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Method Article

A novel plasmid, pSAA0430-08, from *Streptococcus anginosus* subsp. *anginosus* strain 0430-08

Atsushi Tabata[a], Douglas Deutsch[b], Seiya Otsuka[c], Kathleen Verratti[d], Toshifumi Tomoyasu[a], Hideaki Nagamune[a], Vincent A. Fischetti[b,*]

[a] Department of Bioengineering, Division of Bioscience and Bioindustry, Graduate School of Technology, Industrial and Social Sciences, Tokushima University Graduate School, Tokushima, Japan
[b] Laboratory of Bacterial Pathogenesis and Immunology, The Rockefeller University, New York, NY, USA
[c] Department of Biological Science and Technology, Life System, Institute of Technology and Science, Tokushima University Graduate School, Tokushima, Japan
[d] Applied Physics Laboratory, Johns Hopkins University, Baltimore, MD, USA

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ABSTRACT

Mobile genetic elements (MGEs) are the genetic material often involved in the interspecies and intraspecies genetic transduction in bacteria. However, little is known about MGEs in the Anginosus group of streptococci (AGS), one of the streptococcal groups found in the oral cavity of humans. We looked for the presence of MGEs in *Streptococcus anginosus* subsp. *anginosus* (SAA), a representative species belonging to AGS, and found a novel plasmid from SAA strain 0430-08. This plasmid was 7038 bp and ~31% G/C content which we named pSAA0430-08, and examined its genetic structure and characteristics. Open reading frame (ORF) prediction revealed that pSAA0430-08 was composed of 10 ORFs including a putative plasmid replication protein (ORF1) and a putative toxin-antitoxin system (ORF9 and ORF10). Between ORF10 and ORF 1, four tandem repeats of 22 bp each, generally termed as iteron, were also observed. Using variant plasmids of pSAA0430-08, we confirmed that both ORF1 and iteron were necessary for replication in host cells. Interestingly, the region from ORF4 to ORF7 showed homology with a genomic DNA segment of *S. gordonii* strains. Thus, this plasmid may travel between the different species in Streptococci, *i.e.*, *S. gordonii* and *S. anginosus*.

1. Introduction

The Anginosus group streptococci (AGS) is one of the representative bacteria found in the oral cavity of humans. According to the latest classification, AGS is composed of three species including five subspecies, *Streptococcus anginosus* subsp. *anginosus* (SAA), *S. anginosus* subsp. *whileyi*, *S. constellatus* subsp. *constellatus* (SCC), *S. constellatus* subsp. *pharyngis*, *S. constellatus* subsp. *viborgensis*, and *S. intermedius* (Jensen et al., 2013). Generally, it was believed that the pathogenicity of AGS was negligible and strains belonging to AGS were strictly commensal and not human pathogens. However, it is now recognized that AGS is an opportunistic pathogen, documented by increases in clinical reports and epidemiological studies (Bert et al., 1998; Claridge et al., 2001; Elhussein and Hutchison, 2014; Haidar et al., 2015; Jacobs et al., 1995; Ng and Mukhopadhyay, 2009; Noguchi et al., 2015; Siegman-Igra et al., 2012; Suzuki et al., 2016; Tanaka et al., 2015; Terzi et al., 2016; Tran et al., 2008; Whiley et al., 1992.). Although the clinical importance of AGS has been recorded in such reports, our understanding of the pathogen is still considerably lagging compared to Groups A/G/C β-hemolytic streptococci (such as *S. pyogenes* and *S. dysgalactiae*), Group B streptococci (*S. agalactiae*), and *S. pneumoniae*. Therefore, the exact pathogenic potential of AGS is unclear.

In AGS, strains exhibiting β-hemolysis are observed on blood agar plates. Except for strains belonging to *S. intermedius* that produce a cholesterol-dependent cytolysin named intermedilysin (Nagamune et al., 1996), a family of peptide hemolysin named streptolysin S (SLS) has been shown to be the sole hemolytic factor of β-hemolytic AGS (Asam et al., 2013; Tabata et al., 2013, 2014). Interestingly, both β-hemolytic and non-hemolytic strains are present in SAA and SCC (Tabata et al., 2013, 2014). In β-hemolytic strains of SAA and SCC, the homolog of a gene cluster necessary for the production of SLS (*i.e.*, *sag* operon) is inserted in a specific region of the genome of non-hemolytic strains (Tabata et al., 2014), suggesting that certain strains belonging to AGS may have evolved as potential pathogens *via* the acquisition and incorporation of foreign genetic material.

