Genetic analyzer using a dye terminator cycle sequencing kit. The resulting plasmids were then introduced into *E. coli*, and the GST-fusion proteins were expressed. The expressed GST-fusion proteins were affinity purified with Glutathione-Sepharose 4B, GST was removed by a PreScission protease treatment and then finally purified by reapplying to the Glutathione Sepharose 4B column (Lee *et al.*, 2005).

Electroelution of protein from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels

On the Western blot, the proteins of c-st160, c-st164 and c-st165 were reacted with anti c-468 phage serum. In *cst160* and *cst165* genes, their recombinant proteins were obtained as described above, but recombinant proteins could not be produced in the *cst164* gene. Therefore, the *cst164*-encoded protein was extracted from the SDS-PAGE gels of c-st phage particles. The phage particle proteins were separated by SDS-PAGE and the protein bands were visualized by staining with Coomassie brilliant blue (CBB) R-250 (Bio-Rad Lab., Hercules, CA). The *cst164*-encoded protein band was excised and cut into pieces of 1 cm length. Electroelution was performed according to the manufacturer's instructions of the BIOTRAP starter BT1000 kit (Schleicher & Schuell, Dassel, Germany) with Tris/glycine buffer (25 mM Tris, 192 mM glycine) as elution buffer. Elution was carried out at 200 V overnight. After dialysis against 10 mM PBS (pH 7.4), protein was concentrated by an Amicon ultra-4 centrifugal filter (Millipore, Bedford, MA).

Antibody production

Polyclonal antibodies against six recombinant proteins and an electroeluted *cst164*-encoded protein were raised in New Zealand white rabbits. Approximately 500 μg of each protein emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, MI) was injected at multiple intradermal sites. Two weeks later, the same dosage of the proteins emulsified in Freund's incomplete adjuvant

(Difco Laboratories) was injected. Then, after 3 weeks, the rabbits were intravenously boosted with 50 μg of each protein. The final serum samples were acquired 6 weeks after the initial immunizations.

SDS-PAGE and immunoblot analyses

SDS-PAGE and immunoblot analyses were performed as reported previously (Lee *et al.*, 2005). SDS-PAGE was performed on a 12.5% separating gel, and molecular masses were determined using Perfect Protein Markers (6.5–200 kDa; Bio-Rad). Protein bands were stained with CBB. For analysis of phage particle-proteins with SDS-PAGE, 10 μg of phage particles obtained by ultracentrifugation was applied on each gel. In immunoblot analysis, the electro-blotted proteins on polyvinylidene difluoride membranes were blocked with 10% skim milk in phosphate-buffered saline (S-PBS), washed with PBS containing 0.05% Tween 20 (T-PBS) and then reacted for 1 h at 37 °C with 1 : 1000 or 1 : 10 000 diluted rabbit polyclonal anti-c-468 phage serum prepared previously (Oguma *et al.*, 1976) or seven antiproteins sera prepared this time. The membrane was then treated with 1 : 10 000 diluted horseradish peroxidase (HRP)-conjugated goat antirabbit immunogloubulin, and finally detected by an enhanced chemiluminescence-Western blotting detection reagent (Amersham Pharmacia, Uppsala, Sweden). N-terminal amino acid sequence of the main bands on SDS-PAGE was also determined as reported previously (Inoue *et al.*, 1996). The electroblotted bands on polyvinylidene difluoride membranes were stained, cut out and then sequenced with a pulsed-liquid phase protein sequencer.

Immuno-electron micrography

In order to localize the structural proteins of the phage particles, immuno-electron microscopic analysis was performed. Carbon-coated grids were placed on 10 μL of purified phage suspension for 5 min, and then incubated with 10 μL of 1/10 diluted purified seven different antibodies for 1 h at room temperature. After washing with T-PBS, the grids were incubated with 10 μL of the 1/50 diluted colloidal goat antirabbit IgG-gold (E-Y Laboratories Inc., San Mateo, CA) for 1 h at room temperature. The samples were stained with 2% solution of neutral potassium phosphotungstate solution and were examined at 100 kV using an H-7100 transmission electron microscope (Hitachi, Tokyo, Japan) at × 100 000 magnification.

