โครงการ การจำแนกเชื้อสเตร็ปโตคอคคัสมิวแทนส์ที่มีความบกพร่องในการแสดงออกของโปรตีนแอนติเจนโดยวิธีมัลติโลคัสซีเควนซ์ทัยปิง

โดย

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รายงานวิจัยฉบับสมบูรณ์

โครงการ การจำแนกเชื้อสเตร็ปโตคอคคัสมิวแทนส์ที่มีความบกพร่องในการแสดงออกของโปรตีนแอนติเจนโดยวิธีมัลติโลคัสซีเควนซ์ทัยปิง

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สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา

และสำนักงานกองทุนสนับสนุนการวิจัย และมหาวิทยาลัยมหิดล

กิตติกรรมประกาศ

ผู้วิจัยขอขอบพระคุณ สำนักงานคณะกรรมการการอุดมศึกษา และสำนักงานกองทุนสนับสนุนการวิจัย และมหาวิทยาลัยมหิดล สำหรับการสนับสนุนทั้งด้านเงินทุนการวิจัย และความช่วยเหลือด้านต่างๆในระหว่างการรับทุน ซึ่งทำให้ผู้วิจัยมีความสะดวกในการดำเนินงานวิจัย และทำให้โครงการวิจัยประสบความสำเร็จไปได้ด้วยดี อีกทั้งผู้วิจัยขอขอบพระคุณนักวิจัยที่ปรึกษาหลัก Associate Professor Dr. Kazuhiko Nakano และนักวิจัยที่ปรึกษาร่วม รศ. ดร. รัชชพิน ศรีสัจจะลักษณ์ สำหรับการสนับสนุนในด้านความช่วยเหลือ การให้คำแนะนำ และกำลังใจ ตลอดโครงการศึกษาวิจัยนี้

บทคัดย่อ

รหัสโครงการ : MRG5580025

ชื่อโครงการ : การจำแนกเชื้อสเตร็ปโตคอคคัสมิวแทนส์ที่มีความบกพร่องในการแสดงออกของโปรตีนแอนติเจนโดยวิธีมัลติโลคัสซีเควนซ์ทัยปิง

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ระยะเวลาโครงการ : 2 ปี

เชื้อสเตร็ปโตคอคคัสมิวแทนส์แบ่งออกเป็น 4 ซีโรไทป์ คือซีโรไทป์ซี อี เอฟ และเค เชื้อชนิดนี้มีโปรตีนแอนติเจน ซึ่งเป็นโปรตีนขนาดประมาณ 190 กิโลดาลตันที่ผิวเซลล์ โปรตีนดังกล่าวมีความสำคัญต่อกระบวนการเริ่มยึดเกาะของเชื้อที่ผิวฟัน ในการศึกษานี้เราใช้การวิเคราะห์โดยเทคนิคเวสทเทิร์นบลอทเพื่อตรวจสอบการแสดงออกของโปรตีนแอนติเจนในเชื้อสเตร็ปโตคอคคัสมิวแทนส์ จำนวน 750 สายพันธุ์ซึ่งแยกได้จากอาสาสมัครคนไทย 150 คน และพบความผิดปกติในการแสดงออกของโปรตีนดังกล่าวใน 7% ของเชื้อที่ทำการทดสอบ เชื้อในซีโรไทป์เคเป็นเชื้อซึ่งตรวจพบความผิดปกติดังกล่าวมากที่สุด (42%) รองลงมาคือซีโรไทป์เอฟ (41%) แต่ความผิดปกติดังกล่าวพบได้น้อยมากในเชื้อในซีโรไทป์ซี (3%) และอี (5%) การทดสอบทางสถิติแสดงให้เห็นว่าความชุกของความผิดปกติในการแสดงออกของโปรตีนแอนติเจนของเชื้อในซีโรไทป์เอฟและเคมีค่ามากกว่าเชื้อในซีโรไทป์ซีและอีอย่างมีนัยสำคัญ ($P<0.001$) นอกจากนั้นเรายังตรวจพบความหลากหลายของรูปแบบความผิดปกติซึ่งสามารถแบ่งออกได้เป็น 3 แบบคือ แบบ N1 ตรวจไม่พบการแสดงออกของโปรตีนแอนติเจนทั้งในตัวอย่างเซลล์เชื้อและซุปเปอร์นาแทนท์ แบบ N2 ตรวจพบการแสดงออกของโปรตีนแอนติเจนเป็นหลักในซุปเปอร์นาแทนท์ของเชื้อ และแบบ W ตรวจพบการแสดงออกของโปรตีนแอนติเจนเฉพาะในตัวอย่างเซลล์เชื้อแต่ในระดับต่ำกว่าปกติ เมื่อนำเอาซีโรไทป์ของเชื้อมาประกอบการพิจารณา จะพบว่าเชื้อในซีโรไทป์เคมีความผิดปกติในการแสดงออกแบบ N1 เท่านั้น ในขณะที่เชื้อในซีโรไทป์อื่นๆมีความผิดปกติหลายรูปแบบประกอบกัน สำหรับสาเหตุของความผิดปกติในแต่ละรูปแบบนั้น แบบ N1 เกิดจากความผิดปกติในลำดับนิวคลีโอไทด์ รวมถึงกระบวนการทรานสคริปชันของจีนผลิตโปรตีนแอนติเจน ส่วนความผิดปกติแบบ N2 เกิดจากความผิดปกติในลำดับนิวคลีโอไทด์ของจีนผลิตเอนไซม์ซอร์เตส สำหรับความผิดปกติแบบ W มาจากการสร้างเอ็มอาร์เอ็นเอของจีนผลิตโปรตีนแอนติเจนในระดับต่ำกว่าปกติ จากการศึกษาโดยวิธีมัลติโลคัสซีเควนซ์ทัยปิง เราพบโคลนของเชื้อสเตร็ปโตคอคคัสมิวแทนส์ที่มีความผิดปกติในการ

แสดงออกของโปรตีนแอนติเจน พบว่าสายพันธุ์เชื้อประมาณ 65-100% ซึ่งจัดอยู่ในโคลนดังกล่าวมีความผิดปกติในการแสดงออกของโปรตีนชนิดนี้ ยังพบอีกว่าซีโรไทป์ของเชื้อในโคลนเหล่านี้ส่วนใหญ่เป็นซีโรไทป์เอฟและเค ดังนั้นโดยสรุป การศึกษานี้อธิบายถึงสาเหตุของความผิดปกติในการแสดงออกของโปรตีนแอนติเจนในเชื้อสเตร็ปโตคอคคัสมิวแทนส์ และผลจากการศึกษายังเน้นย้ำให้เห็นถึงความสัมพันธ์ใกล้ชิดในรูปแบบโคลนเชื้อของเชื้อซึ่งมีความผิดปกติเกี่ยวกับโปรตีนแอนติเจน และเชื้อในซีโรไทป์เอฟและเค

คำหลัก : เชื้อสเตร็ปโตคอคคัสมิวแทนส์ โปรตีนแอนติเจนขนาด 190 กิโลดาลตัน ความผิดปกติในการแสดงออก ซีโรไทป์ มัลติโลคัสซีเควนซ์ทัยปิง

Abstract

Project Code : MRG5580025

Project Title : Multilocus sequence typing analysis of *Streptococcus mutans* strains with defect of protein antigen expression

