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## NVS and Staphylococci in the Oral Cavity – A Cause of Infective Endocarditis

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### 1. Introduction

Oral streptococci including viridans streptococci and nutritionally-variant streptococci (NVS) along with *Staphylococcus* species including *Staphylococcus aureus* and coagulase negative staphylococci (CoNS) are two main bacterial groups known to be causative of infective endocarditis (IE). These bacteria are not pathogenic in principal for healthy individuals, and streptococci are regular commensal occupants of the oral microflora as well as the gastrointestinal tract and female genital tract. *Staphylococcus* species also comprise normal human microflora of the skin, nasal cavity, and gastrointestinal tract. The infectious routes for these pathogenic bacteria entering the bloodstream are not identified in half of examined IE patients. In addition to oral streptococci, *staphylococcus* species are generally isolated from dental plaque and saliva (Smith et al., 2001; Ohara-Nemoto et al., 2008a), thus the oral cavity is considered to be a common habitat for the two main pathogenic bacteria of IE. These findings suggest that peroral bacterial transmission to the bloodstream should be examined as a cause of IE, while systematic studies of the relationships between IE and oral conditions, such as extent of periodontal disease, dentate state (dentate, edentulous, denture wearing), and oral hygiene, are also needed to elucidate the causative role of oral bacteria. From the point of disease due to peroral bacteremia, it is of interest that a substantial part of staphylococcal arthritis is considered to originate from the oral cavity (Jackson et al., 1999). This review focuses on causative bacteria of IE that mainly colonize the oral cavity and their pathogenicity.

### 2. Causative bacteria of infective endocarditis

IE, an infection of the endocardium of the heart, is an uncommon but life-threatening disease with a high mortality rate ranging from 10-24% (Ferreiros et al., 2006; Yoshinaga et al., 2008; Murdoch et al., 2009). The overall incidence rate has been found to range from 4.4 to 11.6 cases per 100,000 person-years (Berlin et al., 1995; Tleyjeh et al., 2005; Fedeli et al., 2011). Cardiac valvular abnormalities that cause eddy- or jet-type vascular flow are strong risk factors (Strom et al., 1998; Nakatani et al., 2003). Abnormal vascular flow around valves causes clotting deposits, and bacteria which enter the bloodstream and become attached to clots then grow by forming biofilm. According to the most recent surveillance data (848 IE cases reported in 2000 and 2001), the characteristics of IE in Japan include mean age of 55±18

years, with most patients aged from 50 to 70 years, with 82% of IE patients complicated with underlying diseases, such as valvular heart disease (65%), congenital heart disease (9%), or particular implantation (3%), whereas in 18% cases IE occurred without any predisposing cardiac diseases (Nakatani et al., 2003). Noticeably, a route of infection was not identified in 53.9% of those cases. Also, patients with etiologically unidentified IE had no prior infectious disease causing bacteremia, such as urinary tract infection, pneumonia, or cellulitis, and no history of invasive procedure or intravenous drug administration. Thus, it is considered especially important to identify the infectious routes of etiologically unidentified IE in Japan.

The second most common etiology in Japanese IE cases was found to be post-dental procedures and oral hygiene-related conditions in those with viridans streptococci infection (35.7%). Viridans streptococci, indigenous bacteria in the oral cavity, are most frequently identified as pathogenic bacteria of IE and in Guidelines presented in 2003 by the Japanese Circulation Society it was noted that dental procedures may cause IE. This medical background in Japan may be different from Western countries, because no link between IE and dental treatment was shown in a population-based case-control study performed in the United States (Strom et al., 1998).

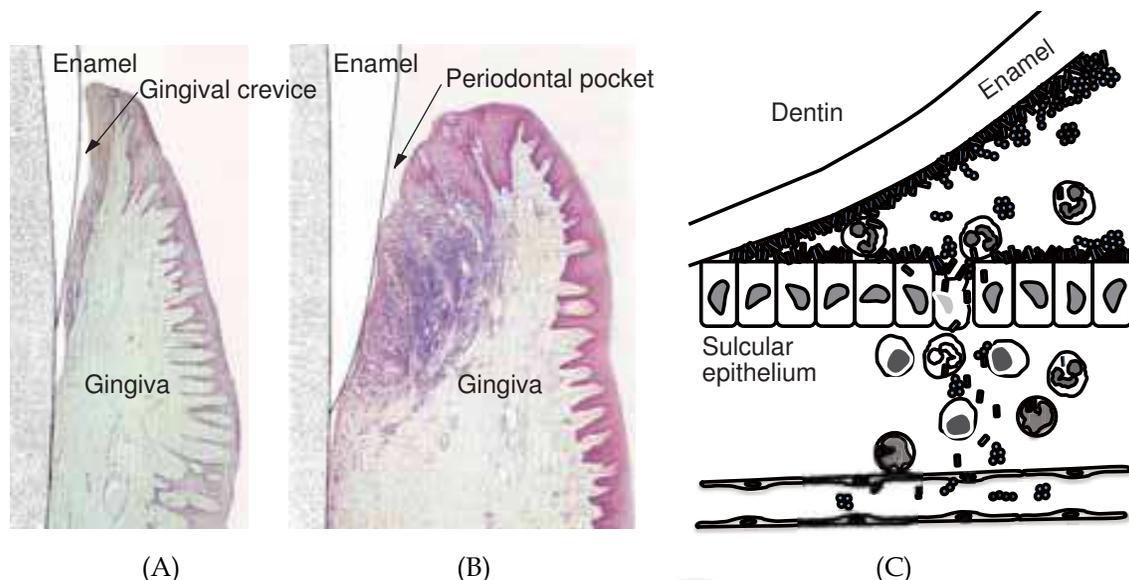


Fig. 1. Histological observation of (A) healthy and (B) diseased periodontal regions. (C) Scheme of dentogingival junction. The periodontal crevice is bathed in the gingival crevicular fluid. In periodontal disease, a crevice becomes a pocket. Polymorphonuclear neutrophils migrate to the crevice, and lymphocytes and monocytes are shown in a connective tissue. Oral microorganisms are expected to enter into the bloodstream through gingival sulcular epithelium and connective tissue

Until recently, viridans streptococci have been considered as the most common causative microorganisms of IE and detected at a range from 30% to 50% (Strom et al., 1998; Nakatani et al., 2003; Murdoch et al., 2004; Tleyjeh et al., 2005; Alshammary et al., 2008). Viridans streptococci are composed of a total of 21 species, including *S. mitis*, *S. anginosus*, *S. salivarius*, and *S. bovis* (Kawamura et al., 1995). Because of their non-pathogenicity in principal and phenotypical characteristic resemblance, clinical isolates of viridans streptococci obtained from patients with various diseases are usually not identified at the bacterial species level, but rather registered as '*Streptococcus viridans*' or viridans

streptococci. For these, though *S. sanguinis* and *S. oralis* are considered to be the most common agents of streptococcal IE, information is limited and molecular based-species identification is needed to clarify their pathogenicity.