Conversion tests

The effect of anti-160 or -165 serum on the toxin-conversion ability of c-st phage was observed. The phage preparation [filtrate of the propagated phage suspension on (C)-AO2] was serially diluted in 10-fold steps (10^1–10^9), and 0.5 mL of each diluted solution was mixed with an equal volume of 10-fold diluted anti-160 or -165 serum. After reacting at 37 °C for 1 h, 0.5 mL of the mixture was inoculated into 2.5 mL of young (C)-AO2 culture and incubated at 37 °C for 3 h. A portion, 0.2 mL, of the mixture was then transferred into 10 mL of cooked meat medium and incubated at 35 °C for 3 days (Oguma, 1976). Just after this inoculation into cooked meat medium, the turbidity of the phage-reacted culture (mixture) was assayed using the Hitachi photometer at 520 nm wavelength, to estimate the extent of lysis. The culture supernatant of cooked meat medium was diluted 10 times, and 0.5 mL was injected intraperitoneally into three mice to detect toxin production (Oguma, 1976). The effect of the sera on plaque formation with phages was not observed because it is very difficult to make a confluent lawn with type C and D indicator strains.

Southern hybridization analyses

The amplified DNA preparations of *cst160* (634 bp) and *cst165* (623 bp) were used as probes, and hybridized with the DNA extracted from different phages according to the procedure reported by Chibani-Azaiez *et al.* (1998). Phage DNA was extracted twice by the phenol–chloroform procedure. After precipitation with isopropanol and ethanol, DNA was treated with RNase H, and then digested with EcoRI. Thereafter, they were electrophoresed on a 1% agarose gel, stained with ethidium bromide and visualized by UV. DNA bands were transferred to Hybond-N plus nylon, and then hybridization and signal detection were performed using the ECL direct nucleic acid labeling and detection system (Amersham pharmacia).

Enzyme-linked immunosorbent assay (ELISA)

The c-st, c-468, c-203, d-1873 or c-st γ phage particles collected by ultracentrifugation and resuspended in 10 mM PBS (pH 7.4) at a concentration of 10 μg mL^{-1} were used as antigens. A portion, 100 μL, of these phage preparations was inoculated into each well of Flat-bottom 96-well plates (Greiner bio-one, Nrtingen, Germany), and kept overnight at 4 °C. Thereafter, the reaction of anti-160 or anti-165 serum to these coated antigens was observed as reported previously (Arimitsu *et al.*, 2004). The sera were diluted serially in 10-fold steps with S-PBS, and then reacted for 1 h at 37 °C. They were then reacted with 100 μL of 1 : 10 000 diluted HRP-conjugated goat antirabbit immunoglobulin for 1 h at 37 °C, and finally reacted with 100 μL of citrate buffer (pH 5.0) containing 0.04% of *o*-phenylene-diamine and 0.02% hydrogen peroxide for 30 min at 37 °C. This reaction was stopped with 100 μL of 2 N H_2SO_4, and the absorbance was measured at 490 nm in a microplate reader (Bio-Rad). The highest dilution of the anti-160, -164 or -165

serum that showed a positive reaction (OD, more than 0.5) was considered as an ELISA titer.

Results and discussion

Analysis of total phage proteins

On SDS-PAGE with c-st phage particles, at least six major protein bands of *c.* 14, 25, 32, 42, 44 and 150 kDa appeared (Fig. 1a). The N-terminal amino acid sequences of 14, 25, 32 and 42 kDa proteins were matched with the deduced amino acid sequences of four ORFs, *cst166*, *cst165*, *cst160* and *cst164*, respectively, of the c-st phage genome (Sakaguchi *et al.*, 2005). The sizes of proteins predicted from *cst166*, *cst165*, *cst160* and *cst164* genes are 61, 25, 25 and 42 kDa, respectively. Therefore, it was speculated that 14 kDa protein is a cleaved fragment of *cst166* product. The *cst160* product (25 kDa) demonstrated a band of 32 kDa. Later, it became clear that the recombinant protein of this gene also showed a band of 32 kDa on SDS-PAGE (this will be discussed later). The N-terminal amino acid sequences of 44 and 150 kDa proteins were not matched with deduced amino acid sequences of any ORF in the c-st genome, suggesting that these proteins might have arisen from the indicator (C)-AO2 cells.

