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Comprehensive characterization of sortase A-dependent surface proteins in *Streptococcus mutans*

[Cell surface protein in *S. mutans*.]

Specified field: bacteriology, pathogenesis

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Conflict of interest

The authors declare no conflicts of interest associated with this manuscript.

Abstract (232 words)

Streptococcus mutans, a cariogenic pathogen, adheres to the tooth surface and forms a biofilm. Bacterial cell surface proteins are associated with adherence to substrates. Sortase A (SrtA) mediates the localization of proteins with an LPXTG motif-containing proteins to the cell surface by covalent binding to peptidoglycan. In *S. mutans* UA159, 6 SrtA-dependent proteins, SpaP, WapA, WapE, DexA, FruA, and GbpC, were identified. Although some of these proteins were characterized, a comprehensive analysis of the 6 proteins has not been reported. In this study, we constructed mutants deficient in each of these proteins and the SrtA-deficient mutant. The SrtA-deficient mutant showed drastically decreased binding to salivary components, biofilm formation, bacterial coaggregation activity, hydrophobicity, and cellular matrix binding (collagen type I, fibronectin, and laminin). The SpaP-deficient mutant showed significantly reduced binding to salivary components and partially increased coaggregation with *Porphyromonas gingivalis*, and decreased hydrophobicity, and collagen binding. The WapA-deficient mutant showed slightly decreased coaggregation with *Fusobacterium nucleatum*. Although the SrtA-deficient mutant showed drastically altered phenotypes, all SrtA-dependent protein-deficient mutants, except the SpaP-deficient mutant, did not show considerable alterations in binding to salivary components. These results indicate that the 6 proteins may coordinately contribute to these activities. In addition, using genomic data of 125 *S. mutans* strains, we compared the amino acid sequences of each surface protein and found many variations among strains, which may affect the

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phenotype of cell surface proteins in *S. mutans*.

List of abbreviations

BHI; brain heart infusion

CFU; colony-forming units

DexA; dextranase

Emr; erythromycin-resistance cassette

FruA; fructanase

GbpC; glucan-binding protein C

Gbps; glucan-binding proteins

OD; optical density

PAc; protein antigen serotype C

PBS; phosphate buffered saline

PCR; polymerase chain reaction

Spcr; spectinomycin-resistance cassette

SrtA; sortase A

TSA; trypticase soy agar

TSB; trypticase soy broth

WapA; wall-associated serotype A

WapE; wall-associated serotype E

Key words

bacterial components, bacteriology, pathogenesis, *Streptococcus mutans*, surface protein

Introduction

Streptococcus mutans is a facultative anaerobic Gram-positive bacterium and is

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known to be a cariogenic pathogen in humans (1, 2). The cariogenicity of *S. mutans* is associated with several virulence factors. This organism produces glucosyltransferases that mediate the synthesis of glucans by metabolizing sucrose as the substrate (1, 2, 3). These glucans are crucial for dental plaque (biofilm) formation. Another factor is that *S. mutans* metabolizes sugars to produce acids (mainly lactic acids), which demineralize calcium phosphate and cause tooth decay (3, 4). In addition, protein antigen serotype C (5) (PAc also named AgI/II (6), SpaP (7), and P1 (8)) form a fibril-like structure to mediate adhesion to pellicles on the cell surface (3, 4, 9).

Adherence of a bacterium to tissues or cells is the first requirement for colonization and infection of the host. In Gram-positive and Gram-negative bacteria, cell surface molecules, including proteins, sugar-phosphate polymers such as teichoic acids, and lipopolysaccharides, are mainly associated with adherence to the substrate (10-14). In Gram-positive bacteria, several cell surface proteins are anchored to cell wall peptidoglycan. Sortase is a membrane transpeptidase that mediates the covalent link of protein with a sorting signal, the LPXTG motif, to the cell wall peptidoglycan in Gram-positive bacteria (15-17). Additionally, many studies have reported that the lack of sortase significantly decreases virulence (18-21). In *S. mutans*, sortase A (SrtA) has been demonstrated to have this transpeptidase activity (22, 23). In genomic analysis of the *S. mutans* UA159 strain, 6 proteins, SpaP, wall-associated protein A (WapA), wall-associated protein E (WapE), fructanase (FruA), dextranase (DexA), and glucan-binding protein C (GbpC), were identified as SrtA-dependent proteins (24). Although studies of some proteins, especially SpaP, have been performed, comprehensive analysis of these

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cell surface proteins has not been reported. It is important to compare several characters including the binding to salivary components, hydrophobicity of cell surface, and biofilm formation among 6 cell surface-deficient mutants. Therefore, we tried to construct mutants deficient in each cell surface protein together with a SrtA-deleted mutant and then compared the phenotypes among these strains.

Materials and Methods

Bacterial strains and growth conditions. *S. mutans* strains, including UA159 (25) and its mutants, *S. sanguins* GTC217, and *S. salivarius* GTC215, were grown in trypticase soy broth (TSB) (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at 37 ℃ under 5% CO_2. *Porphyromonas gingivalis* W83 and *Prevotella intermedia* SP4 were anaerobically grown in brain heart infusion (BHI) broth (Becton, Dickinson and Company) supplemented with hemin (5 μg/ml) and menadione (1 μg/ml) at 37 ℃. *Fusobacterium nucleatum* ATCC25586 was grown in BHI broth at 37 ℃ anaerobically, and *Aggregatibacter actinomycetemcomitans* HK1651 was grown in TSB containing 5% yeast extract at 37 ℃ under 5% CO_2. When necessary, erythromycin (10 μg/ml) or spectinomycin (500 μg/ml) was added to the medium. The strains used in this study are listed in Supplemental Table 1.

Expression of surface proteins determined by quantitative PCR. The expression of surface proteins in the UA159 strain was investigated during growth. *S. mutans* UA159 was cultured overnight, and an aliquot (10^8 cells) was inoculated into 5 ml of fresh TSB and grown at 37 ℃ under 5% CO_2. Bacterial cells at various phases were collected, and total RNA was extracted using a FastRNA Pro Blue kit (MP Biomedicals, Solon, OH, USA) according to the manufacturer's protocol. A 1

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μg aliquot of total RNA was reverse-transcribed to cDNA using a first-strand cDNA synthesis kit (Roche, Tokyo, Japan). With cDNA as a template, quantitative PCR was performed using a LightCycler ® 96 (Roche). The expression of the target gene was calculated using the delta-Ct method as the relative expression level to the internal control, the *gyrA* gene. The primers used in this assay are listed in Table S2. Three independent experiments were performed, and the mean ± SD was calculated.