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Project Period : 2 years

Streptococcus mutans is classified into four serotypes, *c*, *e*, *f*, and *k*. *S. mutans* possesses 190 KDa cell-surface protein named protein antigen (PA) for initial binding of its cell to tooth surface. In this study, we used Western blot analysis to determine PA expression of 750 *S. mutans* strains from 150 Thai subjects and found that 7% of the strains showed the altered PA expression. The highest prevalence of defect was detected in serotype *k* (42%), followed by serotype *f* (41%). In contrast, the defect was rare in serotype *c* (3%) and *e* (5%). The prevalence of PA expression defect in serotypes *f* and *k* was significantly greater than in serotypes *c* and *e* ($P<0.001$). In addition, the defect patterns were diverse, which were classified into three types; no PA expression on whole bacterial cells and in their supernatant samples (Type N1), PA expression mainly seen in supernatant samples (Type N2), and only low expression of PA found from the samples of whole bacterial cells (Type W). When the serotypes of isolates were taken to be considered, *S. mutans* with serotype *k* showed only the Type N1 defect and mixed types of defect were detected from non-serotype *k* strains. The underlying reasons for defect were proven to be defects in nucleotide alignment of gene encoding PA as well as the transcriptional process of this gene for Type N1, defects in nucleotide alignment of gene encoding sortase (*srtA*) for Type N2, and low mRNA expression of PA for Type W. Moreover, multilocus sequence typing (MLST) could reveal clones of *S. mutans* with PA expression defect. Approximately 65-100% of strains located in these clones showed altered PA expression, and most of the strains in these clones were serotypes *f* and *k*. In conclusion, we described the causes regarding variation defects of PA expression in *S.*

mutans. Furthermore, we also emphasized the strong association between PA expression defect and serotypes *f* and *k S. mutans* as well as the clonal relationship among these strains.

Keywords : *S. mutans*, 190-kDa Protein Antigen, expression defect, serotype, MLST

เนื้อหางานวิจัย

Introduction

Streptococcus mutans, a gram-positive facultative anaerobic bacterium, is generally known as a major causative pathogen of dental caries, one of the most important infectious diseases in dental field (Hamada and Slade, 1980; Selwitz *et al*., 2007). As for medical field, *S. mutans* is also known to be associated with bacteremia and infective endocarditis (IE) found in some patients with heart disorders (Moreillon and Que, 2004). *S. mutans* is the most prevalent species of mutans streptococci group found in human oral cavity (Hung *et al*., 2005; Ota *et al*., 2006). The others species in this group are *Streptococcus sobrinus*, *Streptococcus cricetus*, *Streptococcus rattus*, *Streptococcus ferus*, *Streptococcus macacae* and *Streptococcus downei*. *S. mutans* has been classified into 4 serotypes, *c*, *e*, *f*, and *k*, based on the differences of the chemical composition in rhamnose-glucose antigens on its cell wall (Linzer *et al*., 1986, Nakano *et al*., 2004a). Approximately 70-80% of oral isolated *S. mutans* strains belong to serotype *c*, followed by serotype *e* (approximately 20%), whereas the distribution frequencies of serotype *f* and *k* are extremely low (each less than 5%) (Beighton *et al*., 1987; Holbrook *et al*., 1987; Nakano *et al*., 2004b).

As for protein antigens, 190-kDa cell surface protein-PA, is well known in *S. mutans* (Okahashi *et al*., 1989). This protein is also designated as antigenI/II, PAc, P1, and SpaP (Crowley *et al*., 1999; Forester *et al*., 1983; Lee *et al*., 1989). The PA is important for pathogenesis of dental caries, since it has been shown to be correlated with sucrose-independent initial adhesion of *S. mutans* to saliva components coated on tooth surfaces (Banas, 2004; Okahashi *et al*., 1989). Nevertheless, the detection of *S. mutans* strains without PA expression was also reported (Nakano *et al*., 2006; Nakano *et al*., 2008). The strains with such defect were frequently found in serotypes *f* and *k*, the minor serotypes of *S. mutans* in the oral cavity (Nakano *et al*., 2008). However, various pieces of evidence pointed out the relationship between the minor serotype strains and pathogenesis of bacteremia and IE (Nakano *et al*., 2004a; Nakano *et al*., 2007b; Nakano *et al*., 2008). Thus, the association of PA expression defect with minor-serotype *S. mutans* is of interest. In addition, phagocytosis susceptibility of *S. mutans* strains without PA expression was lower than that of the normal PA

expression strains (Nakano *et al*., 2008). Low susceptibility to phagocytosis could provide long survival duration of strains in bloodstream, and then provide a chance for such organisms to circulate and encounter with impaired heart valves. In fact, most of blood isolated *S. mutans* showed low level of susceptibility to phagocytosis (Nakano *et al*., 2004a; Nomura *et al*., 2007). Furthermore, the study using animal model also revealed that *S. mutans* strain without PA expression caused longer duration of bacteremia and more severe systemic inflammation than the PA expression strain (Nakano *et al*., 2006).

Recently, multilocus sequence typing (MLST), a molecular genotyping method based on nucleotide sequences of multiple housekeeping genes, is popular and accepted worldwide for indicating the existence of hypervirulent clones, clone-associated virulence factors, and for bacterial population studies (Brueggemann *et al*., 2003; Coffey *et al*., 2006; Feil *et al*., 2000; Feil *et al*., 2001; Jolley *et al*., 2000; Luan *et al*., 2005; Pullinger *et al*., 2006,). Our previous studies based on MLST scheme of *S. mutans* could exhibit *S. mutans* clones that might be hypervirulent for IE, since *S. mutans* strains in such clones have strong binding ability to collagen, a component protein of heart valve extracellular matrix (ECM) (Lapirattanakul *et al*., 2011). Therefore, it seems valuable to clarify relationship of *S. mutans* strains with PA expression defect by MLST analysis.

Thus, in this study, we determined the prevalence of *S. mutans* with defect of PA expression among Thai isolates and clarified causes of such defect. Moreover, the association between PA expression defect and minor serotype strains was also reconfirmed using these Thai *S. mutans* strains. Finally, MLST method was utilized to find clonal relationship among *S. mutans* strains with PA expression defect.

Methods

Bacterial strains

Two categories of *S. mutans* strains were used in this study. The first group consisted of 129 strains from Finnish and Japanese people (Lapirattanakul *et al*. 2008; Nakano *et al*. 2007a).

The other group contained 750 *S. mutans* strains (TLJ series) isolated from saliva of 150 Thai subjects (Lapirattanakul *et al*., 2011). In addition, strain MT8148 (Ooshima *et al*., 1983) was used as the control *S. mutans* strain with normal PA expression. Serotype information of all strains determined in our previous studies (Lapirattanakul et al., 2008; Lapirattanakul *et al*., 2011; Nakano et al., 2007a) was also utilized in this study.

