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Identification and nucleotide sequence determination of the gene responsible for Ogawa serotype specificity of *V. cholerae* 01

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The gene encoding a protein of 27 kDa, which is specifically expressed in *Vibrio cholerae* of serotype Ogawa, was identified and its nucleotide sequence determined. The plasmid carrying this gene was found to convert serotype specificity from Inaba to Ogawa when introduced into the *Escherichia coli* DH5(pVCI112) cell which harbors a cloned 20-kilobase genomic DNA fragment of *V. cholerae* NIH35A3 and expresses the 01 antigen of Inaba serotype.

O-antigen; Serotype Ogawa; *V. cholerae*; Biosynthesis of lipopolysaccharide; Sequence; Deletion plasmid

1. INTRODUCTION

Vibrio cholerae of serogroup 01 is the most common pathogen causing epidemic cholera. The serogroup 01 is further subdivided into 2 main serotypes, Ogawa and Inaba, on the basis of type-specific antigens. A third serotype, Hikojima, containing both Ogawa- and Inaba-specific antigens has also been reported [1–3]. Comparisons of the sugar compositions of lipopolysaccharide (LPS) molecules carrying these serotypes have been reported [4,5,6]. However, their structural differences have not been clarified yet. We have cloned two types of 5.6 kb genomic DNA fragments from *V. cholerae* strains NIH41 and NIH35A3 which determine Ogawa and Inaba serotype specificity respectively. It was shown by cell-free system of protein synthesis that a protein of 27 kDa is synthesized specifically when the 5.6 kb DNA fragment derived from *V. cholerae* NIH41 (01 serotype Ogawa) was used as a template. This protein was found to be encoded on the 2.1-kb *Hin*dIII fragment within the 5.6 kb DNA [7]. In this paper, we describe identification of a novel gene (*tsf*B, standing for type specific factor B(Ogawa) by nucleotide sequence determination.

2. MATERIALS AND METHODS

2.1. *Manipulation of DNA and DNA sequencing*

Standard methods of DNA manipulation were employed as described by Sambrook et al. [8]. Plasmids, pUC18, pUC118, pACYC177 and pACYC184 were used as cloning vectors. *E. coli* DH5 and MV1184 were used as hosts for transformation. All the plasmids used in this study were constructed from cosmid clones pVCO101 (derived from *V. cholerae* NIH41, serotype Ogawa) and pVCI102 (derived from *V. cholerae* NIH35A3, serotype Inaba) as described before [7]. Deletion mutants of pVCO181 were constructed using a Kilo Sequence Deletion Kit (Takara Shuzo Co., Ltd.). The nucleotide sequencing was carried out with the dideoxynucleotide method described by Sanger et al. [9], using synthetic oligonucleotides as primers (see Fig. 4A).

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2.2. *Protein synthesis in vitro*

DNA-directed protein synthesis was carried out by the method of Zubay [10] with slight modification using *E. coli* Q13 as a source of reaction extract. Reaction was carried out in the presence of ^{14}C-amino acid mixtures (Amersham, Japan), and radiolabelled proteins were fractionated by electrophoresis in an 11% SDS-polyacrylamide gel. The gel was soaked twice in dimethyl sulfoxide (DMSO) for 30 min, then in 22.2% diphenyloxazole in DMSO for 3 h. After washing with water, the gel was dried and exposed to Kodak XAR-5 film.

2.3. *Immunoassays*

E. coli cells harboring plasmids were grown on L-agar plates containing suitable amounts of antibiotics at 37°C for 15–16 h. Five ml of PBS(–) was added to each plate. After cells were scraped off the plate and transferred to a tube, formalin was added at a concentration of 1.5%, followed by incubation at room temperature for 4–5 h. After centrifugation, cells were resuspended in PBS(–) and used as antigens. Formalin-fixed cells (2×10^7 cells) were added to each well of poly-L-lysine treated ELISA plates, and reacted with 0.5% glutaraldehyde for 15 min to immobilize them onto the plates. Monoclonal antibodies (100 μl) specific for Ogawa or Inaba antigen [11,12] were added to each well and incubated for 1 h. After washing 3 times with PBS/Tween, alkaline-phosphatase conjugated anti-mouse IgG was added, followed by incubation for 1 h at room temperature. Wells were washed again with PBS/tween, then 200 μl of substrate solution (0.1 M glycine-NaOH (pH 10.4), 1 mg/ml *p*-nitrophenyl phosphate, 1 mM $MgCl_2$, 1 mM $ZnCl_2$) was added to each well. The absorbance at 405 nm was measured after 1–1.5 h incubation.