Construction of knock out mutants. Surface protein (*spaP, wapA, wapE, dexA, gbpC,* and *fruA*) and SrtA (*srtA*)-deficient mutants of *S. mutans* were constructed according to the following protocol (Fig. S1). Briefly, for generation of single mutants, two fragments corresponding to approximately 500 bp of the upstream and downstream sequences of this gene were generated by polymerase chain reaction (PCR) using KOD plus (Toyobo, Tokyo, Japan) with primer pairs for upstream fragments and downstream fragments (Table S2). Each of the reverse-up and forwards-down primers incorporated 9-11 bases that were complementary to the erythromycin-resistance cassette (Em^r), which was cloned using pBlueScript-Em (Stratagene, La Jolla, CA) (26). The Em^r gene was amplified by PCR using primers for Em^r. All PCR amplicons were purified using the Qiagen PCR Purification Kit (Qiagen KK, Tokyo, Japan), and the corresponding upstream and downstream amplicons were mixed in a 1:1:1 ratio with the Em^r PCR product. The amplicon mixture was then used as a template for a second PCR using appropriate forward primers for the upstream fragment and reverse primers for the downstream fragment. The resulting PCR products were then transformed into UA159, as described elsewhere (26). For construction a double-deficient mutant, upstream and

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downstream fragments were mixed in a 1:1:1 ratio with the spectinomycin-resistance cassette (Spc^r) PCR product cloned from pBSSK-Spc (26). This mixture was then used as a template for a second PCR using appropriate forward upstream and reverse downstream primers. The resulting PCR products were also transformed into single deficient mutants with Em^r to construct double deficient mutants. The mutation was verified by PCR. Then, we investigated the growth of each mutant. Overnight culture of each strain was inoculated into fresh TSB and incubated at 37 ℃ under 5% CO_2. The optical density (OD) was monitored at 1-hour intervals for 12 hours.

Binding assay of *S. mutans* to saliva-coated disks. Saliva was collected from volunteers (the ethics committee of the Kagoshima University Graduate School of Medical and Dental Sciences No. 701). An equal volume of phosphate buffered saline (PBS) was added and mixed well. After centrifugation at 4,400 rpm for 15 min, the supernatant was sterilized by a membrane filter (pore size: 0.45 μm). The clarified saliva was used for this assay. Overnight culture of the *S. mutans* strain was inoculated into fresh TSB and incubated at 37 ℃ under 5% CO_2. After the OD reached 0.8 to 0.9, the bacterial cells were collected and washed with PBS three times. The bacterial suspension was adjusted to 2 x 10^7 cells/ml with PBS. A cell disk (cell disk LF1: Sumitomo Bakelite Co., Ltd, Tokyo, Japan) was soaked in 500 μl of clarified saliva using a 24-well culture plate at 4 ℃ for 24 h. After three washes with PBS, a bacterial suspension (10^7 cells/500 μl) was added and incubated at 37 ℃ under 5% CO_2 for 1 h. The bacterial suspension was subsequently removed, and the cell disk was washed with PBS three times. Next, the disk was transferred into a tube with 1 ml of PBS containing trypsin (100

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μg/ml) and left for 15 min at room temperature. After the suspension, the bacterial suspension was diluted, and the appropriate dilutions (100 μl) were plated on trypticase soy agar (TSA) plates. After 2 days of incubation at 37 ℃ under 5% CO_2, the colony-forming units (CFUs) were counted. More than three independent experiments were performed, and the mean ± SD was calculated. The data were analysed for statistically significant differences compared to wild-type UA159 by Welch's *t*-test or one-way ANOVA followed by Dunnett's post hoc test.

Salivary aggregation assay. Salivary aggregation assay was performed by the method described elsewhere (27). Overnight culture of *S. mutans* strains was used. Bacterial cells were collected and washed with aggregation buffer twice. Aggregation buffer components were as follows: 1.5 mM KH_2PO_4, 6.5 mM Na_2HPO_4, 2.7 mM KCl, and 137 mM NaCl (pH 7.2). Then, the bacterial suspension was adjusted to OD_{660}=1.0 with aggregation buffer. Clarified saliva was prepared by the method described above. The bacterial suspension (1 ml) was mixed with 100 μl of saliva and incubated at room temperature. The OD at 550 nm of the bacterial suspension was measured at 30 min intervals for 2 hours. Three independent experiments were performed, and the mean ± SD was calculated. The data were analysed for statistically significant differences compared to wild-type UA159 by one-way ANOVA followed by Dunnett's post hoc test.

Coaggregation assay. Coaggregation assay was performed with the modified method described elsewhere (28, 29). *S. mutans,* including the wild-type and its mutants, *P. gingivalis*, *P. intermedia*, *F. nucleatum, S. sanguins, S. salivarius* and *A. actinomycetemcomitans* were used for this assay. Preliminarily, the coaggregation of *S. mutans* UA159 with other oral bacteria was investigated. Each bacterium was

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grown in liquid culture under appropriate conditions. Bacterial cells were collected and washed twice with aggregation buffer. The bacterial suspension was adjusted to OD_{660}=1.0 with aggregation buffer. Equal volumes (500 μl) of two bacterial suspensions were mixed and incubated at room temperature. The OD at 550 nm of the bacterial suspension was measured at 1-hour intervals for 3 hours. We performed the coaggregation assay using *S. mutans* and its mutants with three independent experiments, and the mean ± SD was calculated. The data were analysed for statistically significant differences compared to wild-type UA159 by one-way ANOVA followed by Dunnett's post hoc test.