Western blot analysis of PA expression

PA expression of all 879 *S. mutans* strains from Finnish, Japanese, and Thai subjects were carried out by Western blot analysis using specific antibody, as described previously (Nakano *et al*., 2006). Since PA is normally expressed as a cell-associated protein on the surface of *S. mutans*, whole cell sample of each bacterial strain was prepared. The organisms grown in 10 ml of brain heart infusion (BHI; Difco Laboratories, Detroit, MI, USA) broth at 37°C to OD_{550} = 1.0 were collected and dissolved in sodium dodecyl sulfate gel loading buffer and used for the analysis. As for *S. mutans* strains without PA expression found in the whole bacterial cell samples, supernatant samples of those strains were prepared from culture supernatants concentrated by ammonium sulfate precipitation (50%). An equal amount of each protein sample was separated and transferred onto polyvinylidene difluoride membrane (Immobilon, Millipore, Bedford, MA). The transferred protein bands were reacted with rabbit antibody against the PA, then visualized using alkaline phosphatase-conjugated anti-rabbit immunoglobulin G antibody (New England Biolabs, Beverly, MA) and 5-bromo-4-chrolo-3-indolylphosphate-nitrobluetetrazolium substrate (Moss Inc., Pasadena, MD). For all experiments, *S. mutans* strains MT8148 was used as the positive control.

Analysis of mRNA expression of gene encoding PA

Reverse transcriptase polymerase chain reaction (RT-PCR) was utilized to examine the transcription of gene encoding PA, which has been indicated as a cause of PA expression defect in *S. mutans* from Finnish and Japanese subjects (Nakano et al., 2008). All Thai isolates of *S. mutans* with altered PA expression revealed by Western blot analysis were evaluated with this process. Total RNA was prepared from the tested strains using a FastPrep Cell Disrupter (Model FP100A, Q-Bio gene, Carlsbad, CA) in combination with a Fast RNA Pro Blue kit (Q-Bio

gene), according to the manufacturer's instructions. Then cDNA was synthesized from mRNA using High Capacity cDNA Reverse Transcription kits (Applied Biosystems, Foster City, CA). Finally, successive PCR assay was performed using the following primers: PAtF, 5'-TAG TAA AAC ACT GTG TGG TGC TGT-3' and PAtR, 5'-CCA GCT TGG TTT GAC TTT GTT CAG-3' (Nakano *et al*., 2008). For all experiments, genomic DNA from each strain was used as the positive control and RNA samples from which reverse transcriptase were omitted as the negative control. In addition, 16s rRNA was also amplified from each cDNA sample in order to confirm the success of cDNA preparation using primers published previously (Matsumoto-Nakano and Kuramitsu, 2006)

Nucleotide sequence analysis of gene encoding PA

Nucleotide alignment of gene encoding PA was determined in order to clarify the causes of PA expression defect found among PA defect strains of Thai subjects, since such analysis was successfully used for explanation of defective PA expression in gene level of *S. mutans* strains from Finnish and Japanese people (Nakano et al., 2008). Briefly, the gene encoding PA was separately amplified with three pairs of specific primers: PA1F, 5'-TTT GTG CTT TAG AAT TAA TGT TGG-3' and PA1R, 5'-GAC AAT TTT AGA AAT CTT TTT ACC-3'; PA2F, 5'-GGG ATC GGT ACT TTT AGA GCG CGG-3' and PA2R, 5'-CAC TGT TGG ATA AAT CGT TGC CAC-3'; PA3F, 5'-AAC TAA TAC AGT CAC CTT CAA GGC-3' and PA3R, 5'-GCT CAA TCT GTG ATT TAT CGC TTC-3'. The amplified fragments were cloned into a pGEM-T Easy vector (Promega) and their nucleotide alignments were determined using a dye terminator reaction with a DNA sequencing system (ABI Prism 310 genetic analyser; Applied Biosystems) and a BigDye terminator cycle sequencing kit. To obtain the complete sequence, internal primers (PA1mid, 5'-CTG CAA ATG CTG CCA GTA AA-3' PA2mid, 5'-CTA TCC GCA TGT CTG GTC CT-3' and PA3mid, 5'-GAC CGC TCT TCA GCA GAT AC-3') were also utilized. Sequence assembly was performed with software CodonCode Aligner (CodonCode Corporation, www.codoncode.com). Nucleotide sequence data gained were compared with nucleotide sequence of the gene encoding PA of complete genome sequenced *S. mutans* strain, UA159 (Ajdic *et al*., 2002) using CodonCode Aligner and Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Nucleotide sequence analysis of gene encoding sortase

Analysis for nucleotide alignment of sortase gene (*srtA*) was done in *S. mutans* strains of which PA was detected in supernatant samples. Specific primers for amplification of this gene (SRT-fp, 5'-GGC ACT TCA CTA AAT AAT AAT GAA AAT-3' and SRT-rp, 5'-TGC TCA TGA GAC CTC ACA GAA T-3') were designed based on nucleotide sequence of sortase gene retrieved from the complete genome sequenced UA159 (Ajdic et al., 2002). The amplified fragments were then cloned into a pGEM-T Easy vector and determined for nucleotide alignments. To obtain the complete sequence of some strains with internal insertions, internal primers (RCsortasemid, 5'-AGA TTA GTG CTA AAC CAA TC-3') were also employed. Nucleotide sequence data gained were compared with nucleotide sequence of the gene encoding sortase enzyme of strain UA159 by CodonCode Aligner and BLAST.

MLST process

All 129 *S. mutans* strains from Finnish and Japanese subjects have already been classified by MLST method in our previous studies (Lapirattanakul *et al*. 2008; Nakano *et al*. 2007a). Therefore, the information concerning MLST analysis of these strains have already accumulated in the oral streptococcus MLST databases (http://pubmlst.org/oralstrep/). As for MLST analysis of *S. mutans* with PA defect from Thai subjects, one representative of isolates showing identical Western blot and RT-PCR profiles was randomly selected from each subject possessing such defect strains for MLST analysis. Unexpectedly, most of these strains were subjected to MLST process since our recent work (Lapirattanakul *et al*., 2011), thus in this study only 4 strains (TLJ10-3, TLJ12-1, TLJ74-2, and TLJ127-1) remained to be analyzed by the similar procedure.

Briefly, internal fragments of eight housekeeping genes: transketolase (*tkt*), glutamine synthetase type I (*glnA*), glutamate synthetase (*gltA*), glucose kinase (*glk*), shikimate 5-dehydrogenase (*aroE*), glutamate racemase (*murI*), signal peptidase I (*lepC*) and DNA gyrase A subunit (*gyrA*) were amplified and determined for nucleotide sequences. The sequences for each gene fragment were then compared with accumulated nucleotide sequences in the oral

streptococcus MLST databases to assign allele numbers. Eight allele numbers for each strain defined the allelic profile, and consequently, sequence type (ST) of the strain.

Allelic profiles of four strains determined in this project, together with our previous MLST data (Lapirattanakul et al., 2008; Lapirattanakul et al., 2011; Nakano et al., 2007a), were analyzed with computational programs as follows. The START (sequence type analysis and recombinational tests) tools (http://pubmlst.org/software/analysis/start2) (Jolley et al., 2001) were used to produce a matrix of pairwise differences in the allelic profiles and a dendrogram was constructed from the matrix using unweighted pair group method with arithmetic mean UPGMA). Next related STs were grouped into lineages or clonal complexes using the eBURST (enhanced based upon related sequence types) programme, version 3 (http:// eburst.mlst.net) (Feil et al., 2004). Two or more independent isolates sharing identical alleles at six or more loci were defined as members of a lineage. Each lineage was a 'group' or 'clonal complex', which designates STs that have diversified very recently from a common ancestor.

Statistical analysis

Statistical analyses were performed using the software SPSS statistical software package (SPSS Corporation, Chicago, Ill, USA). The proportion of PA expression defect between each serotype was compared using Fisher's exact probability test. For all analyses, *P* values less than 0.05 were considered to be statistically significant.

