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CHARACTERIZATION OF GLYCOSYLATION-ASSOCIATED PROTEIN, GAP2, REQUIRED FOR THE BIOGENESIS OF *STREPTOCOCCUS PARASANGUINIS* FIMBRIAE-ASSOCIATED PROTEIN 1, FAP1

by

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A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirement of the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2014

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CHARACTERIZATION OF GLYCOSYLATION-ASSOCIATED PROTEIN, GAP2, REQUIRED FOR THE BIOGENESIS OF *STREPTOCOCCUS PARASANGUINIS* FIMBRIAE-ASSOCIATED PROTEIN 1, FAP1

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Microbiology

ABSTRACT

Streptococcal species possess a multitude of adhesins that facilitate adherence to a wide range of substrates; this is the first step necessary for the development of disease. *Streptococcus parasanguinis* is a primary colonizer of the oral cavity and adheres to the tooth surface using long fimbriae, which are composed of Fap1, a serine-rich repeat glycoprotein (SRRP). SRRPs are conserved in many Gram-positive bacteria and play a role in bacterial adhesion, fimbrial formation, biofilm formation, and bacterial pathogenesis. Although SRRPs play such an important role, the exact mechanism of their biogenesis remains a mystery. For Fap1, an eleven gene cluster is required for Fap1 biogenesis. The exact function of the three glycosylation-associated proteins (Gap) within this cluster remains unknown. Mutations of *gap1* or *gap3* produce an immature Fap1, suggesting that these Gap proteins are involved in Fap1 biogenesis. The effect of Gap2 on Fap1 biogenesis and its function is unknown. In this study, we focused on elucidating the function of Gap2 by understanding the interactions between Gap2 and Gap1 and Gap3 and how these interactions affect Fap1 biogenesis. Gap2, like Gap1 and Gap3, is required for production and export of mature Fap1, with direct effects on fimbrial assembly and bacterial adhesion. Gap2 interacts with Gap1 and Gap3 to form a stable protein complex, in which Gap2 can bind in a reversible manner to a tightly formed Gap1/3 complex. Gap2 and Gap1 protect Gap3 from being targeted selectively by ClpE

ATPase to be degraded by ClpP protease. Gap2 is protected by Gap1 from being targeted by ClpC and ClpE ATPases for degradation by ClpP protease. Deletion of *clpP* or *clpE* has no apparent effect on Fap1 biogenesis if any of the three Gap proteins are absent-indicating that all three Gap proteins play a direct role in mature Fap1 biogenesis. Our studies demonstrate that the three Gap proteins work in concert in Fap1 biogenesis and reveal a new function of Gap2. This insight will help us elucidate the molecular mechanism of the biogenesis of SRRPs and can provide us possible drug targets to alter the adhesion of pathogens and thereby prevent disease.

Keywords: Streptococcus, protein-protein interactions, ClpP, adhesin

DEDICATION

I would like to dedicate this dissertation to all those who have helped me pave my path. To my father, who gave me the courage and the initiative to begin this journey. To my mother, who gave me the knowledge and confidence to excel. To my husband, who gave me the support and strength to continue this endeavor. To my mentor, who gave me the wisdom and guidance to finish.

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CHAPTER 1

INTRODUCTION

SERINE-RICH REPEAT PROTEINS

Bacterial adhesion is often the first step required for pathogens to cause disease. Bacteria can adhere to a wide range of substrates, which is mediated by a multitude of adhesins on their cell surface. Thus, understanding the mechanisms involved in the production and maintenance of these adhesins is paramount to understanding bacterial pathogenesis. One type of adhesin is the growing family of serine-rich repeat glycoproteins (SRRPs), which are conserved in many Gram-positive bacteria including streptococci, staphylococci, and lactobacilli (1). The first identified SRRP was fimbriae-associated protein 1 (Fap1), which comprise the fimbriae of *Streptococcus parasanguinis*. Since the discovery of Fap1 (2, 3), other SRRPs have been identified (Fig. 1). These include GspB and Hsa of *Streptococcus gordonii* (4-6), SraP of *Streptococcus sanguinis* (7), PsrP of *Streptococcus pneumoniae* (8), Srr-1 and Srr-2 of *Streptococcus agalactiae* (9, 10), SrpA of *Streptococcus cristatus* (11), SraP of *Staphylococcus aureus* (12), and FimS of *Streptococcus salivarius* (13).