Biofilm formation. Biofilm formation assay was performed with the method described elsewhere (30). An overnight culture of *S. mutans* strains was adjusted to an OD_{660} of 1.0, and the bacterial culture was diluted 100-fold (10^7 cells/ml). Ten microlitres of bacterial culture was added to each well containing 100 μl of TSB with 1% sucrose in a 96-well plate. The plates were incubated at 37 ℃ with 5% CO_2 for 24 h. The medium was removed, and the wells were washed with distilled water three times. Finally, biofilm cells were stained with 0.1% crystal violet for 10 min. After three washes with distilled water, 200 μl of 30% acetic acid was added and left for 10 min. The acetic acid solutions were transferred to a new 96-well plate. Biofilm quantification was performed to evaluate the absorbance of each well at 595 nm. Five independent experiments were performed, and the mean ± SD was calculated. The data were analysed for statistically significant differences compared to wild-type UA159 by one-way ANOVA followed by Dunnett's post hoc test.

Hydrophobicity assay. Hydrophobicity assay was performed with the method

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described elsewhere (31). Overnight cultures (5 ml) of *S. mutans* strains were collected and washed with PBS buffer. The bacterial cells were suspended in PBS to OD_{600}=0.8. The bacterial suspension (2 ml) was mixed with hexadecane (0.7 ml). After 1 min of vigorous suspension, the mixture was allowed to stand for 10 min to observe the separation of phases. The OD_{595} of the lower, aqueous phase was measured. Adsorption to the hexadecane phase was calculated as the percentage loss in OD relative to that without hexadecane. More than five independent experiments were performed, and the mean ± SD was calculated. The data were analysed for statistically significant differences compared to wild-type UA159 by Welch's *t*-test or one-way ANOVA followed by Dunnett's post hoc test.

Binding to cellular matrix. An overnight culture of *S. mutans* was pelleted via centrifugation (3000 *g* for 10 min). The pellet was then washed and resuspended in PBS. The bacterial suspension was adjusted to 10^8 bacterial cells/ml, and 100 μl of the suspension (10^7 cells for the adhesin assay with ECM-precoated plates) was applied to ECM-precoated 96-well plates (type I collagen, #356407; laminin, #354410; fibronectin, #354409; all from Corning, NY, USA). For the adhesion assays, cells cultured in 96-well plates were washed three times with PBS, after which 0.25% trypsin was added to remove the adherent bacterial cells from the plastic dish. After addition of 100 μl of PBS, the bacterial suspension was diluted appropriately, plated on TSA, and incubated for 3 days at 37 ℃ under 5% CO_2. Colonies were counted as adhesive bacterial cells. Three independent experiments were performed, and the mean ± SD was calculated. The data were analysed for statistically significant differences compared to wild-type UA159 by student's *t*-test or one-way ANOVA followed by Dunnett's post hoc test.

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Genetic analysis. Whole-genome sequences of 125 *S. mutans* strains were generated with the Illumina MiSeq instrument as previously described (32). The nucleotide sequences of *spaP, wapA, wapE, dexA, gbpC,* and *fruA* of each strain were identified using those of UA159 as references. The amino acid sequences of the same protein among strains were aligned, and phylogenetic trees were generated with the maximum likelihood method based on the JTT matrix-based model, and the bootstrap support values were calculated from 100 replicates using MEGAX (33). Each phylogenetic tree was further classified into clusters with a cutoff threshold of 0.005. One representative amino acid sequence of each cluster was selected and aligned with those from the other clusters of the same protein using the Muscle algorithm in MEGAX, and then multiple sequence alignments were edited in Jalview v2.10.5 (34).

Results

Expression of surface proteins during growth.

We first investigated the growth of UA159 wild type strain and its mutants and found that the growth curve was almost similar among strains (Fig. S2). The expression of six surface-protein genes was evaluated at the exponential phase, stationary phase, and late-stationary phase (16 hours). The data indicated that *wpaA* expression was the most highly expressed in all phases (Fig. 1). A comparison of the gene expression in each phase showed that the expression level was different among strains. Briefly, the expression of *wapA* tended to increase in the late phase, while *dexA* and *srtA* expression was higher in the late-stationary phase than in the mid-exponential phase. In contrast, the expression of *spaP* and *wapE* tended to decrease in the late exponential phase.

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Binding of wild-type and mutants to saliva-coated disks. Using single mutants, we investigated the binding affinity of *S. mutans* cells to saliva. The binding activity of the *srtA*-deficient mutant was significantly reduced (93.5% reduction) compared to that of the wild-type (Fig. 2a). Among six single surface protein-deficient mutants, the binding activity of the *spaP*-deficient mutant was significantly reduced (93.3%) (Fig. 2a). Additionally, *gbpC*-, *wapE*-, *dexA*-, and *fruA*-single deficient mutants showed 54.4%, 45.1%, 28.1%, and 23.9% reductions, respectively, while *wapA*-deficient mutants showed a similar binding activity to that of the wild-type (Fig. 2a). We next investigated the binding activity using double gene-deficient mutants (Fig. 2b). The single *spaP*- and *gbpC*-deficient mutants exhibited 90.2% and 41.6% reductions, respectively, while the *spaP* and *gbpC* double-deficient mutant exhibited 96.9% reduction.

Salivary aggregation of wild-type and mutants. After identifying the contribution of cell surface proteins to saliva-coated disks, we investigated the salivary aggregation of the wild-type and its mutants. After 2 h of incubation, the OD of the wild-type exhibited a 62.4% reduction, while the *srtA*-deficient mutant exhibited only a 4% reduction (Fig. 3). Among the six surface protein-deficient mutants, salivary aggregation was inhibited only by *spaP* deficiency (10.9% reduction).