3. RESULTS

3.1. *Identification of the coding region for the 27-kDa protein*

In order to identify the coding region for the 27-kDa protein encoded by pVCO181, 6 deletion mutants were

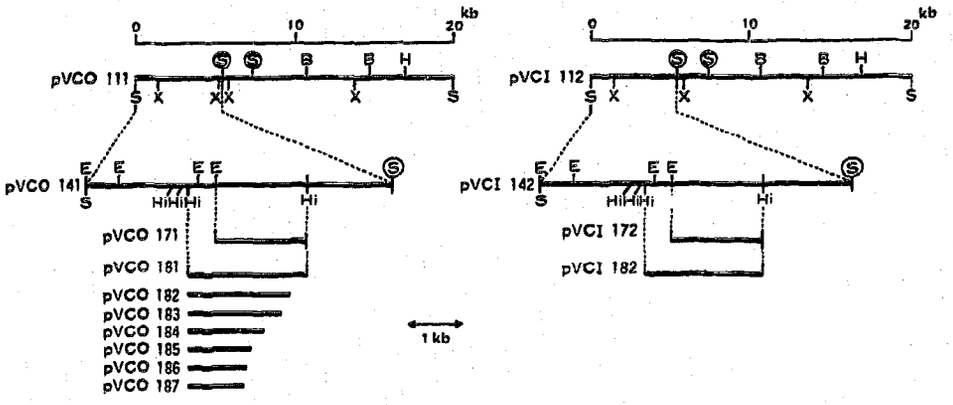


Fig. 1. Restriction maps of pVCO111 (Ogawa-type), pVCI112 (Inaba-type), and their subclones. Abbreviations for restriction enzyme sites: B, *Bam*HI; E, *Eco*RI: H, *Hpa*I; Hi, *Hin*dIII; S. *Sac*I; S, *Sal*I; X, *Xba*I.

contructed (Fig. 1) and subjected to cell-free assay of DNA-directed protein synthesis (Fig. 2). The protein of 27 kDa was synthesized with pVCO182, pVCO183, pVCO184 and pVCO185 as templates. In the case of PVCO186, the protein was produced in a smaller amount thant the other four plasmids. The protein was not produced when pVCO187 was used as a DNA template.

3.2. *Correlated expression of serotype conversion with production of the 27-kDa protein*

The 2.1-kb *Hin*dIII fragment of pVCO141 and pVCI142 were sub-cloned into the vector pACY184, which has a compatible replication origin with that of pUC18; thus, two recombinant plasmids, pVCO434 (Ogawa serotype) and pVCI473 (Inaba serotype) were constructed. They were transformed into the *E. coli* DH5 (pVCI112) which expresses Inaba-type antigen. *E. coli* DH5 (pVCI112, pVCO434) thus obtained was found to express Ogawa-type antigen (Fig. 3A). Then, *Pvu*II fragments containing whole inserts of pVCO185, pVCO186, and pVCO187 were re-cloned into the vector pACYC177, and recombinant plasmids, pVCO185A, pVCO186A, and pVCO187A were obtained respectively. They were transformed into *E. coli* DH5 (pVCI112). Transformants, *E. coli* DH5 (pVCI112, pVCO185A) and DH5 (pVCI112, pVCO186A) thus obtained were found to express antigen of Ogawa serotype, whereas DH5 (pVCI112, pVCO187A) remained with its Inaba-type antigen unchanged (Fig. 3B). Thus, production of 27-kDa protein and serotype conversion correlated each other.