Not only are SRRPs required for bacterial adhesion, but they also play a role in fimbrial formation, biofilm formation, and bacterial pathogenesis (1, 8-10, 12, 14-17). For example, Srr-1 and Srr-2 from *S. agalactiae* are involved in the bacterial pathogenesis of meningitis (18) and neonatal infection (9). PsrP from *S. pneumoniae* is associated with frequency of invasive pneumococcal disease (8). SraP from *S. aureus*

(12) and GspB and Hsa from *S. gordonii* (16, 19, 20) are implicated in the pathogenesis of infective endocarditis. Fap1 and GspB are crucial for the adhesion and biofilm formation of *S. parasanguinis* (14, 17, 21) and *S. gordonii* (22), respectively, to the tooth surface; these bacteria act as a necessary platform for other microbial cells to attach and form a biofilm, including periodontal pathogens such as *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* (23). Thus, understanding the biogenesis of SRRPs will provide insights into the pathogenesis of these species and will have significant implications for the prevention of disease development.

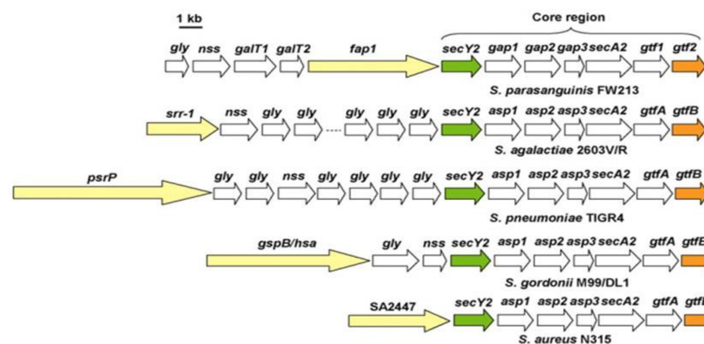

Figure 1. Schematic indicating SRRPs conserved in streptococci and staphylococci. Alignment of selected SRRPs and proteins involved in their biogenesis are depicted. [From "Glycosylation and biogenesis of a family of serine-rich bacterial adhesins" by M. Zhou and H. Wu, 2009, *Microbiology,* 155, p. 317. Adapted with permission].

The SRRP family shares several characteristic regions, which include a long N-terminal signal peptide, a non-repetitive region(s), serine-rich regions, and a C-terminal LPXTG cell wall anchoring domain (1, 6, 22, 24-26); all of these conserved regions are necessary for specifically exporting the SRRP via a specialized system (5, 25-28). For example, Fap1 begins with a 68 amino acid signal sequence at the N-terminus and ends

with a classic (LP(X)TG) cell-wall anchor domain at the C-terminus (3). Notably, Fap1 has a non-repeat region immediately after the signal peptide and is followed by two serine-rich regions that flank a second non-repeat region (Fig. 2). The serine-rich regions contain repeats of SESVSESVSI that constitute 80% of the sequence (1, 28). Similarly, GspB consists of an N-terminal signal peptide, two serine-rich regions, a non-repetitive region between them, and a C-terminal cell wall anchoring domain (5). The NR regions of GspB in *S. gordonii* (16), of SraP in *S. aureus* (12), and of PsrP in *S. pneumoniae* (29, 30) mediate adhesion to platelets and monocytes, to platelets, and to pneumocytes, respectively. Because the NR regions are quite diverse among species, these regions may provide adhesion specificity (1, 31). In both Fap1 (14) and GspB (32), the serine-rich regions are heavily glycosylated; glycosylation of Fap1 (O-linkage to the multitude of serine residues) is involved in fimbrial assembly, bacterial adhesion, biofilm formation, and adherence to the oral cavity (2, 15, 33).

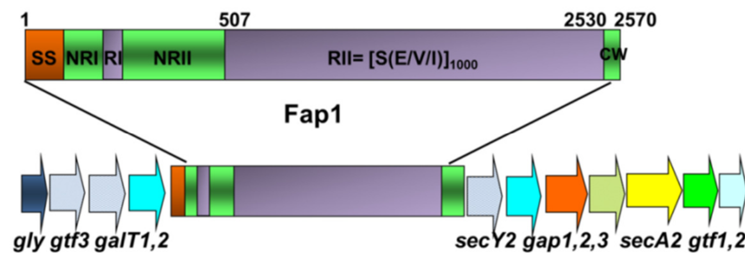

Figure 2. Domains of Fap1, which is surrounded by the loci required for its biogenesis. Fap1 domains include the signal peptide (SS), two non-repetitive regions (NRI and NRII), two serine-rich regions (RI and RII), and a cell wall anchor domain (CW). An eleven gene cluster involved in Fap1 biogenesis is separated into two regions on either side of Fap1: the core region (*secY2*, *gap1-3*, *secA2*, and *gtf1-2*) and the variable region (*gly*, *gtf3*, *galT1*, and *galT2*).

