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Biofilm-Forming Capacity on Clinically Isolated *Streptococcus constellatus* from an Odontogenic Subperiosteal Abscess Lesion

Takeshi Yamanaka[1*], Tomoyo Furukawa[1], Kazuyoshi Yamane[1], Takayuki Nambu[1], Chiho Mashimo[1], Hugo Maruyama[1], Junichi Inoue[1], Maki Kamei[1], Hiroshi Yasuoka[1], Shuji Horiike[1], Kai-Poon Leung[2] and Hisanori Fukushima[1]

[1]*Department of Bacteriology, Osaka Dental University, Hirakata, Japan*
[2]*US Army Dental and Trauma Research Detachment, Institute of Surgical Research, Fort Sam Houston, TX, USA*

Abstract

S. Treptococcus constellatus, a member of the *S. Treptococcus anginosus* Group (SAG), and known as part of indigenous oral microbiota, has been described to cause abscesses in various regions of the body, despite this organism appears to be innocuous, in general at its habitat. In this communication, we report a biofilm-forming capacity of a facultative anaerobic gram-positive coccus isolated as a dominant bacterium in an odontogenic subperiosteal abscess lesiony. The clinical isolate designated as strain H39 formed dense meshwork structures around the cells that are typical for biofilm forming bacteria, and produced viscous materials in its spent culture media. The 16S rRNA gene sequence of strain H39 was 99% homologous to that of *S. Treptococcus constellatus* ATCC 27823, a type strain for *S. Treptococcus constellatus*. Phylogenetic analysis using data sets of *recN, groEL, tuf* and 16S rRNA genes showed a sister relationship between strain H39 and *S. Treptococcus constellatus* ATCC 27823. Dense meshwork-like structures found on strain H39 were observed on *S. Treptococcus constellatus* ATCC 27823, but not on *S. intermedius* ATCC 27335 and *S. anginosus* ATCC 33397. Biofilm assay using 96-wells polystyrene microtiter plates revealed that *S. Treptococcus constellatus* strains H39 and ATCC 27823 can form dense biofilm on abiotic materials consistently. *S. intermedius* ATCC 27335 was able to form biofilm on microtiter plates at a lesser extent to those of *S. Treptococcus constellatus* strains. *S. anginosus* ATCC 33397 did not form biofilm on an abitotic material. As conclusions, dense meshwork structures around the cells of *S. Treptococcus constellatus*, and the capacity of *S. Treptococcus constellatus* and *S. intermedius* to form biofilm on abiotic materials as observed in this study might be related to the pathogenicity of these two organism and the tropism of organisms in SAG. As recently suggested, phylogenetic analysis could be a powerful tool for differentiating and identifying clinical isolates belong to SAG.

Keywords: *S. Treptococcus constellatus; S. Treptococcus intermedius; S. Treptococcus anginosus;* Subperiosteal abscess; Root canal infection; Biofilm

Introduction

S. Treptococcus constellatus is a part of normal commensal flora in the mouth, oropharynx and gastrointestinal tract, and composes the *S. Treptococcus anginosus* Group (SAG), together with *S. Treptococcus anginosus* and *S. Treptococcus intermedius* [1,2]. *S. Treptococcus constellatus* has been cultured from dental caries and periodontitis lesions, but has also been found to cause purulent infections with abscess formation in various regions of the body, especially in thorax and head and neck regions, despite this organism appears to be innocuous at its natural habitat [3-5]. In contrast, *S. anginosus* and *S. intermedius* in SAG show tropism for abdomen and genitourinary tract, and for liver and Central Nerve System (CNS), respectively. There apparently appears to be species bias to some infections, but mechanisms behind such phenomena are not fully understood [6,7].

In this study, we found that *S. Treptococcus constellatus* strain H39 isolated from a subperiosteal abscess lesion due to the infection of root canal system of a tooth, has a capacity to produce viscous materials in spent culture media and form dense meshwork structures, a typical phenotype for biofilm-forming bacteria [8], around cells on agar plates. This phenotype was also observed on type strains for *S. Treptococcus constellatus*. As far as we retrieved, only a small number of literatures are available regarding biofilm-phenotypic characterization of SAG [4,9,10]. Therefore, we also examined the capacity of this isolate and type strains of SAG, to form biofilm on abiotic materials.