3.3. *Nucleotide sequence analysis of pVCO185*

The nucleotide sequence of the 1121-bp insert of the plasmid, pVCO185 (EMBL accession no. X58834) was determined. Sequencing strategy is illustrated in Fig. 4A. Within the entire insert DNA of pVCO185, only one meaningful open reading frame (longer than 858 bases) was found. The predicted amino acid sequences encoded by the open reading frame are shown (Fig. 4B). Nucleotide sequences corresponding to the concensus sequenses of Shine-Dargarno (SD), Pribnow box (-10 region), and -35 region are underlined. The deletion

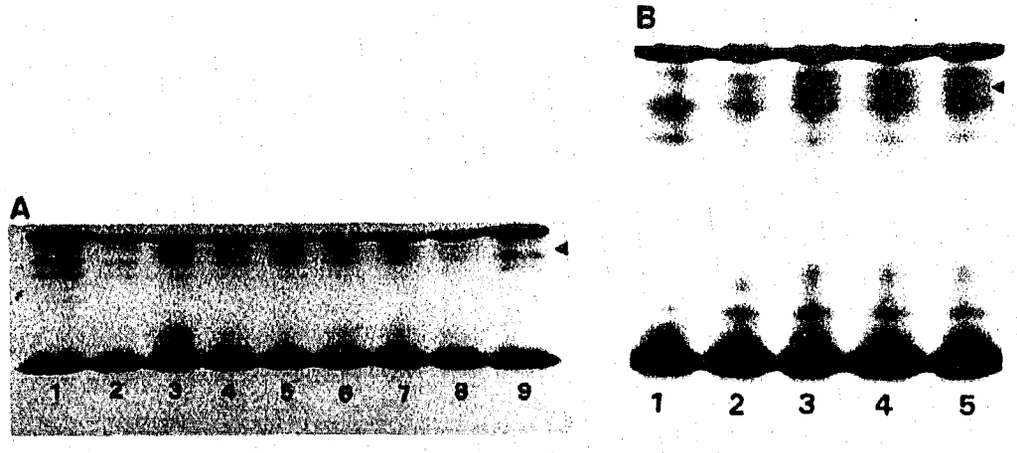


Fig. 2. SDS-PAGE analysis of the product of the cell-free protein synthesis. (A) lanes: 1, pUC18; 2, pVCI182; 3, pVCO181; 4, pVCO182; 5, pVCO183; 6, pVCO184; 7, pVCO185; 8, pVCO186; 9, pVCO187. (B) lanes: 1, pVCO187; 2, pVCO186; 3, pVCO185; 4, pVCO184; 5, pVCO183.

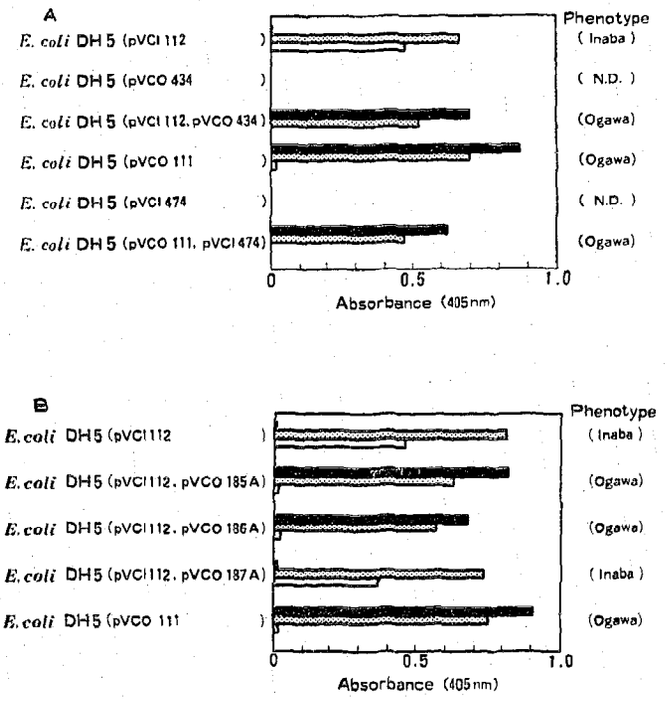
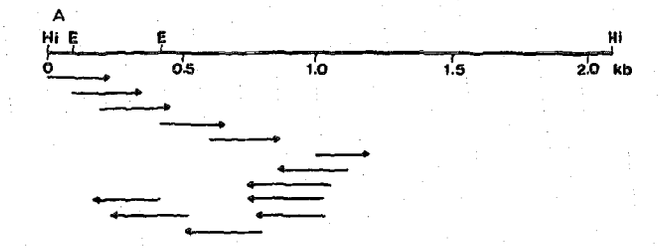


Fig. 3. Reactivities of *E. coli* DH5 cells harboring various combinations of plasmids against 3 monoclonal antibodies. Absorbance data using 3 monoclonal antibodies are presented as follows: closed bar, OF5 (anti-Ogawa antigen); open bar, IH13 (anti-Inaba antigen); and stippled bar, OE5 (anti-Ogawa-Inaba common antigen).

end-points of the plasmids pVCO186 and pVCO187 are also indicated. The location of the open reading frame which starts at nucleotide position 67 and ends at 924, conincides well with the observation that pVCO186 but not pVCO187 was functional in serotype conversion as well as productive of 27-kDa protein in cell-free system.

