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Nonhemolytic *Streptococcus pyogenes* Isolates That Lack Large Regions of the *sag* Operon Mediating Streptolysin S Production▽

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Among nonhemolytic *Streptococcus pyogenes* (group A streptococcus) strains ($n = 9$) isolated from patients with pharyngitis or acute otitis media, we identified three deletions in the region from the *epf* gene, encoding the extracellular matrix binding protein, to the *sag* operon, mediating streptolysin S production.

In clinical laboratories, the property of beta-hemolysis on a blood agar plate is a characteristic used to preliminarily detect pyogenic streptococci such as *Streptococcus pyogenes* (group A streptococcus [GAS]), *Streptococcus agalactiae* (group B streptococcus), and *Streptococcus dysgalactiae* subsp. *equisimilis* (12). GAS produces two hemolysins: oxygen-dependent, labile streptolysin O, encoded by the *slo* gene (8), and oxygen-stable streptolysin S (SLS), encoded by the *sag* operon extending from the *sagA* gene to *sagI* (5). SLS, a potent cytolytic toxin produced by nearly all strains of GAS, is responsible for the zone of hemolysis surrounding GAS colonies grown under routine CO_2 culture conditions.

The *sagA* gene, which is positioned upstream in the *sag* operon, encodes a prepropeptide consisting of 53 amino acid (aa) residues, including a Gly-Gly proteolytic cleavage site that has been predicted to release a propeptide of 30 aa from a 23-aa leader sequence. The propeptide is considered to be the structural element of SLS. The remaining genes in the operon have features consistent with export functions, posttranslational modification of the SLS peptide, and a possible immunity protein (3).

Rarely, nonhemolytic variants of GAS have been isolated from patients with pharyngitis (6, 10), pneumonia (13), sepsis (2, 14), and cellulitis (11). These isolates were probably not producers of SLS, but the molecular cause had previously not been explained. Recently, based on mutational analysis, it was reported that all genetic components of the *sag* operon are required for the expression of functional SLS as an important virulence factor in the pathogenesis of invasive infection (3). In this study, we aimed to determine the reason for nonhemolysis by GAS clinical isolates at the molecular level.

A total of 1,690 samples, including throat swabs ($n = 1,513$) from patients with pharyngitis/tonsillitis and middle ear fluid ($n = 177$) from patients with acute otitis media (AOM), were sent to our laboratory by clinical physicians. Real-time PCR was immediately carried out routinely, in parallel with culturing, for all clinical samples on the day they were received. The real-time PCR used in this study was an application of the methods using molecular beacon probes and primers that we had constructed to detect six pathogens, including GAS, in samples from patients with respiratory tract infection (9). The set of primers and the probe for 16S rRNA genes used for the identification of GAS are as follows: sense primer, 5′-GAGA GACTAACGCATGTTAGTA-3′; reverse primer, 5′-TAGTT ACCGTCACTTGGTGG-3′; and probe, 6-carboxyfluorescein–CGCGATCGCGACGATACATAGCCGACCTGGAT CGCG–Black Hole Quencher 1. DNA extraction with the Extragen II kit (TOSOH, Tokyo, Japan) and subsequent DNA amplification with the Mx3000P system (Stratagene, La Jolla, CA) were performed by our protocol (9). On the following day, when no colonies with hemolysis were observed on the blood agar plate, Gram staining and reexamination by real-time PCR were carried out for some nonhemolytic colonies having different shapes, regardless of the positive PCR results for GAS on the preceding day. Next, colonies were confirmed to have characteristics of GAS by (i) an agglutination test for Lancefield group A antigen (Streptex; Mitsubishi Chemical Medience, Tokyo, Japan), (ii) use of the API Strep system (bioMérieux, Tokyo, Japan), and (iii) evaluation for the pyrrolidonyl arylamidase reaction (Oxoid, Hampshire, United Kingdom) in accordance with the *Manual of Clinical Microbiology* (12).

We finally identified nine nonhemolytic GAS strains from among 818 clinical isolates (1.1%) obtained from patients with pharyngitis/tonsillitis or AOM between November 2006 and March 2009. Colonies of these GAS isolates remained nonbeta-hemolytic under aerobic, 5% CO_2, and anaerobic conditions. Representative examples are shown in Fig. 1. The clinical and epidemiologic features of these isolates, including the *emm* type, the sequence type determined by multilocus sequence typing (MLST), and the type of deletion in the *sag* region, are listed in Table 1.