Materials and Methods

Bacteria and tentative identification

A clinically isolated facultative anaerobic Gram-positive coccus capable of producing viscous materials in its spent culture medium was used. The isolate was recovered in pure culture from an odontogenic subperiosteal abscess lesion, and designated as strain H39. Cultures were maintained by weekly transfer on a trypticase soy agar plate supplemented with 5% sheep blood (TSA-B: BD BBL, Franklin Lakes, NJ, USA) in a 10% CO_2, 10% H_2, 80% N_2 atmosphere at 37°C in an anaerobic chamber (ANX-3, Hirasawa, Tokyo, Japan). Genomic DNA was extracted from strain H39, using MagExtractor (TOYOBO, Osaka, Japan), according to the manufacturer's instructions. The nucleotide sequence of the 16S rRNA gene of the clinical isolate amplified with a primer pair of 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1525R (5'-AAAGGAGGTGATCCAGCC-3') was identical [DNA Data Bank of Japan (DDBJ: http://www.ddbj.nig.ac.jp) accession: AB749301] and 99% homologous to *S. Treptococcus constellatus* ATCC 27823 (accession number: AF104676).

S. Treptococcus constellatus ATCC 27823, *S. intermedius* ATCC 27335 and *S. anginosus* ATCC 33397 were used as the type strains for SAG, and the cultures were maintained as described above.

***Corresponding author:** Takeshi Yamanaka, Department of Bacteriology, Osaka Dental University, 8-1, Kuzuha Hanazono, Hirakata, Osaka 573-1121, Japan, Tel: +81-72-864-3051; Fax: +81-72-864-3151; E-mail: yamanaka@cc.osaka-dent.ac.jp

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Identification by phylogenetic analysis

Identification of organisms in SAG had long been problematic due to the considerable genetic similarity within the group and heterogeneous phenotypes on each species in SAG, but phylogenetic analysis using several housekeeping gene sequences is recently acknowledged as a powerful tool to divide SAG in three species [11-14]. Therefore, one of clinical isolates designated as strain H39 was subjected to phylogenetic analysis, using partial sequences of 16S rRNA, *recN, groEL* and *tuf* genes.

Briefly, strain H39 was grown anaerobically for 48 hours and the genomic DNA was extracted using MagExtractor (TOYOBO), as described above. Gene fragments of r*ecN, groEL* and *tuf* were amplified with primer pairs of Si-*recN*-left (5'-TGAATATGATGTTGGGAAGC-3') and Si-*recN*-right (5'-ACAATGCTTGTCTTGCCTTC-3'), *groEL*-FW (5'-GGNGACGGNACNACNACNGCAACNGT-3') and *groEL*-RV (5'-TCNCCRAANCCNGGYGCNTTNACNGC-3'), and *tuf*-FW (5'-AAYATGATIACIGGIGCIGCICARATGG-3') and *tuf*-RV (5'-CCIACIGTICKICCRCCYTCRCG-3'). PCR was performed using GoTaq Master Mix (Promega, Tokyo, Japan) and MJ Mini Personal Thermal Cycler (Bio-Rad, Tokyo, Japan) for 30 cycles of 30 s at 95°C, 30 s at 50°C, and 2 min at 72°C, with a final extension at 72°C for 7 min. The amplified fragments were subsequently sequenced on the Applied Biosystem 3130xl DNA Analyzer (Applied Biosystems, Tokyo, Japan). The sequence data were assembled with the GENETYX Ver.7 program (Genetyx Ltd., Tokyo, Japan), and deposited in the DDBJ (accession number: AB749302-4). Phylogenetic analyses were performed by using obtained sequences of the housekeeping genes and the 16S rRNA gene and the data sets of *recN, groEL, tuf* and 16S rRNA genes for *streptococci* described elsewhere [11,12,14]. The nucleotide sequences of these genes were aligned by MAFFT (version 6) with FFT-NS-I option [15]. Then, a neighbor-joining tree was constructed using Jukes-Cantor distance matrices for each housekeeping gene.