Results

Detection of *S. mutans* with PA expression defect

Results of PA expression in 129 strains from Finnish and Japanese people were shown as the part of dendrogram (Fig. 1), since the interpretation of these data was performed together with the MLST data. For Thai strains, Western blot analysis revealed PA expression defect *S. mutans* strains in 19 of 150 Thai subjects (13%), in which 53 of 750 *S. mutans* strains (7%) from these subjects showed the altered PA expression. In each serotype, the PA expression defect was demonstrated at the highest prevalence in serotype *k* (42%) and *f* (41%) strains. In

contrast, the defect was rare in serotypes *c* (3%) and *e* (5%) (Table 1). The prevalence of PA expression defect *S. mutans* strains in serotypes *f* and *k* was significantly greater than in the other serotypes ($P<0.001$).

Moreover, PA expression defect patterns were diverse, which were classified into three types; no PA expression on whole bacterial cells and in their supernatant samples (Type N1), PA expression mainly seen in supernatant samples (Type N2), and only low expression of PA found from the samples of whole bacterial cells (Type W) (Fig. 2). The detected 53 *S. mutans* strains with PA expression defect were 30 strains of Type N1 (57%), 7 strains of Type N2 (13%), and 16 strains of Type W (30%).

Evaluation of causes for PA expression defect

The mRNA expression of gene encoding PA by RT-PCR in Thai isolates of *S. mutans* strains with altered PA expression showed that *S. mutans* strains in Type N1 defect showed both positive (40%) and negative (60%) mRNA expression of the gene encoding PA, while Type N2 defect showed only the positive expression result. As for Type W defect, RT-PCR showed the weak mRNA expression in all *S. mutans* strains classified into this type (Fig. 3). When the serotypes of isolates were taken to be considered, *S. mutans* with serotype *k* showed only Type N1 defect without mRNA expression of gene encoding PA by RT-PCR (N1RT-) and mixed types of defect was detected from non-serotype *k* strains (Fig. 4). The statistical association between serotypes and types of PA expression defect was proven ($P<0.001$).

Nucleotide sequence analysis indicated various alterations of gene encoding PA in Type N1 defect strains compared with the normal PA expression strain, UA159 (Table 2). For Type N1 showing positive mRNA expression by RT-PCR (N1RT+), the transcription of gene encoding PA into mRNA could be processed. However, nonsense mutation, a point mutation that created premature stop codon and frameshift mutation either by one-base deletion or ten-base insertion were primarily sources of protein expression defect. Moreover, a point mutation at start condon, from ATG to ATA, was also identified in one of N1RT+ strains (TLJ57-2). As for N1RT-, the origin of PA expression defect was in transcriptional level, since no mRNA of gene encoding PA was present. Nevertheless, several mutations as well as large insertions and deletions were

often observed in the gene encoding PA of N1RT- strains. One insertion region of NTRT- strains (TLJ60-1) showed high identity (99%, E-value = 0.0) with the insertion sequence (IS) element, IS199.

In contrast, nucleotide sequence analysis of gene encoding PA could not explain the reasons of defects in Type N2 and Type W strains (Table 2). All nucleotide alignments of gene encoding PA in Type N2 and Type W strains showed high identity to those of UA159 (98-99%, E-value = 0.0). Putative amino acids of the strains with PA expression in Type N2 and Type W ranged from 1563 to 1567, which indicated a high identity to UA159 (97-99%, E-value = 0.0). However, the frameshift mutations caused by insertions were demonstrated in nucleotide sequences of sortase gene for all Type N2 strains. In strain TLJ10-3, this frameshift mutation caused by one-base insertion, while the alignments of inserted parts in strains TLJ74-2 and TLJ127-1 were found to be IS elements. (99-100% identity to ISSmu1, E-value = 0.0).

Clones of *S. mutans* with PA expression defect revealed by MLST analysis

MLST analysis of Thai *S. mutans* strains with PA expression defect showed only one new ST (ST174) for strain TLJ10-3. Allelic profile of this ST174 was defined by of eight allele numbers as follows; 2 for *tkt*, 2 for *glnA*, 5 for *gltA*, 1 for *glk*, 2 for *aroE*, 3 for *murI*, 27 for *lepC*, and 1 for *gyrA*. As for STs of the other remaining strains, the data were as shown in Table 2. Then MLST profiles of all 19 Thai strains with the PA defect were analyzed together with the profiles of 129 Finnish and Japanese strains retrieved from the oral streptococcus MLST databases. As presented in Fig. 1, the dendrogram of all 148 strains included 120 classified STs. Thirty-five of these 148 strains showed PA expression defect by Western blot analysis. The eBURST programme revealed 13 clonal complex groups (clones) containing 91 *S. mutans* strains, whereas the remaining 57 strains were located as 47 singletons, the STs that were unrelated to any other STs. Among the 13 groups, groups 5, 9, and 13 showed close relationship to the strains with PA expression defect, since approximately 65-100% of strains located in these groups showed altered PA expression. While serotype *f* was the predominant serotype of strains in group 5, strains in groups 9 and 13 were only serotype *k*.

Discussion

High prevalence of PA expression defect in serotypes *f* and *k S. mutans* from Thai population confirmed the strong association between these serotypes and PA defect firstly observed in Finnish and Japanese strains (Nakano *et al*., 2008). Since PA is essential surface protein for *S. mutans* adhesion (Banas, 2004), the defect of PA expression frequently found in serotypes *f* and *k* might explain why these strains are minor population in oral cavity. In our previous work, we found three different patterns of PA expression in 100 *S. mutans* strains from Japanese subjects (Nakano *et al*., 2006). While 93 from 100 strains show normal PA expression, no PA expression as Type N1 was seen in four strains. The remained three strains were designated as Type L, which showed the positive expression of PA in the lower molecular weight than the normal expression. The Type L, however, was not detected from the strains of these 150 Thai subjects. Surprisingly, the Type N2 and Type W defects were the novel patterns of PA expression defect initially detected in this study. Furthermore, we also examined the causes of such defects by determining nucleotide alignment and mRNA expression of gene encoding PA. Two explanations were defined for Type N1 defect. Both point and frameshift mutations affected PA expression in N1RT+strains, whereas the expression defect was in transcriptional process for NTRT- strains. Further research involving the defective origin in Type NTRT-, such as the defect in promoter region of the gene would be of interest. As for Type W defect, low mRNA expression led to low protein expression of PA was detected. Mechanisms as well as factors concerning this low expression is still remained to clarify.

Unlike Type N1 and Type W defects, the defect of Type N2 PA expression was in the gene encoding sortase enzyme, which is important for attaching protein on cell surface of gram-positive bacteria (Spirig *et al*., 2011). Frameshift mutations present in the sortase gene of Type N2 strains disturbed PA anchoring process, consequently the expression of PA was seen in culture supernatants. In fact, the role of sortase in surface expression of PA has been proven since 2003 (Igarashi *et al*., 2003; Lee and Boran, 2003), then the detection of *S. mutans* strains with defect of sortase gene has been reported (Igarashi, 2004; Lee and McGavin, 2004). In those studies, either a single-base substitution (nonsense mutation) or an 11-base deletion in sortase gene resulted in the production of an incomplete sortase enzyme of strains NG5 and

Ingbritt, respectively. Both of these strains failed to retain their surface proteins including PA on the cell surfaces. As for our discovery in Type N2 defect, the interesting point was the identification of IS element, ISSmu1, as the cause of frameshift mutation in sortase gene. ISSmu1 is one of the IS3 family members identified in *S. mutans* (Old and Russell, 2008). Thus, this study was the first report describing the involvement of IS element in sortase expression, which brought about the defect of PA expression on cell surface of *S. mutans*. Actually, the presence of other IS3 family member, IS199, was also found in gene encoding PA of one of N1RT- strains (TLJ60-1), although it is still unknown whether such presence had an effect on the PA expression or not.