In addition to frequent isolation of viridans streptococci, a finding that edentulous state decreased IE risk (Strom et al., 2000) suggests that the opportunity for transmission and amount of transmitting bacteria through the dentogingival interface are functionally important issues in regard to onset of the disease (Okell et al., 1935; Carmona et al., 2002; Ohara-Nemoto et al., 2008a).

Following the incidence rate of IE by viridans streptococci, infection by *Staphylococcus aureus* is significant and ranges from 17% to 43%, while that in combination with CoNS ranges from 9% to 13%. The changing spectrum from streptococci to staphylococci has been reported in recent epidemiological studies performed in many countries (Sandre & Shafran, 1996; Hoen et al., 2002; Cecchi et al., 2004, Ferreiros et al., 2006; Alshammary et al., 2008; Fedeli et al., 2011). *S. aureus* has also been identified as a leading cause of death cases (Alshammary, et al., 2008; Yoshinaga et al., 2008). Etiologic *Staphylococcus* spp. causing IE are assumed to be acquired via a percutaneous route from skin flora, especially in nosocomial infection cases and intravenous drug abusers. However, its infectious route is often not described in cases in Japan (Niwa et al., 2005) and France (Di Filippo et al., 2006). With this in mind, it is reasonable to speculate that a part of staphylococcal IE is caused by peroral infection as in IE cases with viridans streptococci, because the occurrence of oral staphylococci is significantly higher than generally accepted. For example, the prevalence rate of *Staphylococcus* species was found to be 73% in dental plaque and 84% in saliva (Ohara-Nemoto et al., 2008a). In accordance with the existence of oral staphylococci, Etienne et al. (1986) reported cases of staphylococcal IE resulting from dental extraction.

Microorganisms are not found in approximately 15% of the IE cases in Japan, a part of which might be related to NVS, because NVS organisms scarcely grow in ordinary growth media, and require an L-cysteine or pyridoxal supplement. *Abiotrophia defectiva*, formerly *Streptococcus defectiva*, was first described as a new type of viridans group of streptococci 50 years ago (Frenkel & Hirsch, 1961). Later, according to 16S rRNA sequence findings, the genus *Abiotrophia* as well as genus *Granulicatella* was taxonomically established from NVS (Collins & Lawson, 2000). The genus *Abiotrophia* is composed of one species of *A. defectiva*, while the genus *Granulicatella* is composed of *G. adiacens* and *G. elegans*, which are isolated from humans, and *G. balcaenopterae* from minke whales. Most clinical strains of NVS reported were isolated as agents of subacute IE and accounted for more than 4% of streptococcal IE (Bouvet, 1995). NVS are also constituents of the normal flora of the oral cavity and upper respiratory tract (George, 1974, Ruoff, 1991, Ohara-Nemoto et al., 1997), thus the infection route for NVS causing IE is likely peroral.

### 3. NVS

#### 3.1 Occurrence and molecular identification of NVS in saliva and dental plaque specimens

NVS do not grow on Trypticase soy agar with 5% sheep blood, which can support growth of viridans streptococci, whereas they usually grow on either chocolate or Burucella agar with 5% horse blood (Ruoff, 2007), or in nutritionally rich broth supplemented with L-cysteine or pyridoxal (Fenkel & Hirsch, 1961). Furthermore, NVS generally grow well in medium from

a commercially available culture bottle system for anaerobic bacteria, such as BACTEC PLUS Anaerobic/F culture bottles at 37°C under anaerobic conditions (Ohara-Nemoto et al., 2005).

To isolate NVS from oral or combined infection specimens, a culture method for monitoring the bacteriolytic activity of NVS may be useful. This activity toward *Micrococcus luteus* was demonstrated only with NVS species and not with other viridans streptococci isolated from IE cases or oral bacteria (Pompei et al., 1990). Using this method, we isolated NVS from saliva and dental plaque specimens (Ohara-Nemoto et al., 1997). Briefly, after appropriate dilutions with phosphate-buffered saline, oral specimens were inoculated onto a double-layer nutrient agar plate with a top layer containing heat-killed *M. luteus* ATCC 9341 and cultured overnight. Use of an anaerobic condition raised the growth rate of NVS as compared with the aerobic condition. Isolates exhibiting bacteriolytic activity, shown as a colony surrounded with a clear halo, were NVS. These isolates also demonstrated satellitism with a streak of *S. aureus* on a Todd-Hewitt agar plate. For species identification, molecular based methods, i.e., 16S rRNA sequencing or 16S rRNA PCR followed by restriction fragment length polymorphism analysis (PCR-RFLP) (Ohara-Nemoto et al., 1997; Ohara-Nemoto et al., 2005), were successfully applied. When 92 oral NVS strains were examined, the PCR-RFLP patterns of *G. adiacens* and *A. defectiva* were readily distinguished from each other, as well as from those of other streptococcal and enterococcal species (Fig. 2).

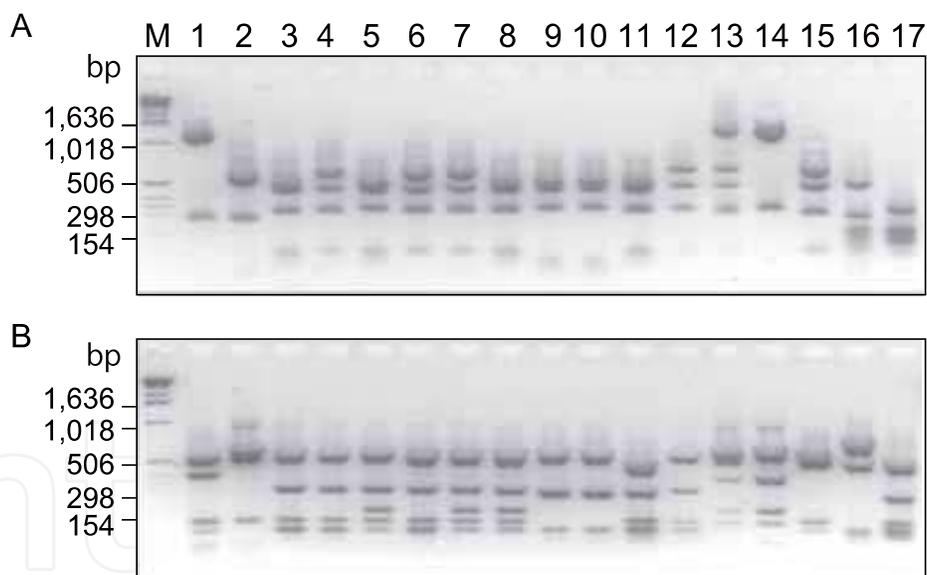


Fig. 2. 16S rDNA PCR-RFLP of *G. adiacens*, *A. defectiva*, and viridans streptococci. The 16S rRNA gene (1.5 kb) was amplified by PCR using a set of universal primers for eubacteria, then cleaved with (A) *Hae*III or (B) *Msp*I. Lanes: M, size marker; 1, *G. adiacens*; 2, *A. defectiva*; 3, *sanguinis*; 4, *S. oralis*; 5, *S. gordonii*; 6, *S. mitis*; 7, *S. salivarius*; 8, *S. bovis*; 9, *S. mutans*; 10, *S. sobrinus*; 11, *S. pyogenes*; 12, *S. pneumoniae*; 13, *S. aureus*; 14, *S. epidermidis*; 15, *Enterococcus faecalis*; 16, *Haemophilus influenzae*; 17, *Escherichia coli*