Measurement of viscosity

The viscous material-producing ability of clinical isolates was evaluated by measuring the viscosity of spent culture media, as described elsewhere [16]. Arbitrarily selected eight clinical strains and type strains of SAG were grown in trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD, USA), supplemented with 0.5% yeast extract (BBL Microbiology Systems) (TSB-Y) for 48 h. The viscosity was measured as shearing stress between a rotor and a rotor shaft at 50 rpm, 20°C, using a rotary viscometer (Toki-sangyo, Tokyo, Japan), in accordance with the manual provided by the manufacturer. The measurement was performed five times on each sample, and the results of four independent cultures were averaged to give the final value. Statistical differences between the bacterial cultures and control medium were determined using an unpaired *t*-test, with the level of significance set at $P<0.05$.

Preparation for Scanning Electron Microscopy (SEM)

For SEM studies, cells scraped from 1-day-old cultures were collected on a piece of filter paper (Glass fiber GA55, Toyo Roshi, Tochigi, Japan), fixed with 2% glutaraldehyde in 0.1 M phosphate buffer (Mitsubishi Chemical Medience, Tokyo, Japan) for 2 hours, and with 1% OsO_4 in 0.1M phosphate buffer for 1 h at 4°C and dehydrated through an ethanol series and 2-methyl-2-propanol, followed by platinum ion coating (E-1030, HITACHI, Tokyo, Japan). Specimens were examined with a scanning electron microscope (S-4800, HITACHI), at an accelerating voltage of 3 kV.

Biofilm formation assay

The ability to form biofilm was determined, as described previously [17]. Briefly, the seed cultures of *S. Treptococcus constellatus* strains H39 and ATCC 27823, *S. intermedius* ATCC 27335 and *S. anginosus* ATCC 33397 were prepared as described above, and diluted to an O.D. of 0.1 at 620 nm in the same medium. Next, 150 µl diluted culture was transferred to three sterile polystyrene microtiter plate wells (IWAKI, Tokyo, Japan) per strain. Sterile TSB-Y was used as a control. The plates were prepared in duplicate and incubated at 37°C for 24 h. Unbound cells were removed by inversion of the microplate and tapping on absorbent paper towel. Adhered cells were then stained with 0.1% crystal violet for 15 min. Excess stain was removed by washing with PBS. The stained wells were recorded photographically using a camera (PC1089, Canon, Tokyo, Japan) set. The crystal violet was then solublized by the addition of 95% ethanol, and absorbance at 570 nm was measured by spectrophotometer U-2000 (Hitachi). Each strain was assayed in three wells on each plate and the whole experiment was repeated three times.

Results

Identification of strain H39

The nucleotide sequence of 16S rRNA gene of strain H39 (accession number: AB749301) was identical and highly homologous to that of *S. Treptococcus constellatus* ATCC 27823 (accession number: AF104676).

Phylogenetic analysis using the partial gene sequences of 16S rRNA gene (accession number: AB749301), *recN* (accession number: AB749304), *groEL* (accession number: AB749303) and *tuf* (accession number: AB749302) deposited in DDBJ (http://www.ddbj.nig.ac.jp) produced trees with a similar topology. A sister relationship between strain H39 and a type strain of *S. Treptococcus constellatus* was strongly supported by high bootstrap values (Figure 1). Therefore, strain H39 was identified as *S. Treptococcus constellatus*.

Viscous material productivity

Strain H39 and type strains of SAG were inoculated into TSB-Y and grown for 48 h. The viscosities of the spent culture media were measured using a rotary viscometer. Strain H39 produced materials *in vitro* that were highly viscous, as compared with the control medium (Figure 2). The viscosity of spent culture medium of *S. Treptococcus constellatus* ATCC 27823 was as high as those of the clinical isolates. The viscosities of *S. intermedius* ATCC 27335 and *S. anginosus* ATCC 33397 were similar to that of control medium, correlated to its cell surface structures (Figure 2).