Western blot analyses using anti-c-468 phage serum demonstrated immunoreactivity with many proteins, but the reaction to 32 and 25 kDa bands was strong (Fig. 1b). Therefore, it was speculated that the products of *cst160* and *cst165* ORFs are phage structural proteins. As the 42 kDa band also weakly reacted to anti-c-468 phage serum, the product of *cst164* was also considered as a candidate for structural proteins. The 14 kDa fragment of the *cst166* gene product showed no obvious reaction with the serum (this will also be discussed later).

Confirmation of 32, 42 or 25 kDa protein as a product of c-st phage specific ORF *cst160*, *cst164* or *cst165*, respectively

Next, immunoblot experiments were performed to confirm that 32, 42 or 25 kDa protein is a product of c-st phage-specific ORF *cst160*, *cst164* or *cst165*, respectively. To overproduce the products of *cst160* and *cst165*, coding sequences of *cst160* and *cst165* were fused to *gst*, overexpressed in *E. coli* and purified using an affinity column of glutathione–Sepharose 4B. On SDS-PAGE analyses of the purified proteins from *E. coli* cultures carrying *cst160-gst* or *cst165-gst* fusion, a protein product of 58 or 51 kDa, respectively, was obtained (data not shown). When these protein preparations were cleaved with PreScission protease and successively purified with glutathione-Sepharose 4B, the 32 and 25 kDa products were obtained (Fig. 2a and c). For unknown reasons, ORF *cst164* was not overexpressed in *E. coli*. Therefore, the 42 kDa protein band was extracted from SDS-PAGE gels of the c-st phage particles. As expected, the extracted 42 kDa protein migrated as a single band after SDS-PAGE analysis (Fig. 2b).

The antibodies against 32, 42 or 25 kDa proteins thus obtained were prepared in rabbits. The titers of these sera, anti-160, -164 or -165 antibodies, measured by ELISA using 32, 42 or 25 kDa protein as antigens, are 1×10^8, 1×10^5 and 1×10^6, respectively. The specific reaction of these antibodies to the purified 32, 42 or 25 kDa protein was further