Coaggregation activity of wild-type and mutants. Preliminarily, the coaggregation activity of wild-type *S. mutans* with several oral bacteria, including *F. nucleatum*, *P. gingivalis*, *P. intermedia*, *S. sanguins*, *S. salivarius*, and *A. actinomycetemcomitans*, was evaluated. *S. mutans* strongly coaggregated with *F. nucleatum* and weakly coaggregated with *P. gingivalis*, while *S. mutans* did not

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coaggregate with other bacteria (Fig. S3). Then, we investigated the coaggregation activity of the wild-type and its mutants (Fig. 4). For coaggregation with *F. nucleatum*, the *srtA*-deficient mutant strongly reduced the coaggregation activity, showing a 35.6% reduction (3 h), while the wild-type showed an 85.4% reduction (3 h). The *wapA*-deficient mutant displayed reduced activity, showing a 73.8% reduction (3 h). The *spaP*-deficient mutant showed a 43.2% reduction (1 h), while the wild-type showed a 67.6% reduction (1 h). After 3 h, the reduction rate of the *spaP*-deficient mutant was similar to that of the wild-type. However, other mutants with a single surface protein deficiency did not show a reduction in coaggregation activity. For coaggregation with *P. gingivalis*, the *srtA*-deficient mutant displayed strongly reduced coaggregation activity, showing a 10.5% reduction (3 h), while the wild-type showed a 32.8% reduction (3 h). The *wapE*- and *spaP*-deficient mutants showed increased activity of 41.3% and 42.8% (3 h), respectively.

Biofilm formation. Compared to the wild-type, the *srtA*-deficient mutant displayed significantly reduced biofilm formation, showing a 96.1% reduction (Fig. S4). However, each protein-deficient mutant showed the same biofilm formation as the wild-type.

Hydrophobicity. For the wild-type UA159 strain, 69.2% of bacterial cells moved to the hexadecane phase (Fig. 5a). The *srtA*-deficient mutant lost its hydrophobicity, and all bacterial cells did not move to the hexadecane phase. The proportions of hydrophobicity of the *spaP*- and *gbpC*-deficient mutants were decreased to 43.8% and 59.1%, respectively. We next investigated the hydrophobicity using double gene-deficient mutants (Fig. 5b). The single *spaP*- and *gbpC*- deficient mutants decreased to 31.1% and 55.1%, while the *spaP* and

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gbpC double-deficient mutant exhibited 25.5%.

Binding of wild-type and mutants to the cellular matrix. Binding activity to collagen type I, fibronectin, and laminin was evaluated. The binding of the *srtA*-deficient mutant to collagen type I, fibronectin, and laminin was significantly reduced compared to that of the wild-type (26.6%, 40.1%, and 33.4%, respectively, Fig. 6). Regarding the binding to individual cellular matrices among surface protein-encoding gene-deficient mutants, in terms of binding to type I collagen, only the *spaP*-deficient mutant showed a significant decrease in binding capacity.

Comparison of the genes coding for surface proteins among 125 *S. mutans* strains. Amino acid sequences of each protein varied among strains, as displayed in the phylogenetic trees. All proteins except WapA were divided into several clusters. The numbers of clusters in SpaP, WapE, GbpC, FruA, and DexA were 5, 17, 3, 8, and 29, respectively, by the cutoff threshold of 0.005 (Fig. S5).

Next, we compared the amino acid sequence of each surface protein from the strains that were randomly selected in each cluster. Regarding SpaP, 5 strains were selected from 5 clusters, and their amino acid sequences were aligned (Fig. 7a). Amino acid alterations were frequently found in the variable region (V). The alanine-rich region (A) was previously reported to be involved in the binding of collagen type I and salivary glycoproteins (35). This region was well conserved among strains, and only two different amino acids were found in 2 strains. In the cell wall anchoring region (CWA), two different amino acids in one strain were observed. In GbpC, the amino acid sequences of 3 strains from 3 clusters were compared (Fig. 7b). Most of the alteration sites were found in the variable region, which was similar to that of SpaP, and the region between the leader peptide and

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variable region. Additionally, one amino acid replacement in LPHTG (changed into LPHAG) was found in UA159. Regarding DexA, the amino acid sequences of 29 strains from 29 clusters were aligned (Fig. 7c). Most of the amino acid variations were present in the leader peptide and two variable regions. In the catalytic region, including the dextran binding domain, several different amino acids could be observed. However, the catalytic sites between the 430F and 441N amino acid residues with the FDGWQGDTIGDN sequence (36) were conserved among the strains. In WapE and FruA, there were multiple variations among strains selected from each cluster (Fig. S6), although their functional regions were not well elucidated.

Discussion

In this study, we comprehensively investigated the phenotype of each surface protein-deficient mutant together with the *srtA*-deficient mutant. In several bacteria, including *S. mutans*, SrtA has been demonstrated to be related to the cell wall peptidoglycan anchorage of cell surface proteins with an LPXTG motif (15, 16). Inactivation of SrtA leads to the loss of correct localization of cell surface proteins on the cell wall (18-21, 37, 38). In *S. mutans*, it was reported that SrtA deficiency resulted in several phenotypic alterations, such as salivary aggregation, biofilm formation, and coaggregation with oral bacteria (19, 22, 39). In this study, we also found that the *srtA*-deficient mutant had decreased saliva-binding activity, salivary aggregation, coaggregation with *F. nucleatum* and *P. gingivalis*, and hydrophobicity. Therefore, SrtA-dependent cell surface proteins are involved in these phenotypes, and it is worth investigating the association of each cell surface protein with these phenotypes comprehensively. In the whole-genome analysis of

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S. mutans UA159, 10 proteins were identified to have the LPXTG motif, and 6 of 10 proteins had this motif at the C-terminus (24). To identify the factors responsible for the activity lost in the *srtA*-deficient mutant, we tried to construct knockout mutants and investigated the association with phenotypic alterations found in the *srtA*-deficient mutant.

Among 6 cell surface-deficient mutants, the *spaP*-deficient mutant showed versatile phenotypic alterations. Consists of 5 distinct regions: the signal peptide at the N-terminus, alanine-rich region (A), variable region (V), proline-rich region (P), C-terminal region (C), and cell wall anchorage region (CWA) (35). Previously, SpaP was demonstrated to be involved in binding to glycoproteins in saliva (35, 40). Amino acid sequences related to binding with glycoproteins were identified as "TELARVQKANADAKAAY (3 times found)" and "TYEAALKQYEADL (4 times found)" (41-43). These sequences were repeatedly found mostly in the collagen I binding region corresponding to the A region. Based on the comparison using the whole-genome sequence of 125 strains, these regions were well conserved, so all *S. mutans* strains analysed in this study may retain these activities. The *spaP*-deficient mutant also showed decreased binding with type I collagen and coaggregation activity with *F. nucleatum*. In *S. gordonii*, SspB, which shows homology with SpaP of *S. mutans*, was demonstrated to bind to *Actinomyces naeslundii* and *P. gingivalis*, and the binding region was reported (44). SpaP also has a region similar to this binding region in SspB so that the binding region to *F. nucleatum* in SpaP may be the same region as SspB. Based on our findings and previous reports, it is well accepted that SpaP has versatile virulence factors that are mainly involved in binding affinity to various substrates.