Species identification performed by PCR-RFLP completely matched that obtained by phenotypic characteristics. Consequently, it was found that the occurrence of NVS in dental plaque and saliva specimens from healthy dental students was relatively high, as 97.8%

(91/93) of the subjects harbored either one of the two bacterial species. In addition, the prevalence of *G. adiacens* was 87.1% and that of *A. defectiva* was 11.8%, which were comparable or even higher than those of viridans streptococci (Kimura & Ohara-Nemoto, 2007). Interestingly, except for one subject carrying both species, all carried only one, suggesting that these two species may be incompatible with each other.

Recently, Shimoyama et al. (2011) developed a rapid and highly sensitive 16S rRNA PCR identification method for NVS using species-specific sets of primers. With this method, *G. adiacens* (including *G. para-adjacens*) was detected in most healthy adult subjects, followed by *A. defectiva* and *G. elegans* (unpublished results). The high level of occurrence of NVS in the oral cavity demonstrated by this and previous reports (Ohara-Nemoto et al., 1997; Sato et al., 1999) is in good agreement with recent results of normal oral flora examined from 5 subjects ranging in age from 23 to 55 years old (Aas et al., 2005). A culture-independent 16S rRNA gene cloning and sequencing method demonstrated that *S. mitis* and related species were most commonly found in all sites of the oral cavity, followed by the two *Granulicatella* species. *G. adiacens* and *G. elegans* were commonly detected in most areas, such as the buccal, vestibule, tongue dorsum, tongue lateral, hard palate, soft palate, tonsils, tooth surface, and subgingival sites in a wide range of ages. Although the detection rate of *A. defectiva* was somewhat lower than that of *Granulicatella* species, this bacterium was also among the top 21 of commonly observed bacteria of 141 predominant species in the oral cavity. Therefore, with the high occurrence of NVS in the oral cavity in mind, a relatively high incidence of NVS-related IE (more than 4% of streptococcal IE) may be reasonable. Elucidation of the prevalence rates and amounts of NVS in the oral cavity, especially in elderly subjects whose incidence rate for IE is remarkably high, may contribute to better understanding of the pathological process of NVS-related IE. Non-culture methods targeting 16S rRNA (Goldenberger et al., 1997; Shimoyama et al., 2011) are convenient and practical for this purpose.

### 3.2 Cases of IE caused by NVS

Most clinical NVS isolates reported were derived from culturing blood obtained from IE patients and up to 100 cases of NVS IE have been presented to date. We treated an IE case with severe mitral and aortic valves insufficiencies caused by *G. elegans* derived from the oral cavity (Ohara-Nemoto et al., 2005). A 53-year-old previously healthy female without anamnesis had undergone a dental procedure at a local clinic two months before consulting with us. The patient had a slight fever and cough, and was diagnosed with chronic heart failure and transferred to an outside facility, where IE associated with aortic and mitral valve vegetation was noted in echocardiography findings. Four consecutive arterial and venous blood cultures were successively performed at other and our facilities with BACTEK PLUS Anaerobic/F culture bottles and Brucella HK agar plates under anaerobic conditions. Two sets of cultures were positive and yielded gram-positive coccoides in short chains. At our hospital, the patient received antibiotic treatment with intravenous benzylpenicillin (1.2 million units per day) and gentamicin (60 mg per day), then cardiac surgery was performed 7 days after admission. The left coronary cups of the aortic valve showed perforation and ulceration with multiple vegetation sites, and the mitral valve anterior leaflets were perforated with large amounts of vegetation. The patient was released from the hospital without a fever after 27 days.



Fig. 3. Oral findings of a patient with IE caused by *G. elegans*. Dental plaque was collected from right upper [34](#) (Ohara-Nemoto et al., 2005)

Bacterial isolates from the patient were identified as *G. elegans* (IMU02b01) by microbiological characteristics and 16S rRNA gene sequencing. An oral examination of the patient one week after surgery showed widespread redness of the gingiva, dental caries, and deposition of a large amount of dental plaque (Fig. 3). The oral state of the patient suggested a risk of bacteremia due to increased dentogingival surface area, with oral *G. elegans* the suspected agent in this case.

Characteristic	Result for <i>G. elegans</i> strain <sup>a</sup> :		
	IMU02b01	IMU02p18	CCUG 26024
Enzyme production	+	+	+
Pyrrolidonyl aminopeptidase	-	-	-
Alkaline phosphatase	-	-	-
Urease	+	+	+
Arginine dihydrolase	-	-	-
$\alpha$ -Galactosidase	-	-	-
$\beta$ -Galactosidase	-	-	-
$\beta$ -Glucuronidase	+	+	+
$\alpha$ -Glucosidase	-	-	-
$\beta$ -Glucosidase	+	+	+
Hippurate hydrolysis	-	-	-
Acetoin production	-	-	-
Acidification of:			
Trehalose	-	-	-
Lactose	-	-	-
Raffinose	-	-	-
Sucrose	+	+	+
Melibiose	-	-	-
Arabinose	-	-	-
Sorbitol	-	-	-
Mannitol	-	-	-
Growth in THB supplemented with:			
L-Cysteine HCL (0.01%)	+	+	+
Pyridoxal HCL (0.001%)	-	-	-

<sup>a</sup> +, positive result; -, negative result

Table 1. Biochemical characteristics of blood- and oral cavity-derived *G. elegans* isolates from an IE patient

To confirm our speculation, a *G. elegans* strain was isolated from a dental plaque specimen obtained from the patient using a selective culture method. As a result, strain IMU03p18 was obtained, which exhibited the same basic phenotypic characteristics as strains IMU02b01 and *G. elegans* CCUG 26024 (Table 1) (Roggenkamp et al., 1998). Among these characteristics, urease production, acidification of raffinose and sucrose, and hydrolysis of hippurate have been reported to be strain dependent (Roggenkamp et al., 1998; Sato et al., 1999; Collins et al., 2000).