The *emm* types of these strains were determined based on DNA sequence homology by comparison of sequences with entries in the CDC database using the Streptococci Group A Subtyping Request Form Blast 2.0 Server (http://www.cdc. gov /ncidod/biotech/strep/strepblast.htm). DNA sequences approx-

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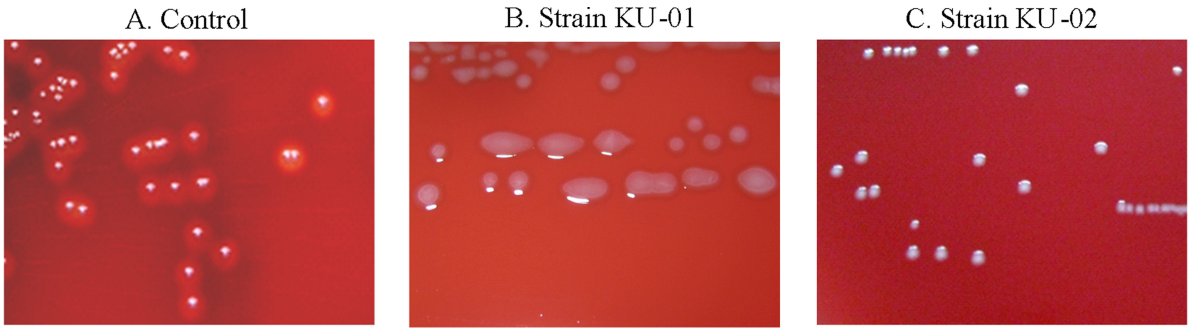

FIG. 1. Nonhemolytic *S. pyogenes* colonies grown on 5% sheep blood agar plates for 18 h at 37°C. (A) Control strain; (B and C) nonhemolytic strains.

imately 14,000 bp in length, extending from the *epf* gene, encoding an extracellular matrix binding protein, to the *sag* operon, encoding SLS, were determined for all nonhemolytic GAS strains. Primers used initially for long-DNA-fragment amplification included a sense primer, 5′-TGTGGATGCCGT TTAGAACA-3′, and a reverse primer, 5′-GAATAGCGACA CGCCTTAGC-3′.

For MLST of the nine GAS strains, DNA sequences from seven housekeeping loci were determined by the methods described by Enright et al. (4), and the sequence results were compared with the data in the *S. pyogenes* database (http://spyogenes.mlst.net/misc/info.asp).

Figure 2 depicts three types of DNA deletions in the region from the *epf* gene to the *sag* operon that were identified among the strains. Deletion type 1 was exhibited by one strain (strain KU-01; *emm1.0*) that lacked a 1,358-bp segment extending from the 3′-terminal region of the *epf* gene to the 5′-terminal region of the *sagB* gene. Six other strains (strains KU-02 to strain KU-07; *emm12.0*) with deletion type 2 lacked a 7,503-bp segment extending from the middle region of the *epf* gene to the *sagD* gene. The remaining two strains (KU-08 and KU-09; *emm1.0*) represented deletion type 3 and had discontinuous deletions in two regions: a 1,709-bp segment in the *epf* gene and another segment of 6,615 bp extending from the middle region of the *epf* gene to the *sagD* gene. All these deletions encompassed the region of the promoter and the *sagA* gene encoding the precursor of SLS.

Two types of *sag* regions have been identified using a DNA database for GAS genomes. In one, the ordinary type, the genes are aligned beginning with the *eno* gene, encoding enolase, and extending through *sagA* to *sagI*. The other type possesses both a *tnp* gene, encoding transposase, and an *epf* gene between the *eno* and the *sagA* genes. GAS strains identified as having *emm2*, *emm3*, and *emm5* represented the former type, while the *emm1*, *emm4*, *emm12*, and *emm28* strains carried the latter type. All nine strains analyzed in this study contained the latter type. Although these unique deletions suggest some associations with a transposon or insertion sequence, such details remain to be clarified.

Transcripts of the *nga* and the *slo* genes are known to be produced by read-through from the *nga* promoter (7). Although the data are not shown here, nucleotide sequences of 4,754 bp in length from the *nga* gene, including the promoter region, to the end of open reading frame of the *slo* gene from the nine strains were identified. No mutations or nucleotide deletions were detected in this region; therefore, the *slo* gene was intact in all strains.

DNA profiles of the nine nonhemolytic strains after pulsed-field gel electrophoresis (PFGE) are shown in Fig. 3. PFGE was performed with the ApaI restriction enzyme (Takara Bio, Kyoto, Japan). The DNA fragments were separated on a 1% agarose gel by using a contour-clamped homogeneous electric field mapper system (Bio-Rad, Tokyo, Japan) for 18 h at 14°C in 0.5× TBE buffer (0.05 M Tris, 0.05 M boric acid, and 1 mM EDTA [pH 8.0]) at 5.7 V/cm with pulse times of 3 to 20 s at an angle of 120° (1). Six strains with *emm12.0* isolated from patients in different regions, i.e., the Chiba and Niigata prefectures, Japan, showed similar DNA restriction patterns. Furthermore, three strains from Gunma prefecture and the Sendai

TABLE 1. Clinical and epidemiologic features of nonhemolytic *S. pyogenes* isolates