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For glucan binding proteins (Gbps), 4 Gbps named GbpA-D were reported (45-49). Among the four Gbps, only GbpC has an LPXTG motif for anchoring to the cell wall. Gbps were reported to have dextran binding affinity of the glucan produced by glucosyltransferases of *S. mutans* (45). Therefore, Gbps are believed to contribute to biofilm formation (47, 50). In this study, we did not find a reduction in biofilm formation in the *gbpC*-deficient mutant. This result is due to the involvement of other Gbps in biofilm formation. However, we found that GbpC was slightly involved in binding to salivary components. Mieher JL et al. reported that GbpC has a structural similarity with SpaP, especially the V region and fibrillar stalk (51). Additionally, the binding of GbpC to salivary glycoproteins was demonstrated (51). Some salivary proteins, such as gp340, have sugar chains (52), so that GbpC may interact with the sugar portion of salivary proteins. GbpC has been reported to be involved in dextran-dependent aggregation under stressed conditions such as sub-MIC of tetracycline or 4% ethanol (53, 54). We reconfirmed the dextran-dependent aggregation was lost in the *gbpC*-deficient mutant, but not in other mutants (data not shown). In addition, the *gbpC*-deficient mutant showed reduced hydrophobicity. Hydrophobicity is also considered to be related to binding to the tooth surface.

Regarding other cell wall proteins, including DexA (55-57), FruA (58, 59), WapA (60-63), and WapE (64), there are several reports for each function. Coby SM et al. reported that dextranase A had dextranase activity, which digested water-soluble glucan produced by *S. mutans* but did not affect sucrose-dependent bacterial aggregation (55), while Yang Y et al. demonstrated that a *dexA*-deficient mutant had increased expression of glucan synthesis-related genes, including *gtfB* and

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gtfD, resulting in increased biofilm formation (57). In addition, DexB was demonstrated to have dextranase activity (65, 66). Since we did not show alterations in biofilm formation in the *dexA*-deficient mutant compared to the wild-type, these complex results suggest that the evaluation of biofilm formation activity in the *dexA*-deficient mutant may depend on the strains and culturing conditions. In this study, we evaluated biofilm formation under the sucrose supplemented medium. Glucosyltransferases (GTFs) played a significant role in biofilm formation due to the synthesis of sticky water-insoluble glucan (3, 4). Although cell wall anchoring proteins were considered to be associated with biofilm formation (67), single deletion of cell surface protein did not strongly affect sucrose-dependent biofilm formation in this study. WapA has been reported to have collagen-binding activity (68). Additionally, it was demonstrated that the *wapA*-deficient mutant showed reduced cell chain length and unstructured biofilm architecture (63). In this study, a reduction in collagen binding was not found in the *wapA*-deficient mutant but was found in the *srtA*-deficient mutant, implying that several factors are involved in collagen binding. In other proteins, FruA was demonstrated to have fructanase activity, which digested sucrose and soluble fructan (58, 59), while the function of WapE has not been elucidated. In this study, we did not find a significant difference in phenotype between the wild-type and the mutants, including the *fruA*-, *dexA*-, *wapA*-, and *wapE*-deficient mutants. However, it is interesting to note that the *srtA*-deficient mutant showed drastically reduced biofilm formation, coaggregation activity, binding activity to the cellular matrix, and hydrophobicity, while each gene-deficient mutant did not show a strong alteration in these activities. In particular, the *srtA*-deficient mutant showed

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drastically reduced biofilm formation, while this activity was not affected in each cell surface protein mutant compared to the wild-type. These results imply that the colocalization of multiple cell surface proteins is required to maintain several functions in *S. mutans*. In addition, it was reported that the C5a peptidase of *S. pyogenes* was a cell wall anchoring protein with an LPTTN motif (not LPXTG motif) (69, 70). We checked the protein with the LPTTN motif in *S. mutans* UA159 strain, but we did not find it. However, there is a possibility that other cell wall anchoring proteins possessing LPXTG-like motifs may be involved in some phenotypes.

In phylogenetic analysis, we found many variations in each cell surface protein (Fig. 7 and Fig.S5). It has been reported that SpaP and GbpC have a variable region at which amino acid replacements have occurred among strains. However, except for the variable region, several differences were also found in SpaP and GbpC, including the functional regions. These results indicate that different amino acid sequences among strains may affect the activity of each factor, which may affect *S. mutans* virulence.

In conclusion, we comprehensively investigated the virulence of each surface protein by the construction of gene-deficient mutants. Except for the significant effect of SpaP on salivary binding, drastic phenotypic alterations were not found, while the *srtA*-deficient mutant showed drastic alterations. Therefore, we considered that cell surface proteins may coordinately contribute to several virulence activities in *S. mutans*.

Disclosure

This study has been approved by the ethics committee of the Kagoshima University

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Graduate School of Medical and Dental Sciences (No. 701).