We found that both IMU02p18 and IMU02b01 were negative for urease, positive for hippurate hydrolysis, and fermented sucrose but not raffinose. In addition, their antimicrobial susceptibility tendencies were identical, as they were highly susceptible to penicillin and other  $\beta$ -lactams, whereas they were intermediate to amikacin and resistant to arbekacin (Table 2). These properties were in accordance with other *G. elegans* isolates from IE patients (Ruoff, 1991; Tuohy et al., 2000). In accordance with observed in vitro antibiotic susceptibility, combination treatment with benzylpenicillin and gentamicin was effective in this case. Pulsed-field gel electrophoresis (PFGE) and arbitrarily primed PCR also demonstrated that the genotypes of the two strains isolated from blood and dental plaque samples obtained from the patient were indistinguishable from each other (Fig. 4). These phenotypical and molecular-based characteristics clearly indicated that they were derived from an identical clone. Thus, our findings suggested peroral infection of *G. elegans* in this case of IE.

Agent	MIC ( $\mu\text{g/ml}$ ) <sup>a</sup>		
	IMU02b01	IMU02p18	CCUG 26024
Penicillin	$\leq 0.06$	$\leq 0.06$	$\leq 0.06$
Ampicillin	$\leq 0.25$	$\leq 0.25$	$\leq 0.25$
Cefazolin	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$
Ceftazidime	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$
Cefozopran	$\leq 0.5$	$\leq 0.5$	$\leq 0.5$
Cefdinir	$\leq 0.12$	$\leq 0.12$	$\leq 0.12$
Cefepime	$\leq 0.12$	$\leq 0.12$	$\leq 0.12$
Imipenem	$\leq 0.12$	$\leq 0.12$	$\leq 0.12$
Gentamicin	$\leq 4$	$\leq 4$	$\leq 4$
Amikacin	32	32	$\leq 16$
Arbekacin	$> 16$	$> 16$	$\leq 4$
Erythromycin	$\leq 0.25$	$\leq 0.25$	$\leq 0.25$
Clarithromycin	$\leq 0.25$	$\leq 0.25$	$\leq 0.25$
Clindamycin	$\leq 0.25$	$\leq 0.25$	$\leq 0.25$
Minocycline	$\leq 2$	$\leq 2$	$\leq 2$
Vancomycin	1	1	$\leq 0.5$
Teicoplanin	$\leq 1$	$\leq 1$	$\leq 1$
Fosfomicin	16	16	16
Levofloxacin	$\leq 2$	$\leq 2$	$\leq 2$
Sulfamethoxazole- Trimethoprim	40	40	40

Table 2. Antimicrobial susceptibility of blood- and oral cavity-derived *G. elegans* isolated from an IE patient. <sup>a</sup>MICs were determined using a micro-dilution method developed by the National Committee for Clinical Laboratory Standards

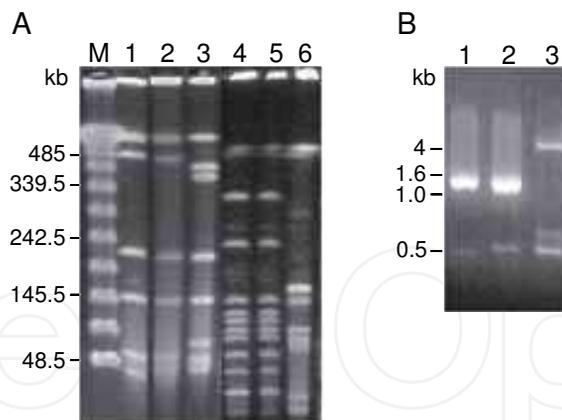


Fig. 4. Molecular based identification of *G. elegans* isolates from blood culture and dental plaque of the IE patient. (A) PFGE was performed after digestion with *Sma*I (lanes 1 to 3) and *Apa*I (lanes 4 to 6). Lanes: M, DNA markers; 1 and 4, IMU02b01; 2 and 5, IMU02p18; 3 and 6, *G. elegans* CCUG 26024. (B) Arbitrarily primed-PCR. Lanes: 1, IMU02b01; 2, IMU02p18; 3, *G. elegans* CCUG 26024

## 4. Staphylococci

### 4.1 Occurrence of staphylococci in the oral cavity

*Staphylococcus* species involving *S. aureus* are considered to be transient bacteria in the oral cavity and the amounts of these organisms in oral specimens ( $10^2$ - $10^4$  cfu/ml in saliva,  $10^3$ - $10^5$  cfu/g in dental plaque) are quite low as compared to those of viridans streptococci ( $10^4$ - $10^6$  cfu/ml in saliva,  $10^7$ - $10^9$  cfu/g in dental plaque). However, it is evident that *Staphylococcus* species, especially *S. epidermidis* and *S. aureus*, are frequently isolated from the oral cavity (Reviewed by Smith et al., 2001; El-Solh et al., 2004; Murdoch et al., 2004; Ohara-Nemoto et al., 2008a). Interestingly, oral staphylococcal possession in adults aged from 20 to over 80 years old ranges from 60% to 88%, with the highest prevalence (88% in saliva) observed with elderly subjects aged from 60 to 79 years (Percival et al., 1991). Furthermore, the prevalence of staphylococci at sites of periodontal disease (59%) was found to be significantly higher than that of healthy subgingival sites: 54% in diseased sites and 29% in periodontally healthy control sites (Murdoch et al., 2004).

In addition to dental interest, oral staphylococci have been suggested to be an infectious source of rheumatoid arthritis. Staphylococci are the most common causes of bacterial arthritis in adults, among which *S. aureus* is the primary agent (Goldenberg, 1998; Ryan et al., 1997). Similar to etiologically unidentified cases of IE, etiological sources are not identified in up to 30% of bacterial arthritis cases (Kaandorp et al., 1997). Thus, it is speculated that oral microflora plays a role in the reservoir of agents related to staphylococcal arthritis. According to the report by Jackson et al. (1999), the occurrence of oral staphylococci was 94% in healthy adults (mean 32 years old) and that in healthy elderly subjects (mean 82 years old) was 100%, with 36% of those elderly subjects found to be colonized by *S. aureus*. Furthermore, in rheumatoid arthritis patients (mean 60 years old), staphylococci were isolated from 96% and the proportion of subjects with oral *S. aureus* was 56%, which was significantly higher than that of the healthy subjects (24%). Jacobson et al. (1997) also demonstrated a higher prevalence of *S. aureus* isolated from the oral cavity of

patients with rheumatoid arthritis. These findings are of particular interest when considering cases of etiologically unidentified staphylococcal IE.