4. DISCUSSION

A novel gene (*tsf*B), responsible for the expression of Ogawa serotype specificity of *V. cholerae* 01 has been identified and its nucleotide sequence determined. A product of *tsf*B gene caused seroconversion of 01 antigen from Inaba type to Ogawa type. It is reported that the main component of *O*-polysaccharide of *V. cholerae* 01 of both serotypes are Glucose,4-amino-4,6,-dideoxy-D-mannose (perosamine); 2-amino-2,6,-dideoxy-D-glucose (quinovosamine); and 2-amino-2-deoxy-D-glucose (glucosamine), and that the main structure of *O*-polysaccharide is α1-2 linked homopolymer of perosamine [13,14]. No characteristic difference was found in the sugar composition between two serotypes [4,5,6]. On the other hand, Redmond [15] has reported that 4-amino-4-deoxy-L-arabinose is found only in the LPS derived from Ogawa serotype, though the finding is not supported by other investigators [4,5,6]. Clarification of the function of the *tsf*B gene product, which may be the enzymatic synthesis of a sugar



B

pVCO185 pVCO186

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          10        20        30        40        50        60
CCTGCATCTGCAAGTTGATTCTGTATGTTATTTTTTACGCTAATATTATTTAAAATTGAG
                  -35                   -10                 SD
                            pVCO187
          70        80        90       100       110       120
GTAGTAATGAAACATCTAATAAAAAACTATGTACAAAAATTAATTAAAACAGAGCTTGAT
      MetLysHisLeuIleLysAsnTyrValGlnLysLeuIleLysThrGluLeuAsp

         130       140       150       160       170       180
GCTATTCAGTCAAAGTCTGTTCATGATAATCGAAACTTCATTTACAATGGAGAGTTTTTA
AlaIleGlnSerLysSerValHisAspAsnArgAsnPheIleTyrAsnGlyGluPheLeu

         190       200       210       220       230       240
ATTCTTGAAAGCGAATTTGGATGGCATTGTTTTCCCAGAGTGCAGTTGAACCATGCTTTA
IleLeuGluSerGluPheGlyTrpHisCysProArgValGlnLeuAsnHisAlaLeu

         250       260       270       280       290       300
AGCTACAAAAACCCAAACTTTGATTTAGGTATGCGTCACTGGATTGTTAATCATTGTAAG
SerTyrLysAsnProAsnPheAspLeuGlyMetArgHisTrpIleValAsnHisCysLys

         310       320       330       340       350       360
CATGACACCACTTATATTGATATCGGTGCAAACGTTGGAACTTTCTGTGGAATCGCTGCT
HisAspThrThrTyrIleAspIleGlyAlaAsnValGlyThrPheCysGlyIleAlaAla

         370       380       390       400       410       420
CGTCATATTACACAAGGAAAAATTATAGCGATAGAACCACTCACAGAAATGGAAAATAGT
ArgHisIleThrGlnGlyLysIleIleAlaIleGluProLeuThrGluMetGluAsnSer

         430       440       450       460       470       480
ATTAGGATGAATGTTCAATTAAATAATCCACTAGTTGAGTTTCATCATTTTGGCTGTGCA
IleArgMetAsnValGlnLeuAsnAsnProLeuValGluPheHisHisPheGlyCysAla

         490       500       510       520       530       540
ATAGGTGAGAATGAAGGGGAAAATATTTTCGAAGTTTATGAGTTTGATAATAGGGTGTCA
IleGlyGluAsnGluGlyGluAsnIlePheGluValTyrGluPheAspAsnArgValSer

         550       560       570       580       590       600
TCATTATATTTTAAAAAAAATACAGACATAGCAGATAAGGTTAAAAATAGCCAAGTTCTG
SerLeuTyrPheLysLysAsnThrAspIleAlaAspLysValLysAsnSerGlnValLeu