The authors declare no competing interests.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure

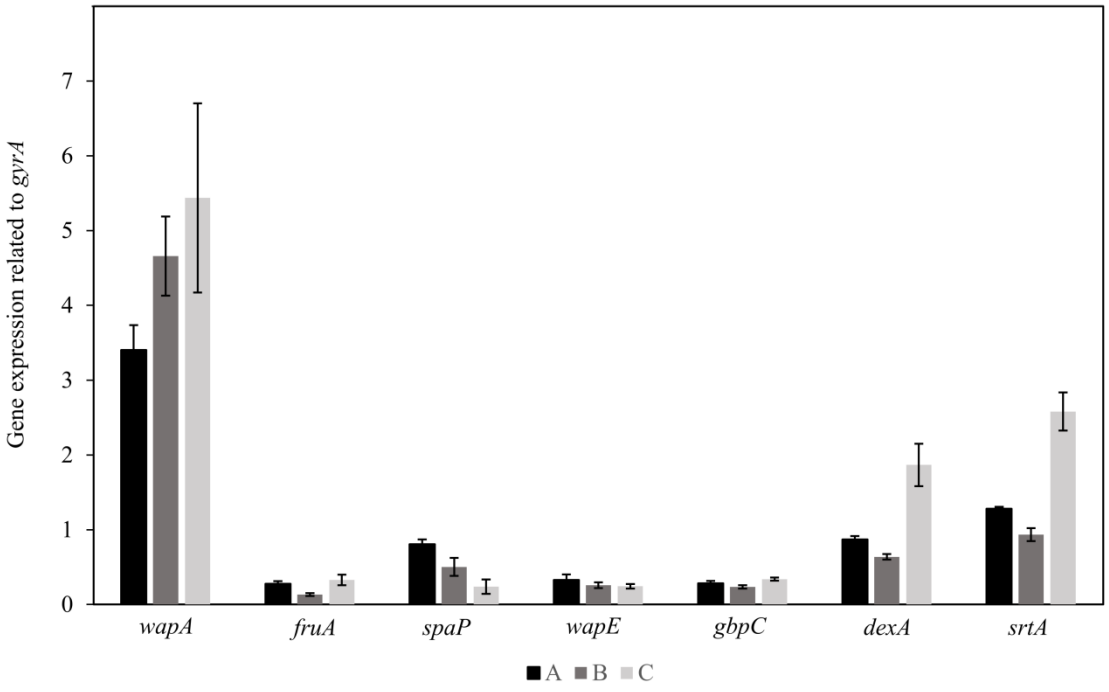

Figure 1. The expression of the genes encoding six surface proteins and sortase A. After aliquots of bacterial cells of UA159 were inoculated into 5 ml of TSB, they were cultured aerobically under 5% CO_2. When the appropriate OD was reached, the bacteria were collected. Next, RNA was extracted and cDNA was synthesized. Gene expression was verified by quantitative PCR. Three independent experiments were performed, and the mean ± SD was calculated. (A): OD_{660}=0.5 (exponential phase), (B): OD_{660}=0.9 (stationary phase), (C): 16 hours- culture (late-stationary

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phase).

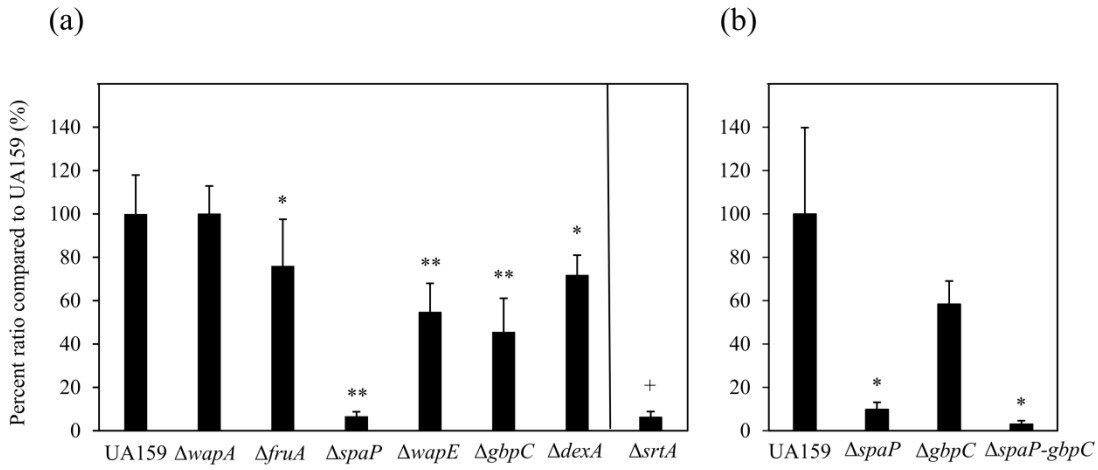

Figure 2. Binding of wild-type *S. mutans* and its mutants to saliva-coated disks.

With single- (a) or double- (b) deficient mutants, the binding affinity of *S. mutans* cells to saliva-coated disks was investigated. Preparation of saliva-coated disks and bacterium was described in the Materials and Methods. Seven (a) and three (b) independent experiments were performed, and the mean ± SD was calculated. The data were analysed for statistically significant differences compared to wild-type UA159 by one-way ANOVA followed by Dunnett's post hoc test (cell surface protein-deficient mutants) or Welch's *t*-test (*srtA*-deficient mutant). *P and $^{+}$P < 0.05, **P < 0.001.

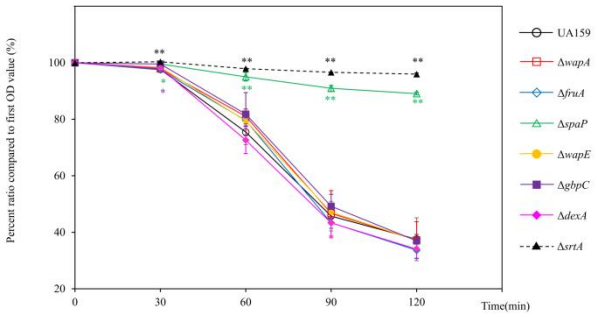

Figure 3. Salivary aggregation of wild-type and mutant *S. mutans*.

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Salivary aggregation of the wild-type and its mutants was investigated for 2 hours at 30 min intervals. Three independent experiments were performed, and the mean ± SD was calculated. The data were analysed for statistically significant differences compared to wild-type UA159 by one-way ANOVA followed by Dunnett's post hoc test. *P<0.05, **P<0.001.

