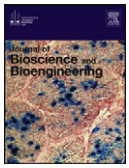

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Organization of the capsule biosynthesis gene locus of the oral streptococcus *Streptococcus anginosus*

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The capsular polysaccharide (CPS) of the important oral streptococcus *Streptococcus anginosus*, which causes endocarditis, and the genes for its synthesis have not been clarified. In this study, we investigated the gene locus required for CPS synthesis in *S. anginosus*. Southern hybridization using the *cpsE* gene of the well-characterized bacterium *S. agalactiae* revealed that there is a similar gene in the genome of *S. anginosus*. By using the colony hybridization technique and inverse PCR, we isolated the CPS synthesis (*cps*) genes of *S. anginosus*. This gene cluster consisted of genes containing typical regulatory genes, *cpsA–D*, and glycosyltransferase genes coding for glucose, rhamnose, *N*-acetylgalactosamine, and galactofuranose transferases. Furthermore, we confirmed that the *cps* locus is required for CPS synthesis using a mutant strain with a defective *cpsE* gene. The *cps* cluster was found to be located downstream the *nrdG* gene, which encodes ribonucleoside triphosphate reductase activator, as is the case in other oral streptococci such as *S. gordonii* and *S. sanguinis*. However, the location of the gene cluster was different from those of *S. pneumonia* and *S. agalactiae*.

[**Key words:** Capsular polysaccharide; *Streptococcus anginosus*; Glycosyltransferase; Oral bacteria; biofilm]

Viridans group streptococci are the primary colonizers of the human tooth surface (1). These bacteria attach to salivary components and form a biofilm that covers the mineral surface through growth and interactions with other bacteria. The capsular polysaccharides (CPSs) of these oral streptococci are known to contribute to biofilm formation on the human tooth surface. In this pathway, the CPSs function as receptors for the lectin-like adhesin protein of other dental plaque inhabitant, *Actinomyces naeslundii* (2,3), which leads to colonization by these bacteria. The presence of the *N*-acetylgalactosamine (GalNAc) β1-3 galactose (Gal) or Galβ1-3GalNAc CPS motif is essential for these interactions (2,4,5). The former type is designated as type Gn, while the latter is designated as type G. The structures of the CPSs of many oral streptococci including *Streptococcus sanguinis*, *S. gordonii*, *S. oralis*, and *S. mitis* have been identified (6–8). These receptor polysaccharides have been divided to 6 groups named 1Gn, 2Gn, 2G, 3G, 4Gn, and 5Gn based on their structural characteristics. The CPS synthesis (*cps*) genes of several oral streptococci have also been reported (3,4,9). These genes tend to be found in *cps* gene clusters such as those of *S. pneumoniae* and *S. agalactiae*, some strains of which cause meningitis (10–17). The typical structure of the *cps* cluster consists of four common regulatory genes in front of a large operon, glycosyltransferase genes for the synthesis of lipid-linked repeating units, a flippase gene for the transport of the repeating units to the outer surface of the membrane, and a polymerase gene for forming polysaccharides from the repeating units.

S. anginosus is another oral streptococcus that is found in the mouths of healthy individuals, predominantly on the surface of the teeth and in the throat, nasopharynx, and feces (18). This strain has been referred to as *S. milleri*, *S. constellatus*, and *S. intermedius*. Generally, streptococci are classified based on their reactivity against Lancefield grouping serum (18). Most *S. anginosus* strains are known to react with group F antiserum. However, unlike other oral streptococci such as *S. gordonii* and *S. oralis*, the structures of the CPS of *S. anginosus* and their synthesis genes are still unclear. In this study, we isolated the *cps* gene cluster of *S. anginosus* and analyzed its structure and function.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids *S. anginosus* ATCC 33397 was purchased from the American Type Culture Collection. This strain was cultured in Brain Heart Infusion Broth (Nissui, Tokyo) under 5% CO_2 at 37°C. The insertional mutant of this strain, in which the *cpsE* gene was mutated, was cultured in the presence of erythromycin (2 μg/ml). *Escherichia coli* DH5α and JM109 were used as the hosts for the genomic DNA library and expression plasmids, respectively. All *E. coli* clones were routinely grown in Luria-Bertani broth containing ampicillin (50 μg/ml).

DNA manipulation The DNA manipulation was performed according to standard procedures. Chromosomal DNA was isolated as reported previously for *S. agalactiae* (10). To detect and isolate the *cps* genes of *S anginosus*, we used the *cpsIaE* gene of *S. agalactiae* Ia as a probe. The probe was amplified from the genomic DNA of *S. agalactiae* using primers (5′-CAATCAAATGACAGGGCTAAT-3′ and 5′-TAAAAC-TAAGGCGTCGCTT-3′) and labeled with [α-^{32}P] dCTP using the BcaBEST™ labeling kit (Takara, Kyoto). PCR was performed with KOD Plus DNA polymerase (Toyobo, Osaka) according to the manufacturer's instructions. Southern hybridization was performed using low stringency buffer (5× SSC, 20% formamide, and 0.1% SDS) at 42°C. Inverse PCR was performed as described previously (16). Briefly, chromosomal DNA (5 μg)

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was digested with restriction enzymes, and the cleaved DNA was then self-ligated at 4°C for 16 h at a concentration of 1 μg/ml. PCR was performed with KOD Plus using 50 ng of ligated DNA and 0.2 μM of appropriate primers. Each inverse PCR product was sequenced to permit the design of primers for the subsequent rounds of PCR.