         610       620       630       640       650       660
GTTAGAAAGTTAAGTAGTTTAGATATATCGCCTACTAACTCTGTAGTTATAAAAATTGAT
ValArgLysLeuSerSerLeuAspIleSerProThrAsnSerValValIleLysIleAsp

         670       680       690       700       710       720
GCTGAAGGCGCAGAAATAGAGATATTAAACCAGATTTACGAATTCACAGAAAAGCATAAT
AlaGluGlyAlaGluIleGluIleLeuAsnGlnIleTyrGluPheThrGluLysHisAsn

         730       740       750       760       770       780
GGAATTGAATATTATATTTGCTTTGAATTTGCAATGGGTCATATACAGAGGTCTAATAGA
GlyIleGluTyrTyrIleCysPheGluPheAlaMetGlyHisIleGlnArgSerAsnArg

         790       800       810       820       830       840
ACTTTTGATGAGATTTTTAACATAATAAACTCAAAATTCGGAAGTAAGGCATATTTTATT
ThrPheAspGluIlePheAsnIleIleAsnSerLysPheGlySerLysAlaTyrPheIle

         850       860       870       880       890       900
CATCCATTATCATCCGCTGAACATCCTGAGTTTAATAAAGCAACGCAGGATATTAATGGG
HisProLeuSerSerAlaGluHisProGluPheAsnLysAlaThrGlnAspIleAsnGly

         910       920       930       940       950       960
AATATCTGTTTTAAATATGTATCATAAAATAATTTAATATATTCCGTATGTCATTGCAAG
AsnIleCysPheLysTyrValSer***

         970       980       990      1000      1010      1020
TTCAACAGACATTTCCGAAGAGTTCACTATACAGTTTAGTATAGCTTTGTGCATAGCGAT


        1030      1040      1050      1060      1070      1080
GTGCTGTGAATTCCTGTCAAAACGTTTGCGAGCATTAATACCTAATTTTTCAACAAAGTTT


        1090      1100      1110      1120
TGTATCCATGTTCTATTTTTAACATTGCATCGGAAAAGCTT
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Fig. 4. (A) strategy of sequencing. (B) nucleotide sequence of pVCO185. Number of the nucleotide position starts at the 5' end of the pVCO185 insert. Concensus sequences of Shine-Dargarno (SD), -10 region, and -35 region and indicated by underline. The 5' end of deletant pVCO186 and pVCO187 are also indicated.

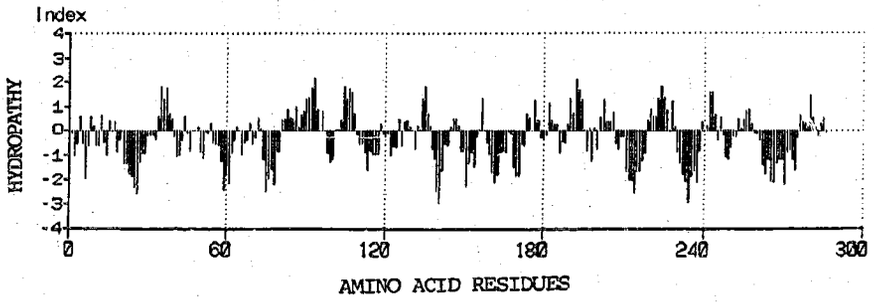

Fig. 5. Hydropathy profiles of the predicted 286 amino acid residues of pVCO185. Program used was Kyte and Doolittle with the window size of 6.

molecule or construction of a polysaccharide structure, will elucidate the molecular difference of O1 antigen of Ogawa and Inaba serotypes.

The protein which is encoded by this gene is predicted to be 33 kDa in size, having a rather hydrophilic profile of amino acid composition (Fig. 5). Homology search of NBRF-PDB (Rel. 27.0) or Swiss Plot (Rel. 15.0) data bank did not reveal any related known protein, suggesting that the protein is novel in its function. Concerning the size of the protein, there was a considerable difference between predicted (33 kDa) and observed (27 kDa) values. A rather small size observed in gel electrophoresis may be accounted for by the non-physiological conditions of transcription and translation using an *E. coli* cell-free system, or it may reflect an unknown physicochemical nature of this protein; that is, it may migrate faster than is expected from its true molecular weight. An alternative possibility is that the protein may be cleaved to the 27-kDa protein and smaller peptides, the latter having been undetectable in gel electrophoresis. Purification of the 27-kDa protein which is now underway will solve these problems in the future.

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