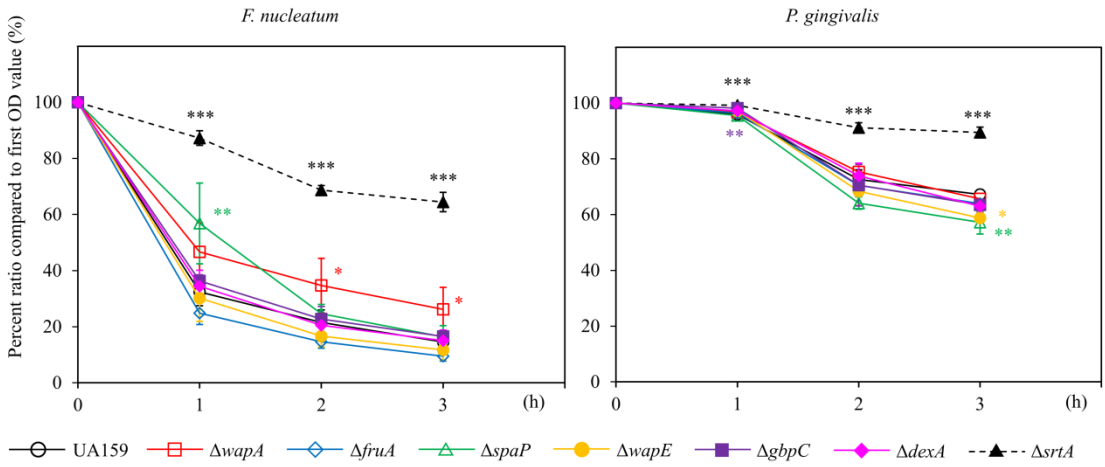

Figure 4. Coaggregation activity of wild-type and mutant *S. mutans*.

Coaggregation of wild-type *S. mutans* and the mutants with *F. nucleatum* and *P. gingivalis* was investigated. Three independent experiments were performed, and the mean ± SD was calculated. The data were analysed for statistically significant differences compared to wild-type UA159 by one-way ANOVA followed by Dunnett's post hoc test. *P<0.05, **P<0.01, ***P<0.001.

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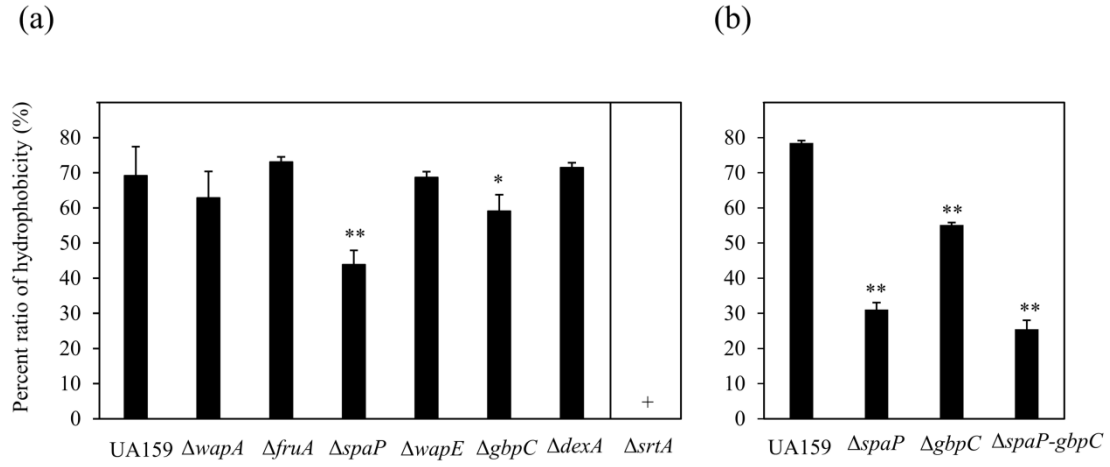

Figure 5. Hydrophobicity.

The hydrophobicity of wild-type and mutant *S. mutans* was investigated. Six (a) and five (b) independent experiments were performed, and the mean ± SD was calculated. The data were analysed for statistically significant differences compared to wild-type UA159 by one-way ANOVA followed by Dunnett's post hoc test (cell surface protein-deficient mutants) or Welch's *t*-test (*srtA*-deficient mutant). *P < 0.05, **P and $^{+}$P < 0.001.

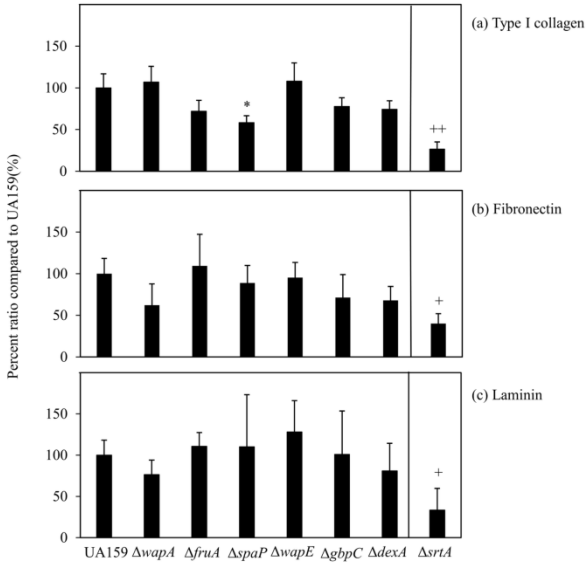

Figure 6. Binding of wild-type and mutant *S. mutans* to the cellular matrix.