For the utilization of MLST to study *S. mutans* strains with PA expression defect, the efficiency of MLST method for strains discrimination could be observed. Most strains with the same STs demonstrated the same patterns of PA expression (Fig. 1). The exception was found for only two STs (ST2 and ST39), in which the members of such STs showed both normal and defective expression of PA. In addition, nucleotide alignments of PA expression defect strains defined as the same STs also displayed the matched sequences of defects in genes encoding PA or sortase proteins (Table 2). For example, the nonsense mutations of gene encoding PA occurred in all three strains of ST140 (TLJ11-2, TLJ85-4, and TLJ137-4). Moreover, the frameshift mutations caused by same insertion events could be detected in gene encoding PA of ST114 (TLJ92-1 and TLJ123-1) and gene encoding sortase of ST2 (TLJ74-2 and TLJ127-1), although the directions of ISSmu1 insertions in sortase gene of TLJ74-2 and TLJ127-1 were opposite (data not shown). Besides its discriminatory power, MLST technique could demonstrate three *S. mutans* clones concerning PA expression defect (groups 5, 9, and 13), since more than 50% of strains with altered PA expression shown in Fig. 1 were present in these clones. Aforementioned, *S. mutans* strains with PA expression defect seemed to be implicated in prolonged bacteremia due to its low susceptibility to phagocytosis (Nakano *et al*., 2006; Nakano *et al*., 2008), these three *S. mutans* clones were of interest in terms of potential strains causing systemic diseases. Surprisingly, our previous studies have shown that the *S. mutans* strains in clonal complex groups 5, 9, and 13 showed strong collagen binding and relatively enhanced abilities to adhere to and invade endothelial cells owing to the possessing of collagen-binding protein genes (Lapirattanakul et al., 2011; Lapirattanakul et al., 2013). Since

the pathogenesis of IE caused by species of streptococci is started by the binding of bacteria in bloodstream to ECM of an impaired heart value (Moreillon, 2002), the presence of both virulent factors enhancing prolonged bacteremia and ECM-binding property in *S. mutans* clones 5, 9, and 13 provided the possibility for such clones as the candidate clones related to IE. Indeed, potential high virulence for IE in *S. mutans* strains with collagen-binding proteins but lacking PA expression was also reported in our recent work (Nomura et al., 2013). Furthermore, since the serotypes associated with groups 5, 9, and 13 were mainly *f* and *k*, these emphasized the strong association of serotypes *f* and *k* with bacteremia and IE. Nevertheless, the additional information of *S. mutans* strains from various sources and origins is still required for more precise prediction of clones appropriate for monitoring IE risk patients

In conclusion, this present study classified types of PA expression defect in *S. mutans* strains as well as clarified the cause of each defect type. In addition, we also highlighted the association between PA expression defect and serotypes *f* and *k S. mutans* as well as the clonal relationship among these strains.

Table 1. Distribution of Thai isolates with PA expression defect based on serotypes

Serotype[a]	No. of strains with normal PA expression[b]	No. of strains with PA expression defect[b]
c	514 (97%)	17 (3%)
e	143 (95%)	8 (5%)
f	26 (59%)	18 (41%)
k	14 (58%)	10 (42%)

[a] Serotyping of all *S. mutans* strains was performed in our previous study (Lapirattanakul *et al*., 2011).

[b] The numbers in parentheses indicate the percentages of strains with normal or defective PA expression in each serotype.

Table 2. Nucleotide alignment summary of genes encoding PA and sortase in each type of PA expression defect

Strain (serotype[a])	ST	Type of defect[b]	Significant deletions, insertions, and mutations in[c]	
			Gene encoding PA	Gene encoding sortase
TLJ9-1 (*f*)	136	N1RT-	171-base deletion (position 721-891 of UA159) 96-base deletion (position 1227-1322 of UA159)	NA
TLJ11-2 (*k*)	140	N1RT-	Nonsense mutation caused by one-base substitution (C to T) at position 2218 of UA159	NA
TLJ12-1 (*c*)	39	N1RT-	No detection	NA
TLJ60-1 (*k*)	125	N1RT-	Frameshift mutation caused by 1223-base insertion (IS199) at position after 180 of UA159	NA
TLJ85-4 (*k*)	140	N1RT-	Nonsense mutation caused by one-base substitution (C to T) at position 2218 of UA159	NA
TLJ129-3 (*f*)	124	N1RT-	198-base deletion (position 2621-2818 of UA159)	NA
TLJ137-4 (*k*)	140	N1RT-	Nonsense mutation caused by one-base substitution (C to T) at position 2218 of UA159	NA
TLJ24-4 (*e*)	118	N1RT+	Nonsense mutation caused by one-base substitution (C to T) at position 529 of UA159	NA
TLJ57-2 (*c*)	111	N1RT+	Missense mutation caused by one-base substitution (G to A) at position 3 of UA159 (the region of start codon)	NA
TLJ92-1 (*c*)	114	N1RT+	Frameshift mutation caused by 10-base insertion at position after 2959 of UA159	NA
TLJ115-2 (*e*)	120	N1RT+	Frameshift mutation caused by one-base deletion (position 10 of UA159)	NA
TLJ123-1 (*c*)	114	N1RT+	Frameshift mutation caused by 10-base insertion at position after 2959 of UA159	NA
TLJ10-3 (*e*)	174	N2	no detection	Frameshift mutation caused by one-base insertion at position after 623 of UA159
TLJ74-2 (*c*)	2	N2	no detection	Frameshift mutation caused by 1440-base insertion (ISSmu1) at position after 47 of UA159
TLJ127-1 (*c*)	2	N2	no detection	Frameshift mutation caused by 1439-base insertion (ISSmu1) at position after 48 of UA159
TLJ53-1 (*f*)	122	W	no detection	NA
TLJ72-3 (*f*)	123	W	no detection	NA
TLJ82-1 (*c*)	112	W	no detection	NA
TLJ109-1 (*f*)	122	W	no detection	NA

[a] Serotyping of all *S. mutans* strains was performed in our previous study (Lapirattanakul *et al*., 2011).

[b] Type of PA expression defect based on Western blot and RT-PCR analyses; N1RT-, Type N1 defect with negative RT-PCR result; N1RT+, Type N1 defect with positive RT-PCR result; N2, Type N2 defect with positive RT-PCR result; W, Type W defect with weak expression by RT-PCR.

[c] When compared with the complete genome sequenced strain, UA159. For deletion and insertion that did not cause any mutations, only the large deletion and insertion of more than 95 nucleotides were indicated.