Our previous study of the occurrence of oral staphylococci also aimed to identify a potential peroral route of staphylococcal IE (Ohara-Nemoto et al., 2008a). *Staphylococcus* species were isolated from saliva and supragingival dental plaque specimens obtained from systemically and dentally healthy adults (n=56, mean 27.1±5.3 years old) using a culture method. Consequently, along with 99 *S. aureus* isolates and 235 isolates of *S. intermedius* and CoNS species, at least 9 of 15 *Staphylococcus* species known to colonize in humans were observed in the oral cavity (Table 3). The isolation frequencies of staphylococci were 83.9% in saliva and 73.2% in dental plaque. Furthermore, *S. epidermidis* (60.7%) and *S. aureus* (46.4%) were the species most frequently isolated from plaque and saliva, respectively, followed in order by *S. hominis*, *S. warneri*, *S. intermedius*, *S. capitis*, and *S. haemolyticus* (12.5-7.1%) from both locations. In contrast, *S. gallinarum* and *S. lugdunensis* were rarely isolated (1/56, 1.8% of all cases) (Table 3). The prevalence tendency of oral staphylococci was similar to that of specimens obtained from the nasal cavity. Staphylococci-positive subjects (n=47) harbored from 1 to 5 species (mean 2.3±1.0) (a portion of those results is shown in Table 4). In addition to *S. aureus* and *S. epidermidis*, *S. capitis*, *S. hominis*, *S. lugdunensis*, and *S. warneri* have been implicated in IE.

Species	No. of positive subjects (% isolation frequency)		
	Saliva <sup>a</sup>	Plaque <sup>a</sup>	Nasal swab <sup>b</sup>
<i>S. aureus</i>	26 (46.4)	19 (33.9)	8 (44.4)
<i>S. capitis</i>	5 (8.9)	5 (8.9)	3 (16.7)
<i>S. epidermidis</i>	23 (41.1)	34 (60.7)	13 (72.2)
<i>S. gallinarum</i>	1 (1.8)	0 (0)	0 (0)
<i>S. haemolyticus</i>	4 (7.1)	3 (5.4)	1 (5.6)
<i>S. hominis</i>	7 (12.5)	7 (12.5)	4 (22.2)
<i>S. intermedius</i>	5 (8.9)	5 (8.9)	1 (5.6)
<i>S. lugdunensis</i>	1 (1.8)	0 (0)	0 (0)
<i>S. warneri</i>	6 (10.7)	5 (8.9)	3 (16.7)
Total staphylococci	47 (83.9)	41 (73.2)	17 (94.4)

Table 3. Occurrence of staphylococci in saliva, dental plaque, and nasal samples. <sup>a</sup>None of the subjects had received antibiotic medication within the previous 3 months (n=56, aged 22-43 years old, 27.1±5.3 years; 37 males, 19 females). <sup>b</sup>Nasal swab samples were taken from 18 (32.7±2.6 years: 12 males, 6 females) of 47 oral staphylococci-positive subjects

The genetic relatedness of these staphylococcal isolates was examined by PFGE and the results revealed nasal-oral trafficking of *Staphylococcus* species (Fig. 5), as PFGE patterns indicated that clinical staphylococcal isolates from each subject were identical clones or close relatives. Furthermore, a longitudinal examination over a 2-month period demonstrated that a single identical or same combination of *Staphylococcus* species was continuously isolated. Thus, *Staphylococcus* species found in the oral cavity are regular residential composers of oral microflora or may be continuously provided from the nasal cavity.

Table 4. Staphylococcal species isolated from oral and nasal cavities. Genetic relatedness of the isolates in bold was confirmed by PFGE. ND, not detected

Subject no.	Saliva	Plaque	
1	<b><i>S. aureus</i></b>	<b><i>S. aureus</i></b>	<i>S. a</i>
2	<b><i>S. aureus</i></b> , <i>S. epidermidis</i> , <i>S. hominis</i>	<b><i>S. aureus</i></b> , <i>S. epidermidis</i> , <i>S. hominis</i>	<i>S. a</i>
3	<b><i>S. aureus</i></b> , <i>S. intermedius</i>	<b><i>S. aureus</i></b>	<i>S. a</i>
4	<b><i>S. aureus</i></b> , <i>S. warneri</i>	<b><i>S. aureus</i></b> , <i>S. epidermidis</i>	<i>S. a</i>
5	<b><i>S. aureus</i></b> , <i>S. epidermidis</i>	<i>S. intermedius</i>	<i>S. a</i>
6	<b><i>S. epidermidis</i></b>	<b><i>S. aureus</i></b> , <i>S. epidermidis</i>	<i>S. a</i>
7	<i>S. aureus</i> , <b><i>S. epidermidis</i></b>	<b><i>S. epidermidis</i></b>	<i>S. ep</i>
8	<b><i>S. epidermidis</i></b>	<b><i>S. epidermidis</i></b>	<i>S. ep</i>
9	<b><i>S. epidermidis</i></b>	<b><i>S. epidermidis</i></b> , <i>S. capitis</i> , <i>S. haemolyticus</i>	<i>S. ep</i>
10	<b><i>S. epidermidis</i></b> , <i>S. hominis</i> , <i>S. warneri</i>	<b><i>S. epidermidis</i></b> , <i>S. intermedius</i>	<i>S. ep</i>
11	<i>S. aureus</i> , <b><i>S. lugdunensis</i></b>	<i>S. aureus</i> , <b><i>S. epidermidis</i></b> , <i>S. warneri</i>	<i>S. ep</i>
12	<i>S. aureus</i> , <i>S. warneri</i>	<b><i>S. epidermidis</i></b>	<i>S. ep</i>
13	<i>S. capitis</i>	<b><i>S. hominis</i></b>	<i>S. h</i>
14	<i>S. epidermidis</i>	<i>S. epidermidis</i>	<i>S. ca</i>
15	<i>S. capitis</i>	ND	<i>S. at</i>
16	<i>S. epidermidis</i>	<i>S. epidermidis</i>	ND
17	<i>S. intermedius</i> , <i>S. warneri</i>	<i>S. warneri</i>	<i>S. at</i>
18	<i>S. intermedius</i>	ND	<i>S. ep</i>

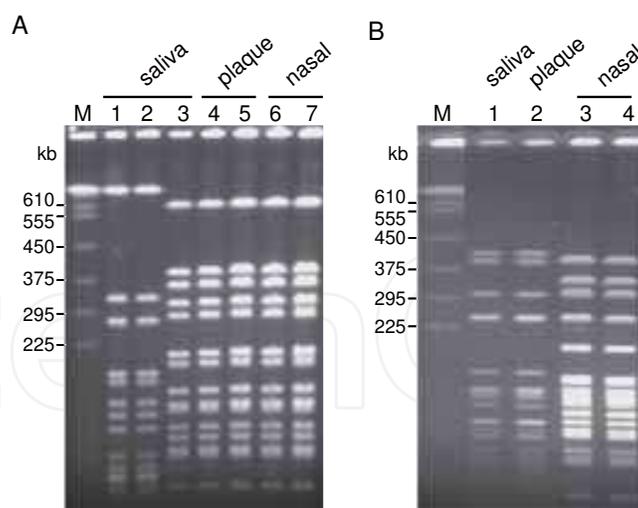


Fig. 5. Genetic relatedness of oral and nasal *S. aureus* (A) or *S. epidermidis* (B) isolates obtained from subjects No. 1 and No. 7 in Table 4, respectively Chromosomal DNA of isolates from oral and nasal specimens was digested with *Sma*I, then separated using PFGE. The relative coefficients of the strains examined were 100% (A, lanes 3-7) and 82.7% (B, lanes 1-4)