Biofilm phenotype on strain H39 and type strains of SAG

SEM observation revealed that strain H39 showed surface-associated dense meshwork-like structures around the cells (Figure 3). Similar surface structure was observed on *S. Treptococcus constellatus* ATCC 27823, but not on *S. intermedius* ATCC 27335 and *S. anginosus* ATCC 33397 (Figure 3).

Biofilm formation assay

The ability to form biofilm was investigated for *S. Treptococcus constellatus* strains H39 and ATCC 27823, *S. intermedius* ATCC 27335 and *S. anginosus* ATCC 33397, using crystal violet microtiter plate assay. Strain H39 was consistently able to form biofilm on flat-bottomed polystyrene microtiter plates (Figure 4). Type strains of *S. Treptococcus constellatus* and *S. intermedius* were able to form biofilm on abiotic materials. The capacity to form biofilm on *S. Treptococcus*

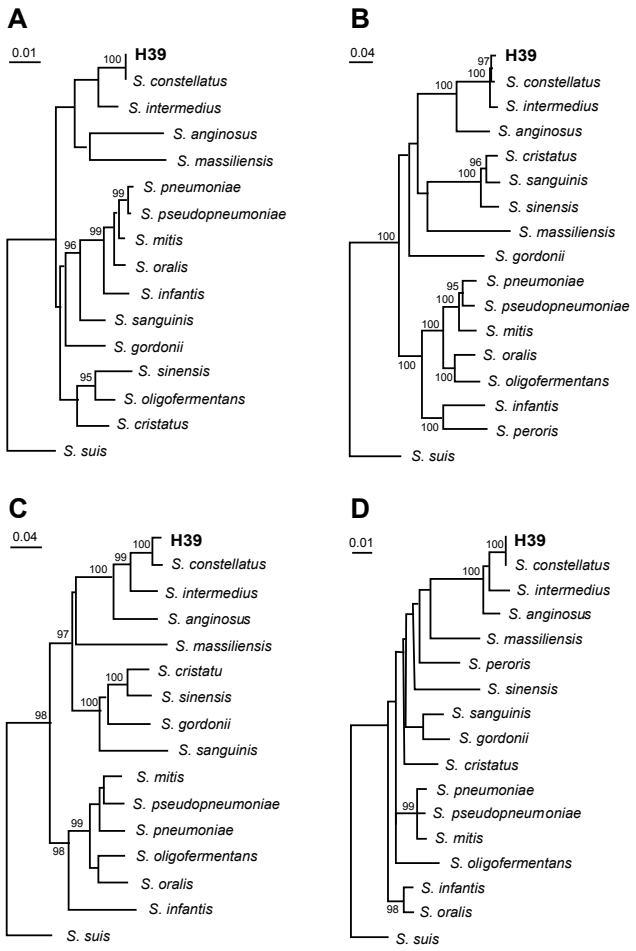

Figure 1: Phylogenetic tree based on partial sequences of 16S rRNA (A), *recN* (B), *groEL* (C) and *tuf* (D) genes. Phylogenetic analyses using these four housekeeping genes produced trees with a similar topolygy. A sister relationship between strain H39 and a type strain of *S. Treptococcus constellatus* was strongly supported by high bootstrap values.

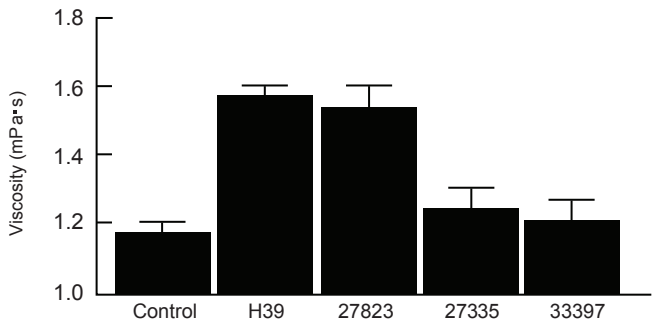

Figure 2: Viscosity of the spent culture media obtained from *S. Treptococcus constellatus* strains H39 and ATCC 27823, *S. Treptococcus intermedius* ATCC 27335 and *S. Treptococcus anginosus* ATCC 33397. Viscosity of the enriched tryptic soy broth medium was measured as a control. Bars indicate standard deviations.