DNA sequencing The DNA sequences of both strands of each PCR product were determined using an ABI 310 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). The sequencing data were compared with those in the DDBJ, EMBL, and GenBank databases using the BLAST network service at the National Center for Biotechnology Information (National Institute of Health, Bethesda, MD, USA). Multiple sequence alignments were performed using GENETYX (Software Development Co., Tokyo).

Construction of the expression plasmid To construct the expression plasmid, pE, the *cpsE* gene was directly amplified with chromosomal DNA from *S. anginosus* ATCC 33397 using primers (5′-CATGAGCTCGATTAGCATTTAGGAGTAGGGA-3′ and 5′-CATGGTACCAGTAGAACTACTTCGCTCCG-3′, restriction sites are underlined). The resultant PCR products were digested with restriction enzymes to cleave the recognition sites within each primer sequence and ligated into the pBluescript II SK (+) vector (Stratagene, La Jolla, CA, USA). In consequence, the plasmid, pE, in which the *cpsE* gene was under the control of the *lac* promoter of the pBluescript II SK (+) plasmid was obtained and was then introduced into *E. coli* JM109. The membranes of recombinant *E. coli* cells were isolated 2 h after induction with 1 mM IPTG for the preparation of CpsE protein, as described previously (10,19).

Glycosyltransferase assay After the enzyme reaction between the membrane fraction of the recombinant *E. coli* JM109 and UDP-[^{14}C]glucose had finished, the reaction product (lipid carrier) was extracted with extract solution (1.5 ml chloroform, 25 ml methanol, 23.5 ml water, and 0.183 g KCl) and analyzed using a liquid scintillation counter to assess the enzyme activity of the *cpsE* gene product, as described previously (10,17,19). *E. coli* JM109 cells harboring pFGH, the pBluescript II SK (+) plasmid containing the *cpsF* to *cpsH* genes and the truncated *cpsE* gene, were used as a negative control.

Construction of an insertional mutant To produce an insertional mutant of *cpsE*, the 5′- and 3′- regions of the gene were amplified using primers (5′-CATGGTACCATACACCACCGATTGGACTG-3′ and 5′-CATCTCGAGACTGCATAAAA-AGCACTGT-3′ for the 5′- region, and 5′-CATCTGCAGGTTGGACCATTTGGTCAG-3′ and 5′-CATGGATCCATCAAAGTGCCCCCAAG-3′ for the 3′- region, restriction sites are underlined) and then introduced into the pBluescript II KS vector. Then, the erythromycin resistance gene (*ermC*) was amplified from the pTV32-OK streptococcal vector (20) and inserted between the 5′- and 3′- regions of *cpsE*. The temperature sensitive replication initiator gene (*repA-ts*) and the replication origin of the broad-host-range lactococcal plasmid (21,22) were also amplified from pTV32-OK using primers (5′-CATTCTAGATTTCGTTTAGTTATCGGCA-3′ and 5′-CATGGGCCCAAAA-TAAAAACCCCCTTCG-3′) and introduced into the vector. The constructed plasmid was then introduced into *S. anginosus* by electroporation, and allelic exchange mutagenesis was performed as described previously (23,24). The transformants were obtained at 30°C by erythromycin selection, and the temperature was shifted to a nonpermissive temperature (40°C) in order to select the insertional mutants. The replacement of *cpsE* was confirmed by detecting the insertion of *ermC* and the truncation of *cpsE*. The location of the *ermC* gene in the insertional mutant was verified by the amplification of specific PCR products across the upstream and downstream boundaries of the *ermC* insertion, using primers for upstream and downstream chromosomal sequences that were extraneous to those present in knockout plasmid construct.

ELISA CPS production of *S. anginosus* was analysed by ELISA using antiserum prepared from a rabbit. The antiserum was prepared by six times of intravenous injections of formaldehyde (0.4%)-fixed *S. anginosus* to the rabbit. An ELISA plate was coated with 10 μg/ml poly-D-lysine. Wild type and mutated strains of *S. anginosus* were placed on the plate. After drying up the plate, it was treated with 5% acetic anhydride in saturated sodium carbonate to quench non-reacted amino groups of poly-lysine. Blocking reaction was performed using PBS containing 5% skim milk. As a primary antibody, the antiserum against *S. anginosus* was used at a dilution of 1:200,000. As a secondary antibody, horse radish peroxidase-conjugated goat antibody against rabbit IgG (sc-2030, Santa Cruz, CA, USA) was used at a dilution of 1:5,000. All animal experiments were performed according to the ethical guidelines for animal experimentation of Nagoya University.