Although the composition and proportions of oral microflora in adults are rather stable over long periods, it is notable that occurrence rates of staphylococci, mainly *S. aureus*, tend to increase with age, which is possibly associated with xerostomia (decrease in saliva flow) and denture wearing. The occurrence of staphylococci (88%) was found to be significantly high in denture plaque (Marsh & Martin, 2009), presumably because of their capability of adherence to prosthetic materials. Similarly, *S. aureus* and *S. epidermidis* are the most common pathogens isolated from disease sites of late prosthetic joint infections (Maderazo et al., 1988). On the other hand, the occurrence rates of *S. mutans* and other viridans streptococci are consistent over time after tooth eruption (Percival et al., 1991). This event may be explained by the finding that *S. mutans* colonizes the tongue coat of elderly individuals after loss of teeth, while the prevalence of *Porphyromonas gingivalis*, a major agent of chronic periodontitis, was shown to be closely related to the presence of teeth with periodontal pockets (Kishi et al., 2010). Therefore, when considering that IE as well as rheumatoid arthritis is age-related disease, we speculate that microflora of dental plaque and possibly denture plaque serve to harbor *Staphylococcus* species that cause these diseases. Oral staphylococci are more important for high-risk IE subjects when they are dentate (presence of dentogingival interface) and have poor oral hygiene. Furthermore, periodontal diseases may increase the risk of staphylococcal bacteremia due to increases in dentogingival surface area and bacterial numbers of *S. aureus*. Notably, a recent study also reported a correlation between staphylococci from the oral cavity and developments of atherosclerosis and cardiovascular diseases (Koren et al., 2011).

## 4.2 Pathogenicity factors of staphylococci

### 4.2.1 Biofilm formation

A biofilm is a multi-layered membranous aggregate of microorganisms attached to a biotic or abiotic surface. Oral bacteria that colonize on tooth surfaces and soft epithelial tissues are considered to grow primarily based on their ability to form biofilm, as this attachment

system is necessary to prevent evacuation by host swallowing. *Staphylococcus* and *Streptococcus* species including NVS form biofilms. For all these except *S. aureus*, which produces potent virulence factors, their ability of biofilm formation plays a role as a major virulence factor of IE. Biofilm components include self-produced polymeric matrix and adhesins, which mediate the primary attachment of bacteria to endocardium and heart valve surfaces, followed by intercellular adhesion. Bacteria in biofilm resist host defenses and antibiotic treatment. Numerous studies have demonstrated that biofilms consist of 4 principal factors; teichoic acids, polysaccharide intercellular adhesins such as PIA from *S. epidermidis* or PNAG from *S. aureus*, extracellular DNA (ecDNA), and proteinaceous adhesins.

Teichoic acid is a cell wall component of gram-positive bacteria, and recently found to be an essential constituent of staphylococcal biofilms (Gross et al., 2001; Sadovskaya et al., 2005). Cell wall and extracellular teichoic acids are a mixture of two kinds of polymers,  $\alpha(1\rightarrow5)$ -linked poly(ribitol phosphate), substituted at the 4-position of ribitol residues with  $\beta$ -GlucNAc, and  $(1\rightarrow3)$ -linked poly(glycerol phosphate), partially substituted with D-Ala at the 2-position of glycerol residue (Vinogradov et al., 2006). Since a large fraction of teichoic acid is located in the 'fluffy'-layer region beyond the cell wall, it is considered that it functions in primary adhesion of bacteria to attached surfaces.

The glucosamine-based extracellular polysaccharides PIA and PNAG (PIA/PNAG) is an identical chemical compound, poly- $\beta(1,6)$ -*N*-acetyl-D-glucosamine, which is responsible for cell-cell attachment (Mack et al., 1996; Cramton et al., 1999). PIA/PNAG is synthesized by enzymes encoded by the *icaADBC* (intercellular adhesin) operon (Heilmann et al., 1996; Götz, 2002) and *icaADBC* mutants of *S. epidermidis* RP62A were shown not to form biofilm (Gerke et al., 1998). However, it has also been demonstrated with many clinical isolates that PIA/PNAG-negative *S. epidermidis* and *S. aureus* exhibit a strong biofilm phenotype (Rohde et al., 2007; Hennig et al., 2007; Boles et al., 2010). Rohde et al. (2007) reported that 27% of biofilm-positive *S. epidermidis* isolates produced PIA-independent biofilms, some of which were possibly mediated by the proteinaceous adhesin Aap, an accumulation-associated protein (Hussain et al., 1997). In addition, other proteins, such as the cell wall lytic enzyme AtlE (Heilmann et al., 1996), biofilm-associated protein Bap (Cucarella et al., 2001), and others are involved in attachment to the polymer surface or host matrix proteins, and related to cell-cell adhesion (Frank & Patel, 2007; Otto, 2009).

It was recently shown that AtlE is responsible for autolysis of *S. epidermidis*, resulting in release of ecDNA, and that ecDNA is a structural component of biofilms formed by *S. epidermidis* and *S. aureus* (Qin et al., 2007; Rice et al., 2007). The ratios of these 3 factors, PIA/PNAG, proteinous, and ecDNA, in biofilm formation seem to be varied for each strain, and become altered by various environmental or culture conditions. The ratios were conveniently semi-quantified *in vitro* by measuring amounts of biofilms formed on polymeric surfaces after incubation with or without dispersin B, trypsin, and DNaseI (Izano et al., 2008). We observed that production of the major extracellular protease GluSE was enhanced under specific culture conditions that also increased biofilm formation (Fig. 6) (Ohara-Nemoto et al., 2002). This observation is quite interesting with considering the relationship to a recent finding that protein-dependent biofilm formation by *S. aureus* was inhibited by expressions of extracellular proteases (Martí et al., 2010). The involvement of proteases in staphylococcal and streptococcal biofilm formation remains to be clarified.