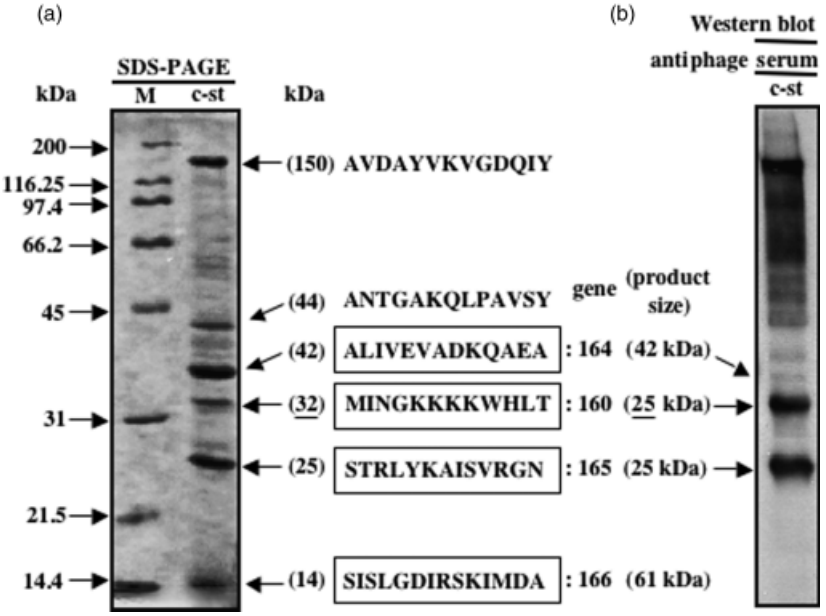

Fig. 1. SDS-PAGE and Western blot of c-st phage particles. Proteins forming c-st phage particles was analyzed by both SDS-PAGE (a) and Western blot (b). On Western blot, the electroblotted bands on the membranes were reacted with anti-c-468 phage serum prepared previously. The N-terminal amino acid sequences of six clear bands on SDS-PAGE were determined and compared with the amino acid sequences predicted from the c-st phage DNA base sequences. The size of the native *cst160*-encoded protein of phage on SDS-PAGE was slightly larger (32 kDa) than that of the calculated value (25 kDa) based on its amino acid sequence.

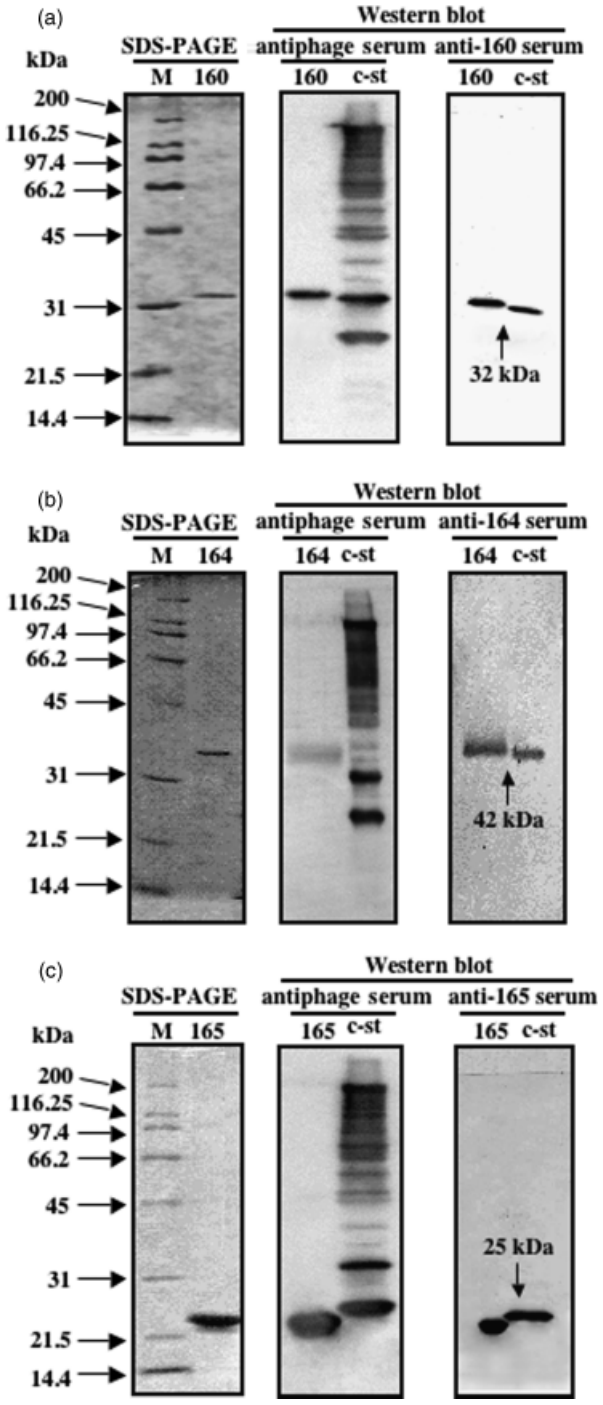

Fig. 2. SDS-PAGE and Western blot of 160, 164 and 165 proteins and their antisera. Recombinant proteins of 160 (a) and 165 (c), and an electroextracted 164 protein (b) were analyzed by SDS-PAGE and Western blot using antisera against each protein and c-468 phage. On Western blot, c-st phage banding profiles reacted with anti-c-468 phage serum shown in Fig. 1b were again used as a control.