SRRP GLYCOSYLATION

Although SRRPs play such an important role in adhesion and pathogenesis, the exact mechanism of SRRP biogenesis is not well understood. The chromosomal region dedicated to SRRP glycosylation and export is quite large and highly conserved in many streptococci, staphylococci, and even eubacteria (Fig. 1). This dedicated locus is separated into two regions: a core region that is conserved in every genome and is involved in export and a variable region that includes several putative glycosyltransferases.

In *S. parasanguinis*, Fap1 has an eleven gene cluster involved in its biogenesis and is separated into two regions: the core region (*secY2*, *gap1-3*, *secA2*, and *gtf1-2*) and the variable region which includes four glycosyltransferases (*gly*, *gtf3*, *galT1*, and *galT2*) (Fig. 2). *gtf1* and *gtf2* and genes from the *gly-gtf3-galT1*-galT2 locus mediate Fap1 glycosylation (2, 34-38); Fap1 is glycosylated in the cytoplasm with several monosaccharides, including glucose, N-acetyl glucosamine, N-acetyl galactosamine, and rhamnose (14, 37). Gtf1 and Gtf2 form a complex that performs the initial glycosylation of Fap1 by transferring GlcNAc to the unmodified Fap1 (38, 39). This is followed by further sugar modification by Gtf3 (glucose), GalT1 (GlcNAc or glucose), GalT2, and Gly (35, 40). Loss of Gtf1 or Gtf2 results in a high molecular mass (HMM) unglycosylated form of Fap1 (36, 38), which is not detected in the cell wall fraction suggesting it is no longer exported (Fig. 3). Deletion of the other glycosyltransferases results in a partially glycosylated Fap1 (2, 35, 40). This partially glycosylated Fap1 exists as two forms: a smaller improperly glycosylated and a HMM—where the former can be exported while the HMM form is trapped in the cytoplasm (Fig. 3). Thus, only the initial

glycosylation is required for export of Fap1. The other glycosyltransferases alter glycosylation patterns, which can affect biofilm formation (2, 35).

Like Fap1, GspB of *S. gordonii* is glycosylated intracellularly, independently of export, by glycosyltransferases Gly, Nss, GtfA, and GtfB (4, 32). Similar to *gtf1* and *gtf2*, deletion of *gtfA* or *gtfB* results in an unglycosylated HMM form of GspB (4). Deletion of the other glycosyltransferases, including *gly* and *nss*, results in a partially glycosylated GspB, which can still be exported (41). Similarly, in *S. agalactiae*, loss of GtfA or GtfB results in an unglycosylated form of SRR1. Loss of the other glycosyltransferases results in altered glycoforms, which are still exported (42). In *S. aureus*, loss of the GtfA/GtfB complex precludes glycosylation of SraP (43).

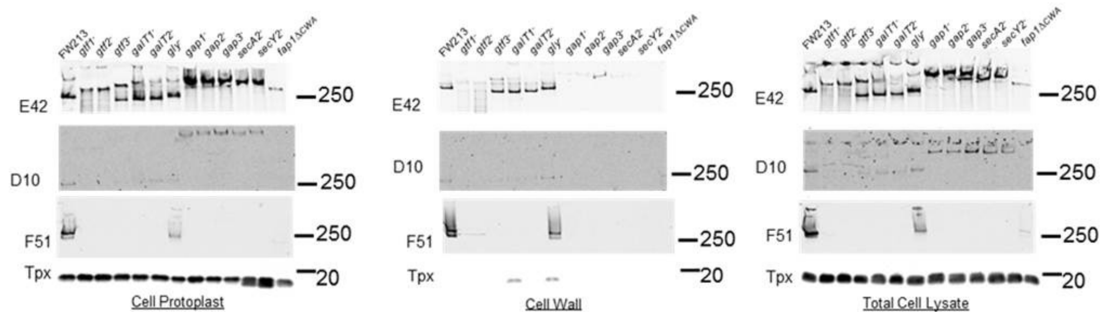

Figure 3. The glycosyltransferases and accessory secretion components are required for Fap1 glycosylation and export. Western blot analysis of total cell lysate and fractions of cell protoplast and cell wall. Strains include wild-type FW213 and mutants of the glycosyltransferases (*gtf1, gtf2, gtf3, galT1, galT2, gly*) and the accessory secretion components (*gap1, gap2, gap3, secA2, secY2*). A strain in which Fap1 lacks the cell wall anchor domain (*fap1Δcwa*) was used as a control. Three Fap1 antibodies that recognize different forms of Fap1 were used—E42 (polypeptide backbone), D10 (specific glycoform), and F51 (mature). Antibody against Tpx (a cytosolic protein) was used as a fractionation control.