In bacteria, it has been shown that mobile genetic elements (MGEs) are responsible for the transfer of virulence genes between organisms (Frost et al., 2005). MGEs have been reported in AGS, such as: Tn*3704*

* Corresponding author at: Rockefeller University, 1230 York Avenue, New York, NY 10065, USA.
E-mail address: vaf@mail.rockefeller.edu (V.A. Fischetti).

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in *S. anginosus* F22 (Clermont and Horaud, 1994), MTnSag1 in *S. anginosus* UCN93 (Gravey et al., 2013), Tn*916S* in an *S. intermedius* isolate (Lancaster et al., 2004), and a genomic region related to phages and chromosomal island in *S. anginosus* and *S. intermedius* (Nguyen and McShan, 2014). Recently, it was reported that MGEs accounted for > 10% of the genome in AGS (Olson et al., 2013). Results from the complete genome sequence of *S. anginosus* J4211 revealed two prophage and multiple MGEs (Rahman et al., 2015). More recently, it was reported that another *S. anginosus* strain, J4206, also has multiple MGEs (Rahman et al., 2016). However, interspecies and intraspecies transfer of genetic material in AGS, including virulence and antibiotic-resistance genes, is not well studied.

In our investigation of MGEs in AGS, we found that an SAA strain 0430-08 with reactivity against Lancefield group F antiserum, contained a novel MGE identified as a plasmid (named pSAA0430-08). We determined the complete sequence of the plasmid and performed a detailed analysis of their genetic properties.

2. Materials and methods

2.1. Bacterial strain and culture condition

Streptococcus anginosus subsp. *anginosus* (SAA) strains 0430-08 and NCTC10713^T were used in this study (Table 1). The strain 0430-08 was isolated from a vaginal swab and reacted against Lancefield group F antiserum. The type strain of SAA, NCTC10713^T, was used for the construction of the variant plasmid of pSAA0430-08 because it is highly transformable in the presence of competence-stimulating peptide (CSP). These strains were maintained as glycerol stocks at − 80 °C, and recovered on Brain Heart Infusion (BHI; Becton Dickinson and Company, Franklin Lakes, NJ, USA) agar plate or BHI agar plates containing 5% (v/v) defibrinated horse blood (purchased from Nippon Bio-Supp. Center, Tokyo, Japan). Cultivation was conducted in the presence of 5% (v/v) CO_2 at 37 °C. *Escherichia coli* DH5αZ1 was used to construct the shuttle vector between *E. coli* and SAA.

2.2. Antibiotic susceptibility test

To evaluate the antibiotic susceptibility of the tested strains, the minimum inhibitory concentration (MIC) of several antibiotics [erythromycin, chloramphenicol, tetracycline hydrochloride, gentamicin sulfate, vancomycin hydrochloride, and bacitracin (Wako pure chemical, Osaka, Japan), and penicillin G potassium salt (Nacalai tesque, Kyoto, Japan)] was determined by the broth dilution method. Briefly, a 2-fold dilution series of antibiotics were prepared by BHI broth and 50 μL-aliquots of each dilution was dispensed into wells of a 96-well plate. An overnight culture of the tested strain was adjusted at OD_{600} to 0.001 using BHI broth, then an aliquot (50 μL) of the bacterial suspension was added to each antibiotics dilution series in the plate wells. After overnight incubation at 37 °C under 5% CO_2, the MIC was determined. The result was shown as the range of MIC obtained from three independent measurements.

2.3. Extra-chromosomal DNA isolation

The isolation of extra-chromosomal DNA from SAA strain 0430-08 was based on a previously reported procedure (Utter et al., 2014) or a modified version of this method.