confirmed by immunoblot analyses (Fig. 2a–c). The anti-c-468 phage serum showed immunoreactivity against c-st phase-specific proteins of 32, 42 and 25 kDa as mentioned above, and this serum demonstrated immunoreactivity to *cst160*-, *cst164*- or *cst165*-encoded protein, which is commigrated to the immunoreactive bands of anti-160, -164 or -165 serum. Collectively, these results indicated that the 32, 42 and 25 kDa proteins are specific products of *cst160*, *cst164* and *cst165*, respectively. On immunoblot, the mobility between recombinant proteins of *cst160, cst164 and cst165* and the cognate proteins presenting in phage particles was somewhat different. It was speculated that the molecular forms of the recombinant proteins and the phage particle-derived proteins are not identical, and therefore they actually showed somewhat different behavior on SDS-PAGE. In addition, the size of the *cst160*-encoded protein estimated by SDS-PAGE (32 kDa) was slightly larger than that of the calculated value (25 kDa) based on its amino acid sequence (Fig. 1). The reason for this phenomenon is not clear. However, as (1) the same result was obtained on SDS-PAGE with c-st phage particles, (2) the nucleotide sequence of the cloned *cst160* gene in plasmid is identical to that of the original in the c-st phage genome and (3) the number of acidic or basic amino acid residues of this protein was not so different from other proteins, it is speculated that this protein may have some resistance to SDS-denaturation as reported by Takeda *et al.* (1993).

Localization of 32, 42 and 25 kDa proteins in c-st phage by immuno-electron microscopy

As reported previously (Eklund *et al.*, 1971, 1972; Oguma, 1976), types C and D converting phages exhibited a very similar shape. They exhibited a hexagonal head 100 nm in diameter and a tail 350–400 nm long and 11–15 nm in diameter surrounded by a sheath 30 nm in diameter (Fig. 3a). Also, the shape of the nonconverting phage c-st γ was quite similar as reported previously (Eklund *et al.*, 1971): a hexagonal head 50–60 nm in diameter and a tail 200–250 nm long and 6 nm in diameter surrounded by a sheath 20 nm in diameter (Fig. 3b). As reported previously, some heads and tails were separated probably because of the artificial stress during the process of purification or concentration of phage particles. Also, the long sheaths were sometimes cut off at different sites, demonstrating tails surrounded by different-size sheaths (Fig. 3a and b). The reaction of the each antiprotein serum to the phage particles was observed under electron microscopy using a secondary antibody labeled with gold particles. The anti-160 serum reacted only with sheath proteins and anti-165 serum reacted only with the head protein (Fig. 3c), whereas, the antibody against 42 kDa protein (164) did not show any immunoreactivity with the phage particles.