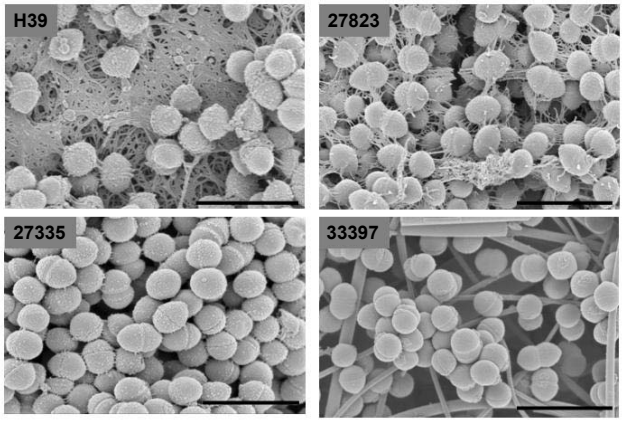

Figure 3: Scanning electron micrographs showing surface structures of *S. Treptococcus constellatus* strains H39 (H39) and ATCC 27823 (27823), *S. Treptococcus intermedius* ATCC 27335 (27335) and *S. Treptococcus anginosus* ATCC 33397 (33397). *S. Treptococcus constellatus* strains H39 and ATCC 27823 had dense meshwork structures surrounding the cell surfaces, but other strains lacked this phenotype. Thick fibers behind *S. anginosus* ATCC 33397 cells are glass fibers of filter paper (33397). Bars=2 μm.

constellatus ATCC 27823 seemed to be comparable to that of strain H39, but *S. intermedius* ATCC 27335 was weaker than that of strain H39. *S. anginosus* ATCC 33397 could not form biofilms on microtiter plates (Figure 4).

Discussion

Clinical studies [3,18] have shown that *S. Treptococcus constellatus* has the capacity to cause invasive purulent infection relatively in superficial areas through the body, while *S. intermedius* and *S. anginosus* in SAG exhibit a marked association with the infection in the central nervous system and gastrointestinal tracts, respectively. This organism is not frequently isolated from abscess lesions deep inside of the body, but several clinical reports showed the association of *S. Treptococcus constellatus* with brain abscesses [19,20], endocarditis [21-23], and sepsis after dental treatment [24]. The remarkable propensity to cause abscess or purulent infection observed on three species in SAG remind us an idea that there might be a unique interaction between the organisms in SAG and human neutrophils. In this regards, Wanahita et al. [25] reported that SAG organisms stimulated less chemotaxis than did *Staphylococcus aureus*, in contrast to viridans Streptococci (*S. Treptococcus mitis, S. Treptococcus parasanguinis and S. Treptococcus sanguinis*), which caused greater chemotaxis than did *S. aureus*. Interestingly, SAG and viridans streptococci were equally engulfed by human neutrophils than *S. aureus*, but killed more slowly and less completely. Our previous studies showed that the resistance of biofilm-forming *Prevotella intermedia* strains to phagocytosis by human neutrophils were more significant than those of strains lacking biofilm phenotype [16,17,26]. We hypothesize that biofilm bacteria, being held together by exopolysaccharides as biofilm matrices, might present a huge physical challenge for phagocytosing neutrophils. As a consequence of these neutrophils being frustrated by their inability to phagocytose this bacterial mass, this might trigger the unregulated release of bactericidal compounds that could cause tissue injury [27], as shown in the inflammatory pathway associated with lung injury or chronic wounds [28,29]. Further investigation is needed to clarify whether inconsistency of biofilm phenotype in SAG gives influence of their interaction with human neutrophils and propensity to cause abscesses.

As described above, only a few studies have done regarding biofilm phenotype on *S. Treptococcus constellatus*. Landrygan-Bakri et al.