NA: Not applicable

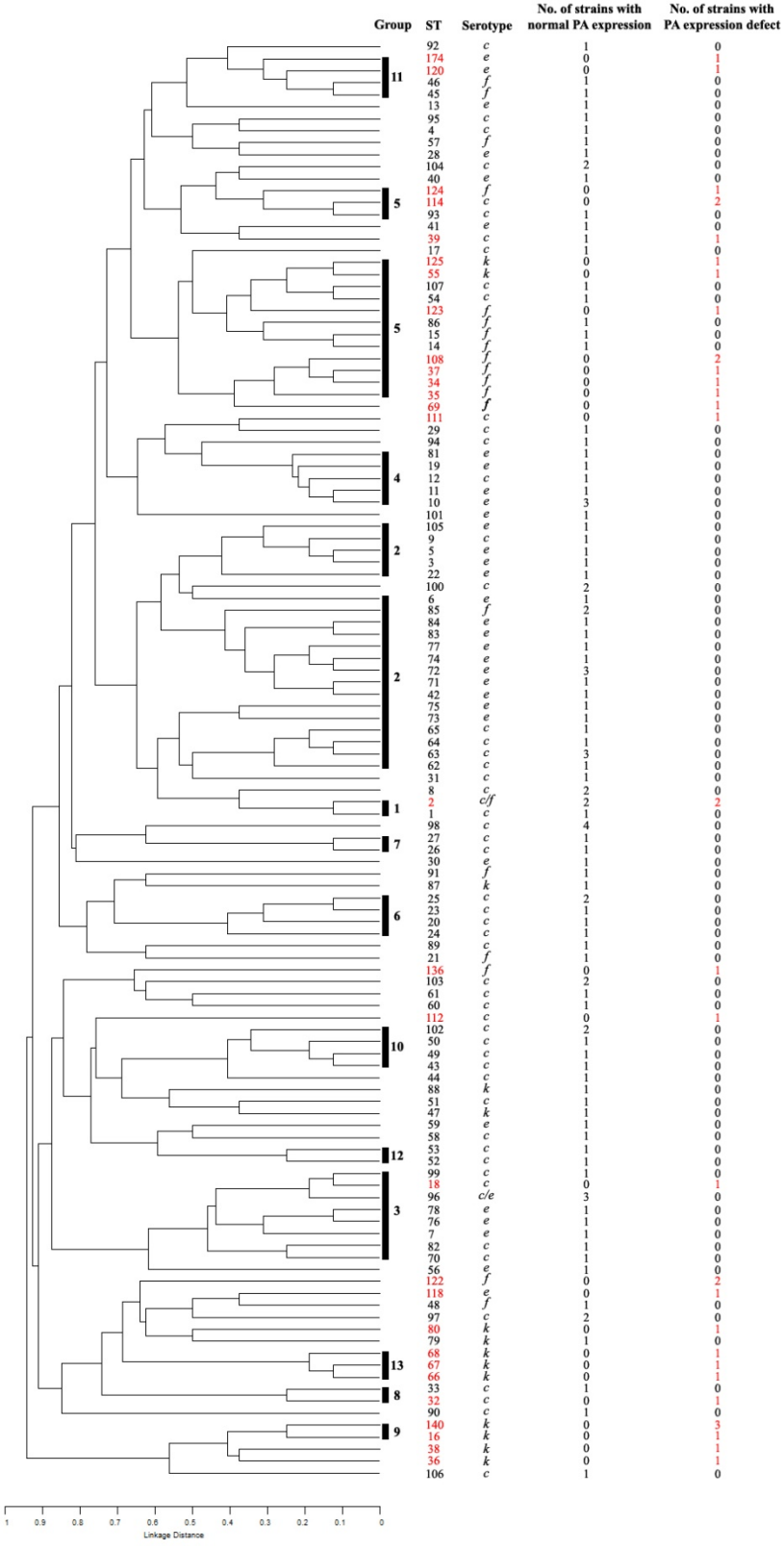

Fig. 1 Dendrogram created by MLST profiles of 148 *S. mutans* strains. Total 120 STs were classified. STs containing strains with PA expression defect revealed by Western blot analysis were indicated by red color.

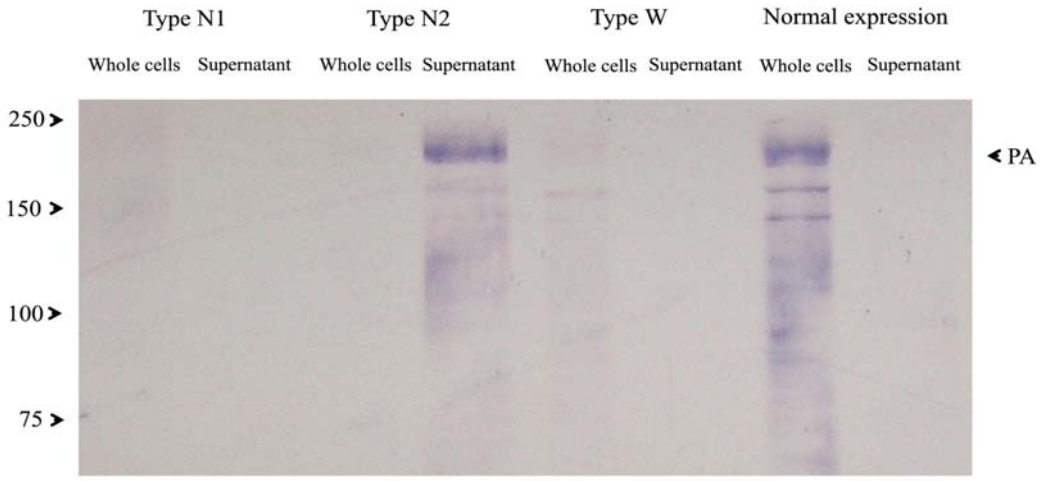

Fig. 2 Three types of PA expression defect detected by Western blot analysis. Type N1: no PA expression on whole bacterial cells and in their supernatant samples, Type N2: PA expression mainly seen in supernatant samples, and Type W: only low expression of PA found from the samples of whole bacterial cells. *S. mutans* strain MT8148 with normal PA expression was used as the positive control.