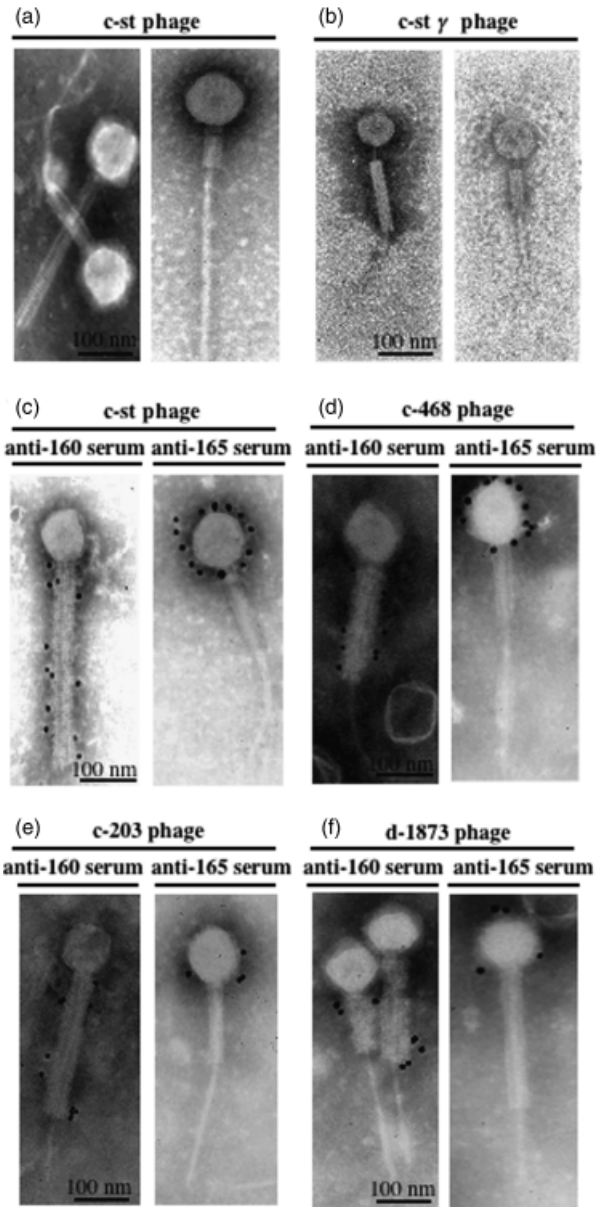

Fig. 3. Immunoelectron microscopy for phages with anti-160 and -165 sera. The reaction of anti-160 or -165 serum to converting phages (c-st, c-468, c-203 and d-1873) and a nonconverting phage (c-st γ) was observed using gold particle-labeled goat antirabbit serum as the second antibody. Representative positively reacted pictures of c-st (c), c-468 (d), c-203 (e) and d-1873 (f) phages as well as control c-st (a) and c-st γ (b) phages that were not reacted with the sera are shown.

Analysis of other gene products

The nucleotide sequences of ORFs *128* to *131* and *162* to *169* in the c-st phage genome exhibited 20–30% similarity to the genes of SPβ, a large (134 kb) temperate phage lysogenized in *Bacillus subtilis* strain 168 (Lazarevic *et al.*, 1999). Although the function of these genes is unknown, it was speculated that some of them might be the structural genes for phage particles as well as *cst160* and *cst165*. Therefore, 11 additional genes (including *cst166* gene) were cloned, and it was attempted to express them in *E. coli*. As four genes, *cst-109*, *-162*, *-163* and *–169*, could be expressed (the *cst166* gene was not expressed), antibodies against the products of these four genes were prepared. The reaction of these sera to the phage particles was analyzed by immuno-electron microscopy, but none showed a positive reaction, suggesting that these proteins may not be phage structural proteins. Of the 11 genes examined, *cst166* was of main interest. On SDS-PAGE, the 14-kDa fragment of this gene was identified. Although this was not reacted with anti-468 phage serum, it was still speculated that this might play an important role in forming phage particles. As the *cst166* gene could not be expressed as a GST fusion protein, it is being attempted to express it as a His tag protein.

Effect of anti-160 or -165 serum on toxin-conversion ability of c-st phage

A nontoxigenic (C)-AO2 strain can be converted to toxigenic by infection with c-st phage (Oguma *et al.*, 1973, 1976). The effects (neutralization) of antisera on this phenomenon were examined. In case of c-st phage, up to a 10^6-fold diluted phage preparation was able to convert nontoxigenic (C)-AO2 to a toxigenic state. After treatment with anti-160 or -165 serum, conversion titers were 10^5 and 10^6, respectively, indicating that antiserum against head protein has no effect on the conversion ability of the phage, but that of sheath protein has a slight effect.

Comparison of *cst160* and *cst165* and their products among five phages

In an attempt to clarify the homogeneity or heterogeneity of *cst160* and *cst165* genes and their gene products (proteins) among the converting phages of c-st, c-468, c-203 and d-1873, and a nonconverting phage c-st γ, Southern hybridization, Western blot and immunoelectron microscopy analyses were performed.

When EcoRI-restriction patterns of five surveyed phage DNA were compared, some similarities were observed between c-st and c-468, and between c-203 and d-1873, but all of these were quite different from that of c-st γ (Fig. 4a).