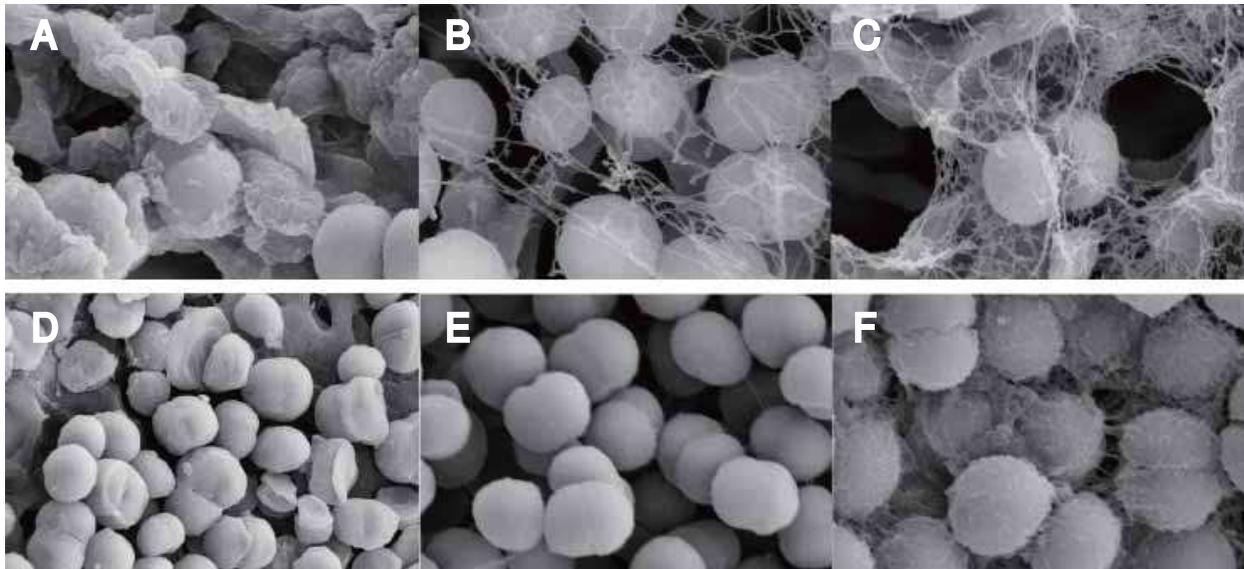


Fig. 6. Culture condition-dependent biofilm formation by *S. epidermidis*. Scanning electron micrographs of 'biofilm-negative' *S. epidermidis* ATCC 12228 (A and D) and 14990 (B and E), and 'biofilm-positive' ATCC 35984 (C and F). Bacteria were cultured in Todd-Hewitt broth (THB) agar (A-C) or THB (D-F). Biofilm formation was clearly demonstrated with strains 12228 and 14990 cultured on THB agar, while it was not evident when these were cultured in THB

#### 4.2.2 Staphylococcal glutamic acid-specific protease

Glutamic acid-specific staphylococcal GluV8-family proteases belong to a serine protease family that possesses a catalytic triad composed of Ser, Asp, and His, forming a competent electron relay. GluV8 from *S. aureus*, first reported by Drapeau et al. (1972) as V8 protease, is related to its bacterial growth *in vivo* and pathogenicity (Coulter et al., 1998). GluV8 processes adhesion molecules that are expressed on the bacterial cell surface and destroy the extracellular matrix of host cells (Karlsson & Arvidson, 2002). GluSE was found as an *S. epidermidis* GluV8 homolog, which is the most abundant extracellular protein (Sasaki et al., 1998), and efficiently degrades host proteins such as elastin, fibronectin, collagen, complement protein C5, and immunoglobulin (Dubin et al., 2001; Moon et al., 2001; Ohara-Nemoto et al., 2002). The gene encoding GluSE is ubiquitously distributed on the chromosome and the protein is expressed in most clinical isolates under *in vitro* culture conditions (Fig. 7). Production frequency was comparable between isolates from patients suffering from IE,



Fig. 7. GluSE production in *S. epidermidis* clinical isolates. Extracellular soluble fractions were subjected to SDS-polyacrylamide gel electrophoresis, followed by immunoblotting with anti-GluSE Ig. Lanes 1-6, isolates from patients: lanes 7-12, isolates from saliva of healthy individuals: 13, purified GluSE

bacteremia, and wound infection (7/10, 70%), as well as in saliva from healthy subjects (44/59, 74.6%) (Ikeda et al., 2004).

As demonstrated by 2D-PAGE followed by protein identification with MALDI TOF-MS and immunoblotting, 28-kDa mature GluSE was observed as the major extracellular protein constituent (Fig. 8A), while another Glu-specific cysteine proteinase, Ecp, was moderately expressed (Ohara-Nemoto et al., 2008b). In a cell wall fraction, limited amounts of 32-, 30-, and 29-kDa proforms of GluSE were observed (Fig. 8B and C), whereas no pro- or matureforms of GluSE were detected in bacterial cytoplasm. These findings indicated that GluSE is immediately secreted after protein synthesis and maturation through cleavage at the Ser<sub>1</sub>-Val<sub>1</sub> bond.

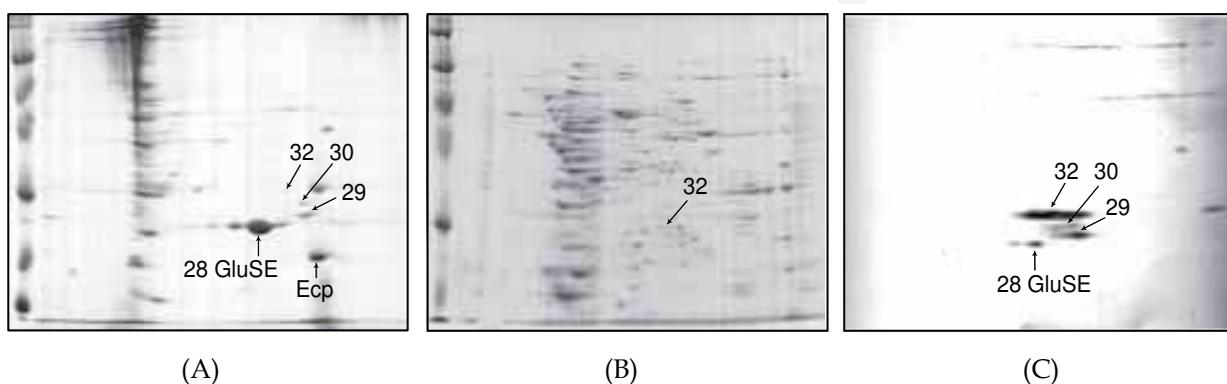


Fig. 8. 2D-PAGE shows GluSE in extracellular and cell wall fractions. The extracellular fraction of *S. epidermidis* (A) and cell wall fractions (B and C) were separated by 2D-PAGE, and developed with Coomassie brilliant blue staining (A and B) or immunoblotting with anti-GluSE Ig (C). Numbers indicate relative molecular weights (kDa)

Genes encoding GluV8 homologs were recently cloned from other CoNS species, and their proteolytic activities and biochemical characteristics were determined (Nemoto et al., 2008; Ono et al., 2010): They are GluSW from *S. warneri*, GluScp from *S. caprae*, and GluScoh from *S. cohnii*, and the order of specific activity was found to be GluV8 >> GluScp > GluSW > GluSE. These GluV8 family proteases may be associated with the survival and spreading of bacteria *in vivo* by cleavage of proteinous molecules involved in host defense. GluV8 degrades  $\alpha_1$ -protease inhibitor, which is the major inhibitor of elastase. Inactivation of  $\alpha_1$ -protease inhibitor then causes activation of elastase released from activated neutrophilic granulocytes, resulting in damage to host tissues (Arvidson, 2006).