For the modified method of extra-chromosomal DNA isolation, we used the QIAGEN Plasmid Midi Kit (Qiagen, Hilden, Germany). Briefly, the recovered colony was inoculated into BHI broth and incubated overnight without shaking. The culture was diluted 1:500 in 500 mL of BHI broth and incubated at 37 °C for 8 h. The bacterial cells were harvested by centrifugation (8000 × *g*, 10 min, 4 °C) and washed with sterilized phosphate-buffered saline (PBS). The bacterial cell pellet was re-suspended in 10 mL of pre-chilled QIAGEN buffer P1 containing 20 μg/mL mutanolysin (Sigma, St. Louis, MO, USA) and mixed slowly, and incubated at 37 °C for 30 min. Ten-milliliter of QIAGEN buffer P2 was added slowly with mixing and incubated for 3 min, then 10 mL of pre-chilled QIAGEN buffer P3 was added and mixed slowly. After 15 min incubation at room temperature, the suspension was centrifuged (8000 × *g*, 20 min, 4 °C, then 20,600 × *g*, 30 min, 4 °C) and the resultant supernatant was used for the purification of extra-chromosomal DNA by QIAGEN-tip 100 midi-prep filter according to the method provided by the manufacturer. The purification of the extra-chromosomal DNA was confirmed by Agarose gel electrophoresis using Tris-acetate-EDTA buffer system (TAE; 40 mM Tris, 19 mM acetate, 1 mM EDTA).

A preparative method of extra-chromosomal DNA purification was conducted using NucleoSpin Plasmid EasyPure (Macherey-Nagel GmbH & Co. KG, Düren, Germany) with some modifications for sample preparation. In order to improve the purification, both lysozyme (final concentration of 2 mg/mL) and mutanolysin (final concentration of 20 μg/mL) were added to buffer A1 and incubated for 30 min at 37 °C. The purification of extra-chromosomal DNA was checked by Agarose-gel electrophoresis.

2.4. DNA sequence determination of extra-chromosomal DNA

The extra-chromosomal DNA was sequenced using the MiSeq next generation sequencer (Illumina, San Diego, CA, USA). In order to close the 5′- and 3′-ends of the draft sequence, a conventional Sanger sequencing was used as follows: Briefly, the fragment for Sanger sequencing was amplified by PCR using purified extra-chromosomal DNA as a template, Phusion High-Fidelity DNA Polymerase (Thermo Scientific, Waltham, MA, USA) with the primers pSaa0430-08_Fw and pSaa0430-08_Bw (primers are listed in Table 2). After purification of the PCR products by QIAquick Gel Extraction Kit (Qiagen), sequencing was conducted by GENEWIZ, Inc. (South Plainfield, NJ, USA).

2.5. Open reading frame prediction

The prediction of open reading frames (ORFs) encoded on the pSAA0430-08 was done using GENETYX Mac (Network ver. 18) and

Table 1
Strains and plasmids used in this study.

Strains and plasmids	Characteristics	Source
0430-08	Lancefield group F strain from vaginal sample and harboring pSAA0430-08	This study
NCTC10713^T	Type strain of *S. anginosus* subsp. *anginosus*	Whiley and Beighton, 1991
DH5αZ1	A strain of *Escherichia coli* for shuttle vector construction	Lutz and Bujard, 1997
pSAA0430-08	The novel plasmid from SAA strain 0430-08	This study
pORF1-10*erm*	pSAA0430-08 variant including *erm* cassette between ORF3 and ORF4	This study
pORF9-3*erm*	pSAA0430-08 variant composed of ORF9 to ORF3 and *erm* cassette	This study
pORF1-3*erm*	pSAA0430-08 variant composed of ORF1 to ORF3 and *erm* cassette	This study
pORF9-1*erm*	pSAA0430-08 variant composed of ORF9 to ORF1 and *erm* cassette	This study
pSVORF1it*cat*	The shuttle vector of *E. coli* and SAA composed of both iteron and ORF1 from pSAA0430-08, p15A *ori*, and *cat* cassette	This study
pSVit*cat*	The shuttle vector of *E. coli* and SAA composed of iteron from pSAA0430-08, p15A *ori*, and *cat* cassette	This study
pSVORF1*cat*	The shuttle vector of *E. coli* and SAA composed of ORF1 from pSAA0430-08, p15A *ori*, and *cat* cassette	This study

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