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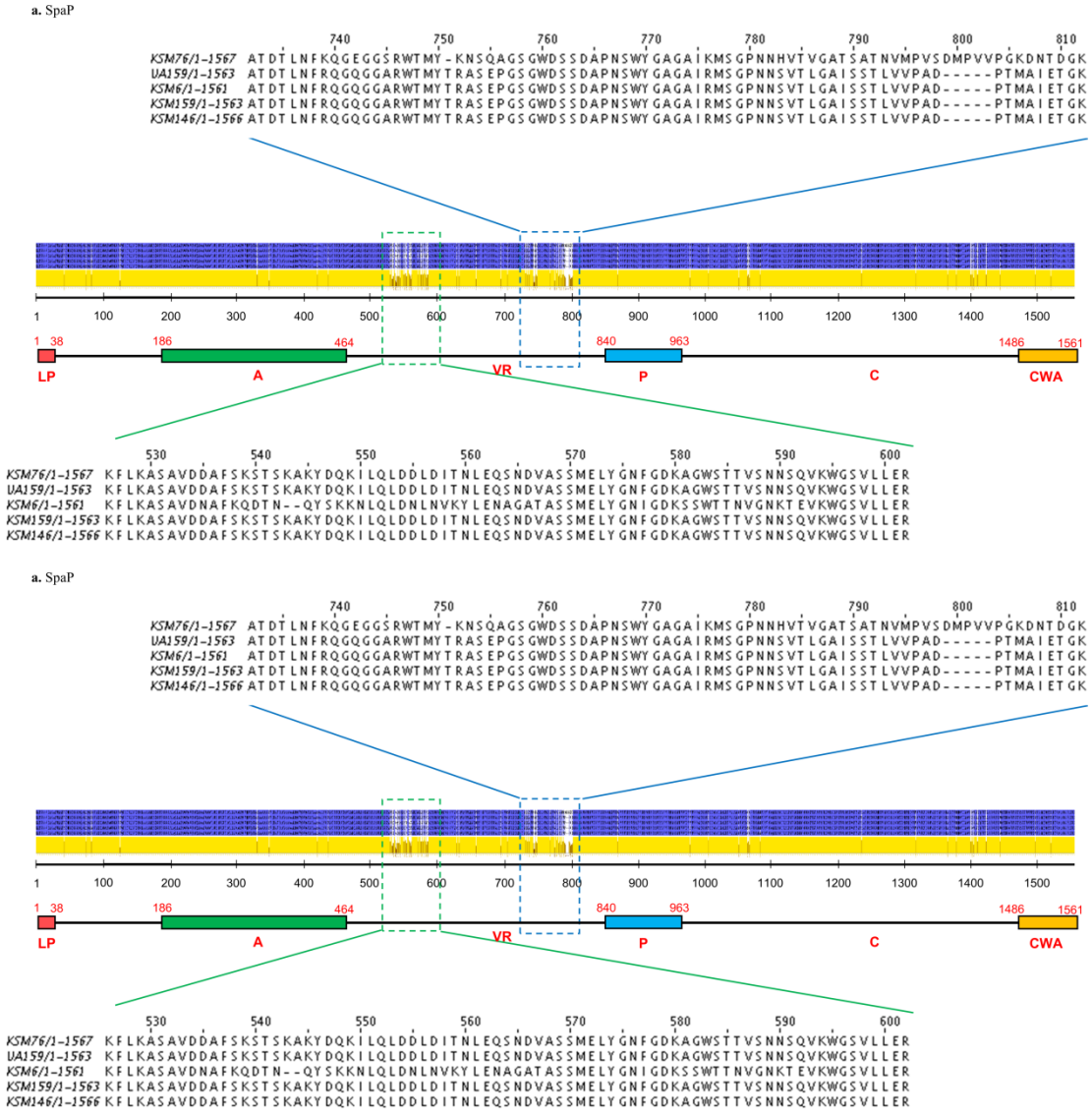
The binding of wild-type *S. mutans* and the mutants to the cellular matrix was investigated. Three independent experiments were performed, and the mean ± SD was calculated. The data were analysed for statistically significant differences compared to wild-type UA159 by one-way ANOVA followed by Dunnett's post hoc test (cell surface protein-deficient mutants) or student *t*-test (*srtA*-deficient mutant). *P and $^{+}P < 0.05$, $^{++}P < 0.005$. (a): Type I collagen, (b): Fibronectin, (c): Laminin.



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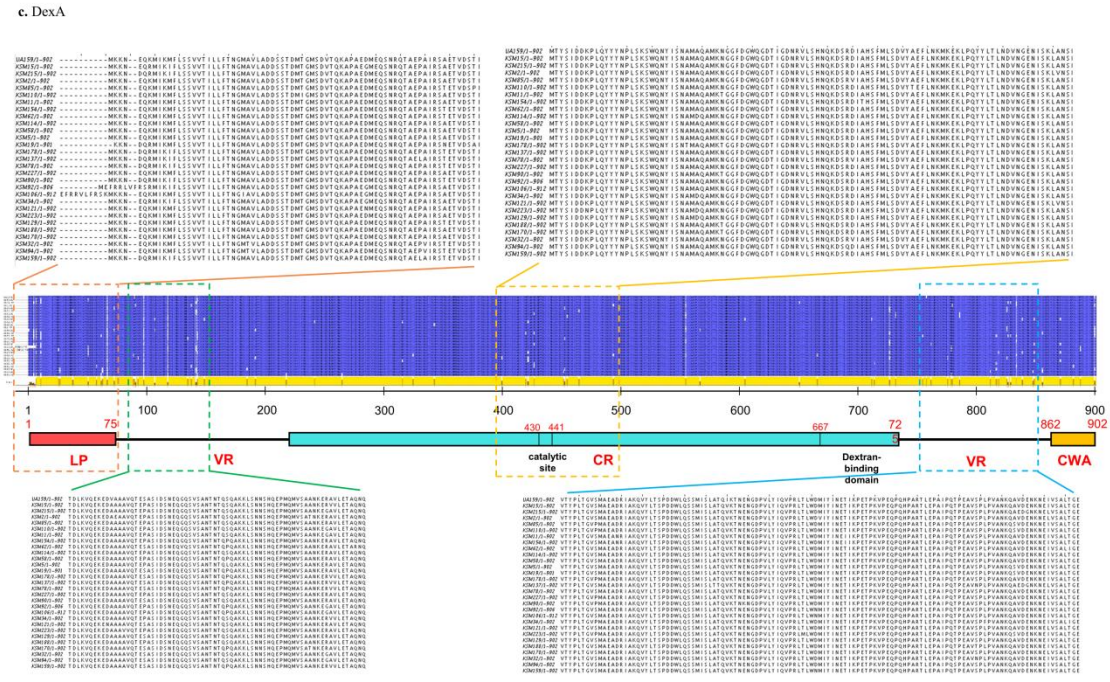

Figure 7. Multiple amino acid sequence alignments of SpaP (a), GbpC (b), and DexA (c).

The representative amino acid sequence of each cluster was selected and compared with those from the other clusters of the same protein. LP, leader peptide; A, Ala-rich repeats; VR, variable region; P, pro-rich repeats; C, C-terminal regions; CWA, cell wall anchorage region; CR, conserved region. Each number corresponds to the position of the residue in the protein sequences.

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