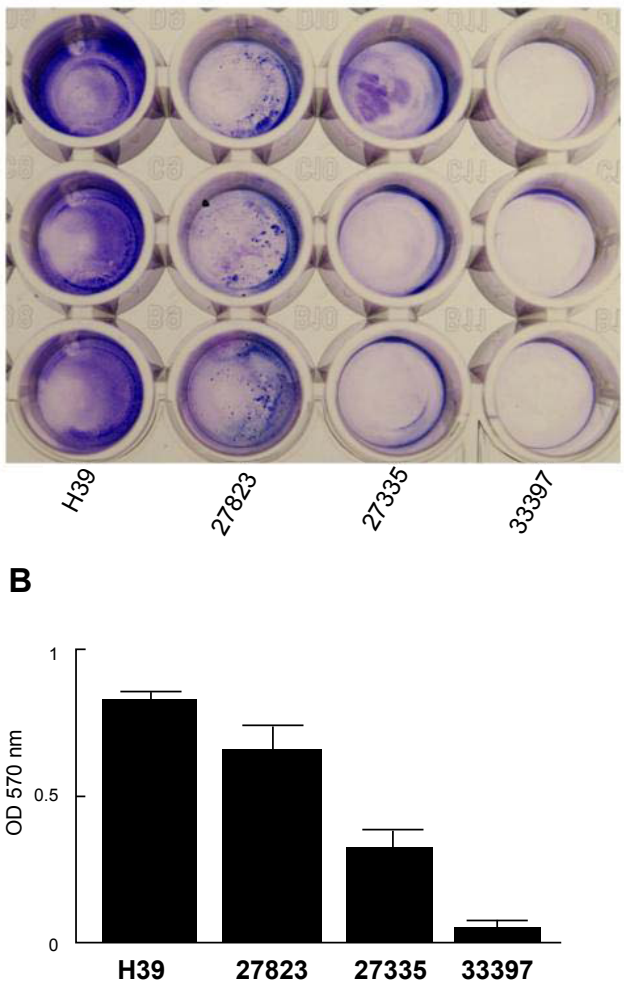

Figure 4: Biofilm formation on microtiter plates. Biofilm production of *S. Treptococcus constellatus* strains H39 and ATCC 27823, *S. Treptococcus intermedius* ATCC 27335 and *S. Treptococcus anginosus* ATCC 33397 on a polystyrene microtiter plate: a set of microtitier plate wells (three wells for each strain) stained with 0.1% crystal violet solution after 24 h of incubation (A). The quantitative analysis of biofilm production as measuring the optical density of destained biofilms at 570 nm (B). The data are from one of three independent experiments. Bars indicate standard deviations.

[10] investigated the adherence of SAGs to the matrix proteoglycans and fibronectin. All tested strains of *S. intermedius* in their study adhered strongly to gingival dermatan sulphate proteoglycans, as well as chondroitin sulphate proteoglycans (except one clinical isolate). In contrast, two strains of *S. anginosus* and all *S. intermedius* test strains showed strong adherence to fibronectin. *S. Treptococcus constellatus* was less adherent to extracellular matrices of human tissue than *S. intermedius* [10]. Regarding the cell surface structures of organisms in SAG, biofilm-like structures were observed on a clinical strain of *S. Treptococcus constellatus* isolated from a severe recurrent form periodontitis lesion [4]. Petersen et al. [9] showed that the quorum sensing mediated by competence-stimulating signaling peptide favored the biofilm mode of growth of *S. intermedius*, in which the organism does not show meshwork structures on the cell surface. In this study, *S. intermedius* ATCC 27335 was able to form biofilm on microtiter plates, but lacked dense meshwork structures. Presumably there might be different systems to induce biofilm growth between *S. Treptococcus constellatus* and *S. intermedius*.

In this study, we examined one clinical isolate and three type strains for SAG, and we found diversity of cell surface structures between the organisms in SAG. In addition to the previously known various properties of SAG which cause ambiguity in determining pathogenic potential of organisms in SAG, the phenotypic difference found in this study might influence their pathogenicity and tropism in the human body. It is known that *S. Treptococcus constellatus* is sometimes co-isolated with *Eikenella* species [30], or *Prevotella* species [31,32], so the capacity to form single/multi species biofilm on *S. Treptococcus constellatus* should be further investigated.

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