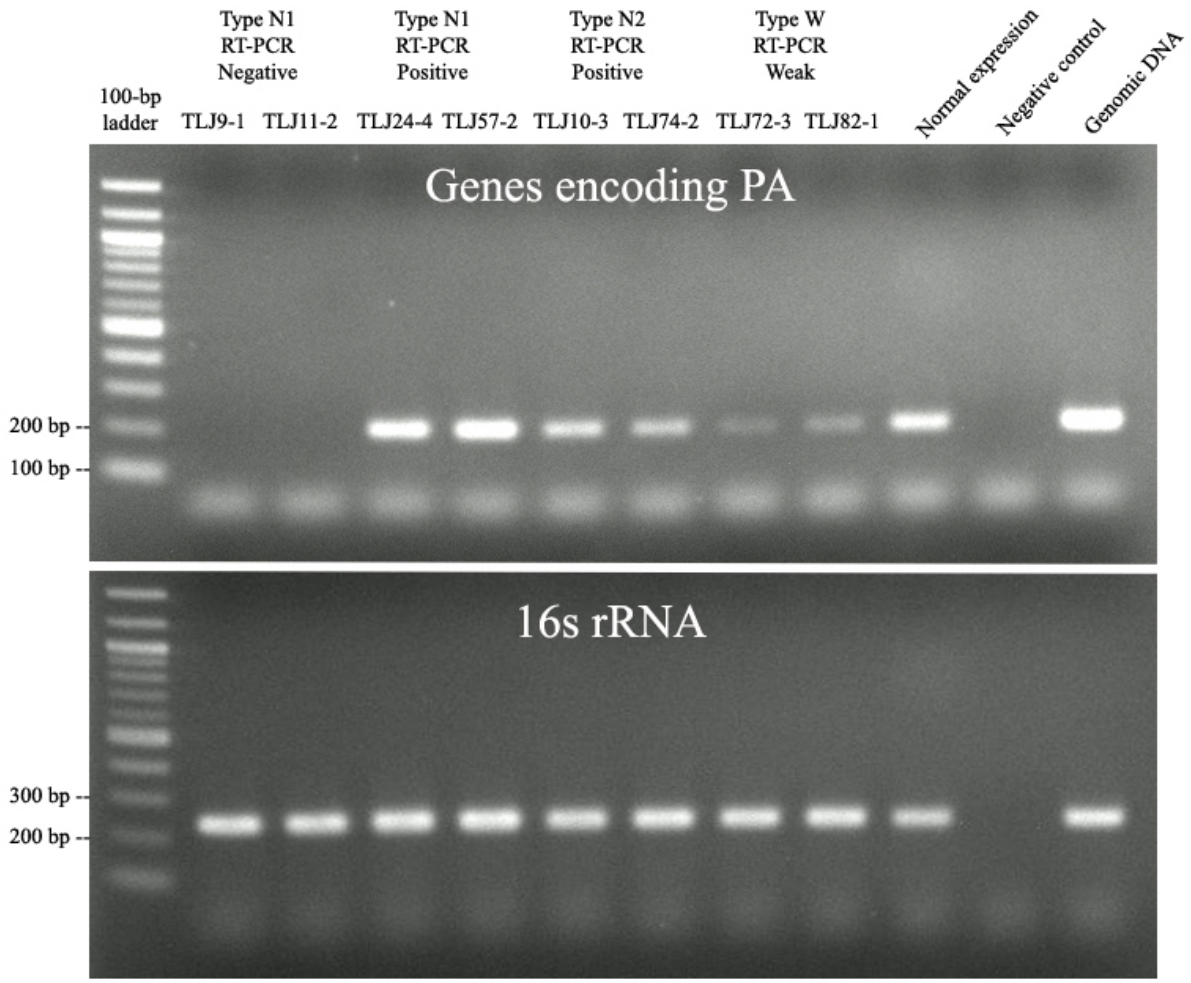

Fig. 3 Representative RT-PCR results of *S. mutans* strains with three types of PA expression defect. Type N1: no PA expression on whole bacterial cells and in their supernatant samples, Type N2: PA expression mainly seen in supernatant samples, and Type W: only low expression of PA found from the samples of whole bacterial cells. *S. mutans* strain MT8148 with normal PA expression was used as the positive control. Genomic DNA of a representative PA defect strain was also demonstrated.

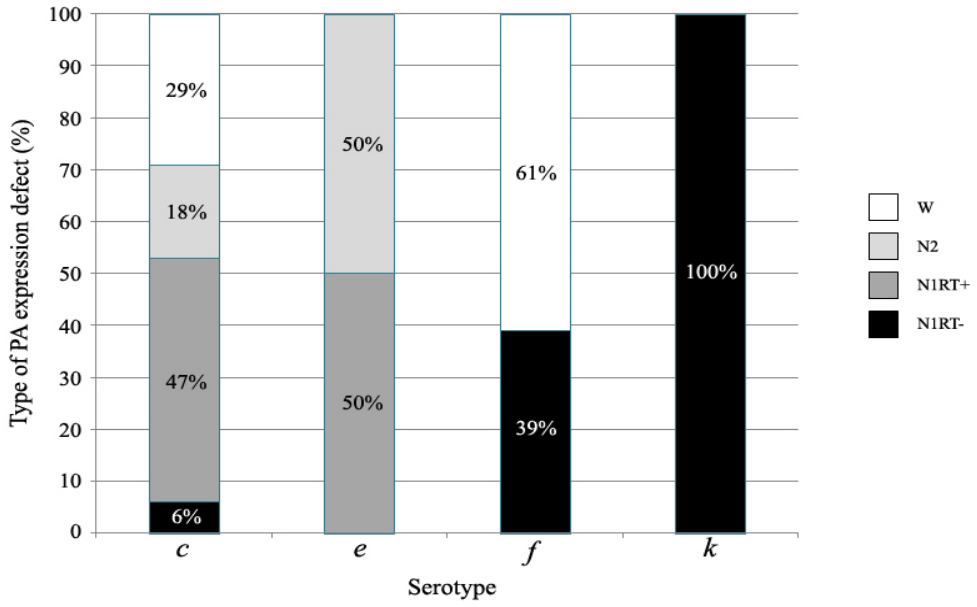

Fig. 4 Type distribution of PA expression defect in Thai *S. mutans* isolates based on serotypes. N1RT-: Type N1 strains showing negative mRNA expression of gene encoding PA by RT-PCR technique, N1RT+: Type N1 strains showing positive mRNA expression of gene encoding PA by RT-PCR technique, N2: Type N2 strains, and W: Type W strains.

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Output จากโครงการวิจัยที่ได้รับทุนจาก สกอ. และ สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

ยังไม่มี

2. การนำผลงานวิจัยไปใช้ประโยชน์

ยังไม่มี

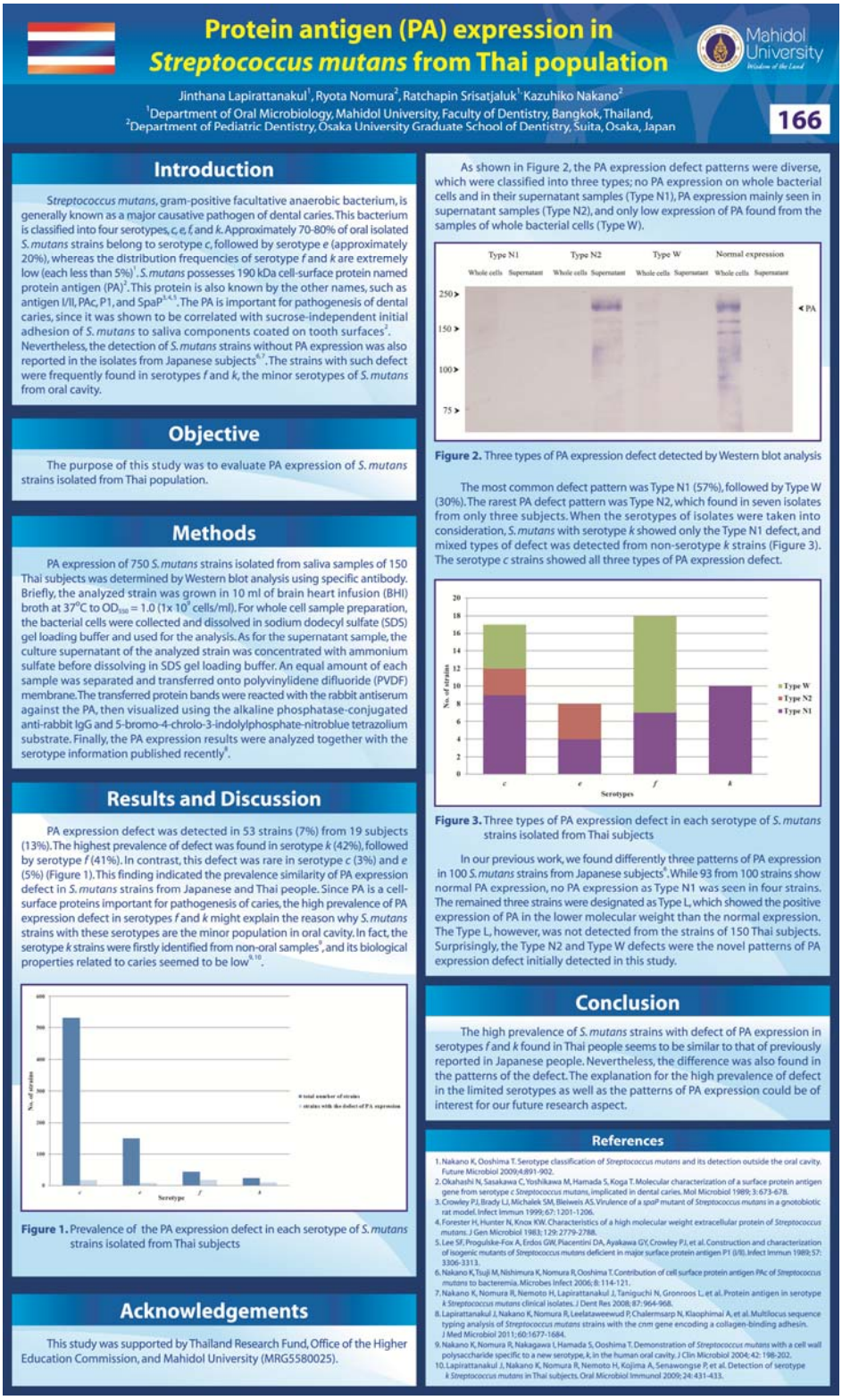
3. อื่นๆ (การเสนอผลงานในที่ประชุมวิชาการ)