Strain no.	Date of isolation	District	Patient			*emm* type	Sequence type	Deletion type involving *sag* operon
			Age (yr)	Gender	Disease			
KU-01	Nov. 2006	Gunma	30	M	AOM	*emm1.0*	28	1
KU-02	Feb. 2008	Chiba	2	M	Pharyngitis	*emm12.0*	36	2
KU-03	Apr. 2008	Chiba	4	F	Pharyngitis	*emm12.0*	36	2
KU-04	May 2008	Chiba	5	F	Pharyngitis	*emm12.0*	36	2
KU-05	May 2008	Chiba	6	M	Pharyngitis	*emm12.0*	36	2
KU-06	May 2008	Chiba	2	F	Pharyngitis	*emm12.0*	36	2
KU-07	Nov. 2008	Niigata	4	M	Pharyngitis	*emm12.0*	36	2
KU-08	Jan. 2009	Sendai	5	F	Pharyngitis	*emm1.0*	28	3
KU-09	Jan. 2009	Sendai	5	M	Pharyngitis	*emm1.0*	28	3

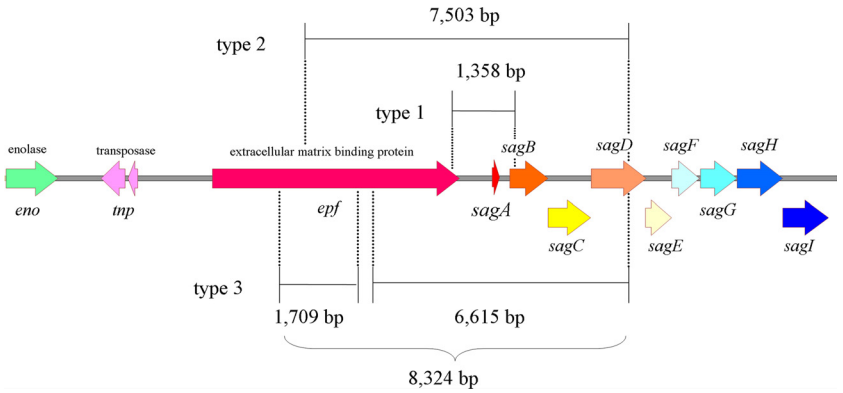

FIG. 2. Three deletion types identified near and/or in the *sag* operon encoding SLS. Large deletions encompassed the regions of the promoter and *sagA*, encoding the precursor of SLS. Deletion type 1, accession number AB518308; deletion type 2, accession number AB518309; deletion type 3, accession number AB518310.

City area identified as *emm1.0* strains showed very similar DNA restriction patterns.

Previously described nonhemolytic GAS strains have included various T antigen types and *emm* types (2, 6, 10, 11, 13, 14). In this study, we analyzed nonhemolytic phenotypes of the *emm1* and *emm12* strains. Evidence suggests that nonhemolytic *emm12* variants spread horizontally among children, considering that several cases occurred in the same area (Chiba prefecture). We also isolated three *emm1* GAS strains, one mucoid type and two nonmucoid types, from samples obtained from different areas. These strains displayed different deletion types in the *sag* operon but showed highly similar PFGE profiles, suggestive of a common origin.

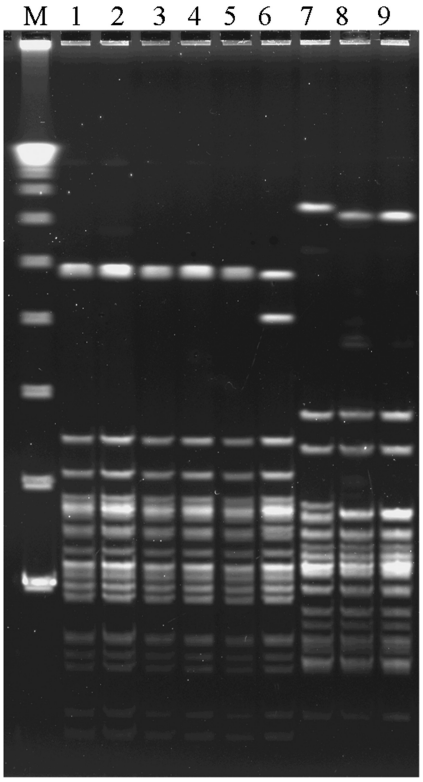

FIG. 3. PFGE patterns for nine nonhemolytic strains. Lanes: M, lambda ladder; 1, KU-02 (*emm12.0*); 2, KU-03 (*emm12.0*); 3, KU-04 (*emm12.0*); 4, KU-05 (*emm12.0*); 5, KU-06 (*emm12.0*); 6, KU-07 (*emm12.0*); 7, KU-01 (*emm1.0*); 8, KU-08 (*emm1.0*); and 9, KU-09 (*emm1.0*).

Emergence of the GAS strains described herein suggests that the routine bioassay poses a risk of missing nonhemolytic GAS colonies on blood agar plates, although nonhemolytic GAS variants are considered to be rare.

Nucleotide sequence accession numbers. The deletion type sequences determined in this study have been deposited in GenBank under the following accession numbers: deletion type 1, AB518308; deletion type 2, AB518309; and deletion type 3, AB518310.

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