In the Southern hybridization test, the phage whole DNA was treated with the probes of *cst160* or *cst165*. Data supporting the above conclusion were obtained. The size of EcoRI digested fragments containing *165* gene seemed to be identical between c-st and c-468, and between c-203 and d-1873. The size of the EcoRI digested *160*–containing fragment also seemed to be identical between c-203 and d-1873. These data support our previous conclusion that c-st and c-468 phages, and c-203 and d-1873 phages form

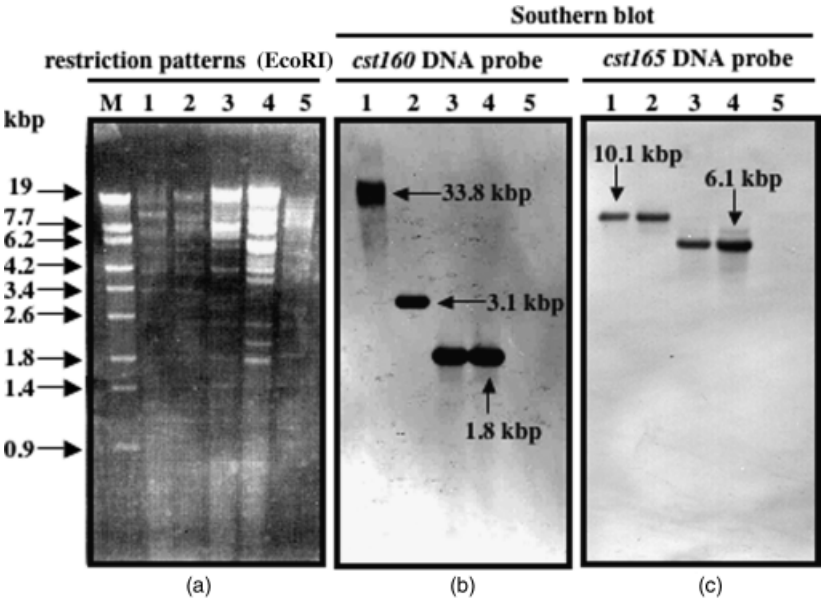

Fig. 4. EcoRI-restriction profile of phage DNA and Southern blot. DNA of phages c-st (lane 1), c-468 (lane 2), c-203 (lane 3), d-1873 (lane 4) and c-st γ (lane 5) were treated with EcoRI and their restriction profiles were observed (a). These bands were then reacted with DNA probes of *cst160* (b) or *cst165* (c).

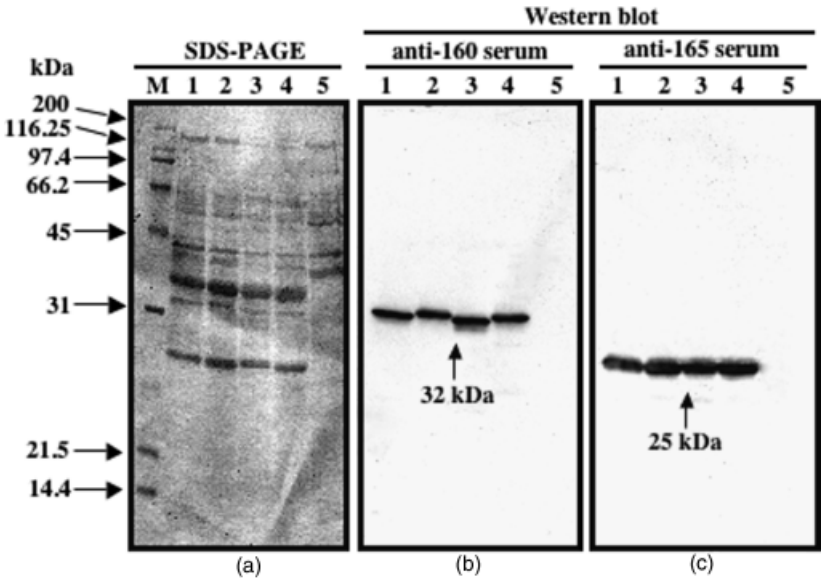

Fig. 5. SDS-PAGE of different phage particles and Western blot with anti-160 and -165 sera. SDS-PAGE was performed with the phage particles of c-st (lane 1), c-468 (lane 2), c-203 (lane 3), d-1873 (lane 4) and c-st γ (lane 5), and thereafter they were reacted with anti-160 (b) or anti-165 (c) serum.

their own groups, but there are some antigenic cross-reactions between the phages of these two groups (Oguma *et al.*, 1976). However, no hybridizing signal was detected with DNA of nonconverting phage c-st γ, suggesting that the c-st γ genome does not carry the homologues of *cst160* and *cst165* (Fig. 4b and c).