In a comparison of amino acid sequence and proteolytic activity, the following essential amino acid residues in the GluV8-protease family were determined. Val<sub>1</sub> is required to exert proper maturation mediated by cleavage between the Xaa-Val bond and for proteolytic activity itself, with Trp<sub>185</sub>, Val<sub>188</sub> and Pro<sub>189</sub> also involved in proteolytic activity (Fig. 9) (Nemoto et al., 2009). The  $K_m$  value of native GluSE harboring a combination of Tyr<sub>185</sub>Val<sub>188</sub>Asp<sub>189</sub> was larger than that of GluV8 with Trp<sub>185</sub>Val<sub>188</sub>Pro<sub>189</sub>. Amino acid substitutions in these three residues decreased  $K_m$  with a constant  $k_{cat}$  value (Table 5). These residues can be involved in substrate affinity, which implicates the mechanism of alteration in proteolytic activity among the members of this family.

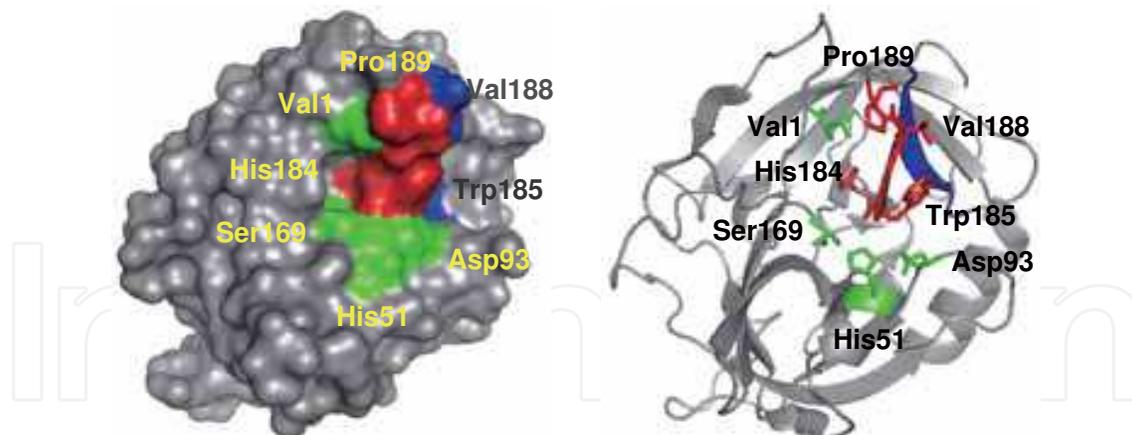


Fig. 9. Amino acid residues involved in proteolytic activity of GluV8 are shown in a three-dimensional structure. The catalytic triad and Val<sub>1</sub> are shown in green, and His<sub>184</sub> to Pro<sub>189</sub> and Glu<sub>191</sub> to Phe<sub>198</sub>, which form an anti-parallel  $\beta$ -sheet, are shown in red and blue, respectively

Protease	$K_m$ (mM)	$k_{cat}$ (s <sup>-1</sup> )
GluV8 (W <sub>185</sub> V <sub>188</sub> P <sub>189</sub> )	0.30±0.08	6147±1117
GluSE (W <sub>185</sub> V <sub>188</sub> P <sub>189</sub> )	0.35±0.13	7695±1200
GluSE (W <sub>185</sub> V <sub>188</sub> D <sub>189</sub> )	2.84±1.49	8863±1765
GluSE (Y <sub>185</sub> V <sub>188</sub> P <sub>189</sub> )	4.15±1.58	7681±3069
GluSE (W <sub>185</sub> A <sub>188</sub> P <sub>189</sub> )	1.32±0.10	9059±4205

Table 5. Kinetic parameters of recombinant GluV8 and GluSE with amino acid substitutions at positions 185, 188, and 189

Recently, Iwase et al. (2010) reported that biofilm formation by *S. aureus* was inhibited by GluSE, which also destroyed pre-existing *S. aureus* biofilms. Accordingly, GluSE enhanced the susceptibility of *S. aureus* colonizing the nasal cavity to host immune system components. These observations suggest the existence of bacterial interference among *Staphylococcus* species mediated by GluV8-family proteases in normal microflora. At present, the molecular mechanism remains unclear. It is not easy to speculate how bacterial proteases harbouring the same substrate specificity degrade opponent factors without affecting the corresponding self-factors. Therefore, investigations on the production regulation of GluV8-family proteases and their target molecules are important. Production of GluV8 together with other virulence factors, such as hemolysins and toxins, is regulated by the well-studied system of the accessory gene regulator (*arg*) locus (Ji et al., 1997; Novick 2003). Recent reports have raised the possibility that the *arg* system as well as GluV8 or other extracellular proteases is involved in biofilm detachment (Yarwood et al., 2004; Boles & Horswill, 2008; Martí et al., 2010). Thus, regulation of the biosynthesis of proteinous factors, especially GluV8-family proteases in *Staphylococcus* species, and their relationship with bacterial interference must be elucidated to better understand the molecular mechanism of onset of staphylococcal IE.

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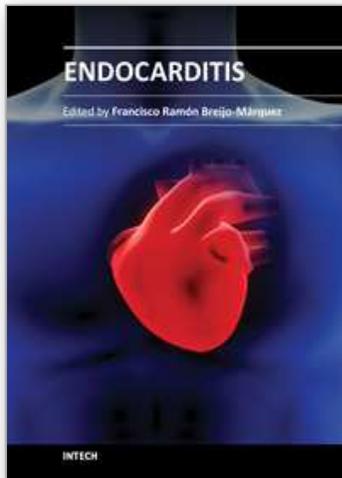
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## **Endocarditis**

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Endocarditis is a disease that occurs as a result of the inflammation of the endocardium. It is an inflammatory process located in the inner lining of the cardiac chambers and native or prosthetic valves. It is characterized by colonization or invasion of the heart valve vegetations composed of platelets forming, fibrin and microcolonies of microorganisms, and occasionally of inflammatory cells. Other structures may also be affected, such as the interventricular septum, chordae tendineae, the mural endocardium or even intra-cardiac implants. The book covers, with scientific rigour, the most prevalent causes and current treatments of endocarditis, as well as the cases when the organs remote from the heart are affected by this disease.

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