Nucleotide sequence accession number The sequence reported here was submitted to the GenBank database through DDBJ under accession no. AB643814.

RESULTS AND DISCUSSION

Cloning of the *cps* genes from *S. anginosus* In order to clone the *cps* genes of the *S. anginosus* ATCC 33397 strain, a DNA fragment containing a section of the *cpsIaE* gene of *S. agalactiae* type Ia, which codes for a glucosyltransferase that transfers glucose onto a lipid carrier, was used as a probe (10). Since this gene is known to be present in the majority of the so far characterized *cps* gene clusters of streptococci (14,15), we thought that it might be useful as a probe for detecting a novel *cps* operon of *S. anginosus*. We performed Southern hybridization against the chromosomal DNA of *S. anginosus* with low stringency and found a specific DNA fragment that showed similarity to the *cpsIaE* gene on the *S. anginosus* chromosome (Fig. 1A). This 2 kb *Hin*dIII DNA fragment was cloned into the pBluescript II SK (+) vector. As a result of the sequencing of this fragment, we found that the gene shares homology with the *cpsIaE* gene of *S. agalactiae* (56% at the amino acid level) and named it *cpsE*. Using the inverse PCR method and colony hybridization, we were able to isolate the whole *cps* operon from *S. anginosus* ATCC 33397.

DNA sequence analysis The DNA sequence of the fragment, which was 23,804 nucleotides long, was completely determined on both strands using overlapping clones that covered the *cps* gene locus. Sequence analysis detected 24 ORFs, which were designated *nrdD*, *orfW*, *orfX*, *orfY*, *nrdG*, *cpsA* to *cpsN*, *orfO* to *orfR*, and *polI*. Homology analysis indicated that the central 14 genes from *cpsA* to *cpsN* were associated with CPS synthesis (Table 1). These *cps* genes were in the same orientation and were separated by short distances. Potential -35 (TTAACT) and -10 (TATAAT) promoter sequences were identified upstream of *cpsA*. A putative Rho-independent transcription terminator sequence was found downstream of *cpsN* ($\Delta G = -24.5$ kcal/mol) (Fig. 1B). These observations suggest that the 14 ORFs from *cpsA* to *cpsN* constitute one polycistronic operon and that transcription starts from *cpsA*. A possible Shine–Dalgarno sequence was identified just upstream of the potential initiation codon of each ORF. All ORFs except for *cpsB* were preceded by ATG codons. *cpsB* was preceded by a TTG codon. The mean G + C content of the sequenced area was 33.5%. The percentage G + C content of the *cps* cluster agreed well with that of the chromosomal DNA of *S. anginosus* (34–39%) (18).

The amino acid sequence of each ORF was deduced, and an overview of all *cps* genes and several genes in their flanking regions together with their properties and translation products are shown in Table 1. The *cpsA* gene product showed a high degree of similarity to the CpsIaA (50% identity) protein of *S. agalactiae* type Ia (10) and the Wzg (79% identity) protein of *S. gordonii* 38 (4,9). The streptococcal CpsA proteins are thought to be involved in the transcriptional regulation of the *cps* genes. However, the mechanism by which these membrane-bound transcription factors regulate their transcription is still unclear.

The *cpsB* to *cpsD* genes were also found downstream of *cpsA*, as is the case in other streptococci. These gene products also showed high degrees of similarity to the corresponding gene products of other streptococci (Table 1). These genes have been suggested to play important roles in the accumulation of polysaccharides on the cell surface through tyrosine phosphorylation and dephosphorylation of CpsD (25).

The *cpsE* gene product showed high similarity to those of another oral streptococcus, *S. gordonii* 38 (59%), and a lactic acid streptococcus, *S. thermophilus* 5. Furthermore, it showed a close similarity to the CpsIaE (56% identity) protein of *S. agalactiae* type Ia (10). CpsIaE was suggested to be a glucosyltransferase that catalyzes the linkage formation between the first sugar (glucose) and a lipid carrier based on the results of glycosyltransferase assays (10); thus, the CpsE of *S. anginosus* might also transfer glucose to the cell surface lipid carrier.

The gene product encoded by *cpsF* showed close similarity to those of the *wchF* genes of *S. pneumoniae* 7f (82%), *S. gordonii* 38 (82%), and *S. oralis* 34 (82%) (Table 1). Moreover, it also showed high similarity to the product of the *Eps5F* gene of *S. thermophilus* 5. Since both the WchF and Eps5F proteins are considered to be rhamnosyltransferases based on the structures of their polysaccharides and sequence similarity (4), the CpsF of *S. anginosus* probably plays the same role. CpsG shares homology with the WcwA protein of *S. pneumoniae* 7f, the WefA proteins of *S. gordonii* 38 and *S. oralis* 34, and the Eps5G protein of *S. thermophilus* 5 (3,4). Thus, it is expected to be an α-1,3-GalNAc transferase or α-1,3-galactosyltransferase.