Upon SDS-PAGE analyses of total proteins, all four converting phages demonstrated very similar profiles with many bands (Fig. 5a). Anti-160 and -165 sera showed immunoreactivity to 32 and 25 kDa protein bands, respectively, of all converting phages (Fig. 5b and c), whereas the nonconverting c-st γ phage demonstrated a different SDS-PAGE banding profile when compared with converting phages, and detectable immunoreactivity was observed with neither anti-160 nor -165 sera (Fig. 5b and c). On ELISA with each phage particle as an antigen, both anti-160 and -165 sera showed high reactivity with four converting phages, while a very weak reactivity was observed with c-st γ (Fig. 6). In the former case, the reaction of anti-160 serum to the four converting phages was similar, but the reaction to anti-165 serum was varied. The reaction of anti-165 serum to c-st and c-468 phages was significantly higher than that to c-203 and d-1873 phages.

Under electron microscopy analyses, anti-160 and -165 sera reacted with the sheaths and heads of the c-468, c-203 or d-1873 phage, respectively, but no positive reaction was

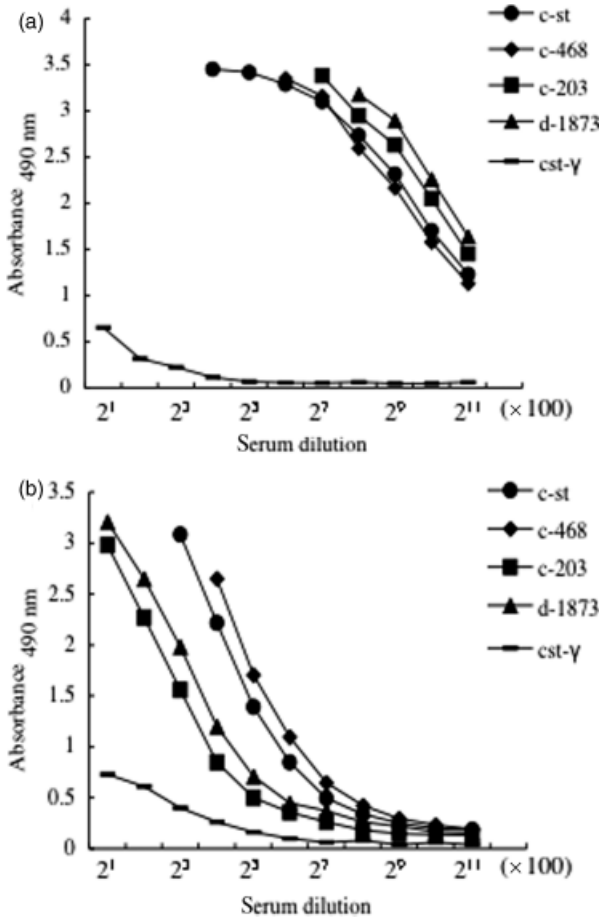

Fig. 6. Titration curves of anti-160 and -165 sera to different phages on ELISA. ELISA was performed by reacting serially diluted anti-160 (a) or -165 (b) serum with five different phage particles coated on 96-well plates.

observed with c-st γ phage particles. Again, in this case, the reaction of anti-165 serum to the heads of c-st and c-468 phages (Fig. 3c and d) seemed to be higher than that of the c-203 and d-1873 phages (Fig. 3e and f), but the reaction of anti-160 serum to the sheaths of all of these four phages seemed to be the same (Fig. 3c–f), indicating that sheath protein is highly conserved compared with the head one. Further nucleotide sequencing analyses of the homologues of *cst160* and *cst165* genes in c-468, c-203 and d-1873 phages should help in clarifying the extent of homology of these genes among these phages.

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