3.1 Poster presentation ในชื่อเรื่อง “Protein antigen (PA) expression in *Streptococcus mutans* from Thai population” ในงานประชุม International Association for Dental Research - Asia Pacific Region (IADR-APR 2013) ในระหว่างวันที่ 21 – 23 สิงหาคม พศ. 2556

3.2 ได้รับการตอบรับให้นำเสนอผลงานในรูปแบบ Oral presentation ในชื่อเรื่อง “Variation of Protein antigen expression defects in *Streptococcus mutans* isolated from Thai population" ในงานประชุม The 12th Research Conference of the Dental Faculty Consortium of Thailand (DFCT 2014) ในระหว่างวันที่ 1 – 3 กรกฎาคม พศ. 2557

ภาคผนวก

1. Poster ที่ใช้ในการนำเสนอผลงาน เรื่อง “Protein antigen (PA) expression in *Streptococcus mutans* from Thai population” ในงานประชุม International Association for Dental Research - Asia Pacific Region (IADR-APR 2013) ในระหว่างวันที่ 21 – 23 สิงหาคม พศ. 2556



Protein antigen (PA) expression in *Streptococcus mutans* from Thai population

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166

Introduction

Streptococcus mutans, gram-positive facultative anaerobic bacterium, is generally known as a major causative pathogen of dental caries. This bacterium is classified into four serotypes, *c*, *e*, *f*, and *k*. Approximately 70-80% of oral isolated *S. mutans* strains belong to serotype *c*, followed by serotype *e* (approximately 20%), whereas the distribution frequencies of serotype *f* and *k* are extremely low (each less than 5%)[1]. *S. mutans* possesses 190 kDa cell-surface protein named protein antigen (PA)[2]. This protein is also known by the other names, such as antigen I/II, PAc, P1, and SpaP[3,4,5]. The PA is important for pathogenesis of dental caries, since it was shown to be correlated with sucrose-independent initial adhesion of *S. mutans* to saliva components coated on tooth surfaces[2]. Nevertheless, the detection of *S. mutans* strains without PA expression was also reported in the isolates from Japanese subjects[6,7]. The strains with such defect were frequently found in serotypes *f* and *k*, the minor serotypes of *S. mutans* from oral cavity.

Objective

The purpose of this study was to evaluate PA expression of *S. mutans* strains isolated from Thai population.

Methods

PA expression of 750 *S. mutans* strains isolated from saliva samples of 150 Thai subjects was determined by Western blot analysis using specific antibody. Briefly, the analyzed strain was grown in 10 ml of brain heart infusion (BHI) broth at 37°C to OD_{550} = 1.0 (1x 10^9 cells/ml). For whole cell sample preparation, the bacterial cells were collected and dissolved in sodium dodecyl sulfate (SDS) gel loading buffer and used for the analysis. As for the supernatant sample, the culture supernatant of the analyzed strain was concentrated with ammonium sulfate before dissolving in SDS gel loading buffer. An equal amount of each sample was separated and transferred onto polyvinylidene difluoride (PVDF) membrane. The transferred protein bands were reacted with the rabbit antiserum against the PA, then visualized using the alkaline phosphatase-conjugated anti-rabbit IgG and 5-bromo-4-chrolo-3-indolylphosphate-nitroblue tetrazolium substrate. Finally, the PA expression results were analyzed together with the serotype information published recently[8].

Results and Discussion

PA expression defect was detected in 53 strains (7%) from 19 subjects (13%). The highest prevalence of defect was found in serotype *k* (42%), followed by serotype *f* (41%). In contrast, this defect was rare in serotype *c* (3%) and *e* (5%) (Figure 1). This finding indicated the prevalence similarity of PA expression defect in *S. mutans* strains from Japanese and Thai people. Since PA is a cell-surface proteins important for pathogenesis of caries, the high prevalence of PA expression defect in serotypes *f* and *k* might explain the reason why *S. mutans* strains with these serotypes are the minor population in oral cavity. In fact, the serotype *k* strains were firstly identified from non-oral samples[8], and its biological properties related to caries seemed to be low[9,10].

Figure 1. Prevalence of the PA expression defect in each serotype of *S. mutans* strains isolated from Thai subjects

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As shown in Figure 2, the PA expression defect patterns were diverse, which were classified into three types; no PA expression on whole bacterial cells and in their supernatant samples (Type N1), PA expression mainly seen in supernatant samples (Type N2), and only low expression of PA found from the samples of whole bacterial cells (Type W).

Figure 2. Three types of PA expression defect detected by Western blot analysis

The most common defect pattern was Type N1 (57%), followed by Type W (30%). The rarest PA defect pattern was Type N2, which found in seven isolates from only three subjects. When the serotypes of isolates were taken into consideration, *S. mutans* with serotype *k* showed only the Type N1 defect, and mixed types of defect was detected from non-serotype *k* strains (Figure 3). The serotype *c* strains showed all three types of PA expression defect.

Figure 3. Three types of PA expression defect in each serotype of *S. mutans* strains isolated from Thai subjects

In our previous work, we found differently three patterns of PA expression in 100 *S. mutans* strains from Japanese subjects[8]. While 93 from 100 strains show normal PA expression, no PA expression as Type N1 was seen in four strains. The remained three strains were designated as Type L, which showed the positive expression of PA in the lower molecular weight than the normal expression. The Type L, however, was not detected from the strains of 150 Thai subjects. Surprisingly, the Type N2 and Type W defects were the novel patterns of PA expression defect initially detected in this study.

Conclusion

The high prevalence of *S. mutans* strains with defect of PA expression in serotypes *f* and *k* found in Thai people seems to be similar to that of previously reported in Japanese people. Nevertheless, the difference was also found in the patterns of the defect. The explanation for the high prevalence of defect in the limited serotypes as well as the patterns of PA expression could be of interest for our future research aspect.

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