Although there is variation in the number and type of glycosyltransferases among different species, the initial glycosylation of the SRRP is required for export in *S. parasanguinis*, S. *gordonii*, *S. agalactiae*, and *S. aureus.* The remaining glycosylation steps are not required for export, but may be necessary for optimal processing of Fap1 as deletion of the remaining glycosyltransferases in *S. parasanguinis* results in two forms of Fap1, only one of which is properly exported; as the steps of Fap1 glycosylation advances, efficiency of processing Fap1 to the mature form increases (Fig. 3). This begs to question what can recognize the various glycosylated forms of SRRPs to regulate proper export. A better understanding of the proteins involved in SRRP export will provide insight into these mechanisms.

SRRP EXPORT

SecA2/SecY2 system

As an adhesin, SRRPs must be exported to the cell surface. In species that have SRRPs, a conserved locus is associated with the export of the SRRP. This locus consists of several genes, including *secA2*, *secY2*, *asp1*, *asp2*, and *asp3* (1). Although the exact role these components play in the export of SRRPs is unknown, several key features have been elucidated.

SecA2 and SecY2 share homology with their counterparts in the canonical Sec system and most likely function in a similar manner. SecA2 is an ATPase that shares structural features with SecA, including the DEAD-like ATPase motor domain and PPXD and IRA1 domains (44, 45). SecY2 shows strong similarity to the SecY transmembrane protein of the canonical protein secretion system (5, 46). While the SecA

and SecY system are a generalized secretion system, the SecA2/SecY2 system is specialized to secrete only the SRRP just downstream of it (44). Indeed, several SRRPs are exported exclusively by the SecA2/SecY2 system (44), including Fap1 of *S. parasanguinis* (37), GspB of *S. gordonii* (5), Srr1 of *S. agalactiae* (42), and SraP of *S. aureus* (47). Moreover, while the number and position of genes involved in the glycosylation of SRRPs from different species varies, the export components are conserved in number and position relative to each other, suggesting that these components, but not necessarily the glycosyltransferases, are essential for proper SRRP export. Indeed, as mentioned above, export of Fap1 is still possible with some variation in glycosylation patterns. However, deletion of *secA2* or *secY2* all but abolishes export of Fap1 (Fig. 3) (5). Similarly, GspB of *S. gordonii* (5), SRR1 of *S. agalactiae* (42), and SraP of *S. aureus* (47) cannot be detected in the cell wall fraction of *secA2* or *secY2* mutants, suggesting that SecA2 and SecY2 play a vital role in export of the SRRPs.

Accessory Secretion Proteins

Besides SecA2 and SecY2, there is no known genetic homology for the remainder of the locus—the accessory secretion proteins (*asp1-asp2-asp3*)—outside of the SRRP family. Through Phyre analysis, Asp1 shares structural homology with glycosyltransferases (48) and Asp2 has some structural homology with a hydrolase (49). Asp3 is predicted to possess a carbohydrate-binding domain, which may have affinity towards the glycosylated SRRP precursor (48). However, to date, no studies have delineated the true purpose of these three proteins.

Although there are no studies determining the exact function of the Asp proteins, current observations suggest that they are involved in SRRP biogenesis. For example, in *S. parasanguinis*, deletion of the *asp* homologs—*gap1* and *gap3*—results in production of a HMM form of Fap1 (15, 33, 48) similar to that seen in the *secA2* and *secY2* mutants, export of which is greatly reduced (Fig. 3) (49). Moreover, the interaction between Gap1 and Gap3 is required for Fap1 biogenesis, further indicating that Gap1 and Gap3 are involved in mature Fap1 biogenesis (33, 48, 50). Similarly, in *S. gordonii* and *S. aureus*, SRRPs are no longer exported upon loss of Asp1 and Asp3 (41, 47).

The model in Figure 4 summarizes what we have determined about the glycosylation and export of SRRPs to date—with focus on Fap1 of *S. parasanguinis*. Fap1 consists of unique features, which, all together, direct export to the accessory secretion system. In the cytoplasm, Fap1 is glycosylated with several glycosyltransferases. Notable, the first glycosylation step (catalyzed by Gtf1/Gtf2) is essential for export. Partially glycosylated Fap1 (i.e. after the initial step) are exported exclusively by the accessory secretion system, where the proteins appear to be processed properly but lack full glycosylation; the efficiency of processing is reduced with fewer sugar modifications on Fap1. Loss of the accessory secretion components SecA2, SecY2, Gap1, and Gap3 results in a greatly reduced ability to export the SRRP, which in some species exist as a HMM form.

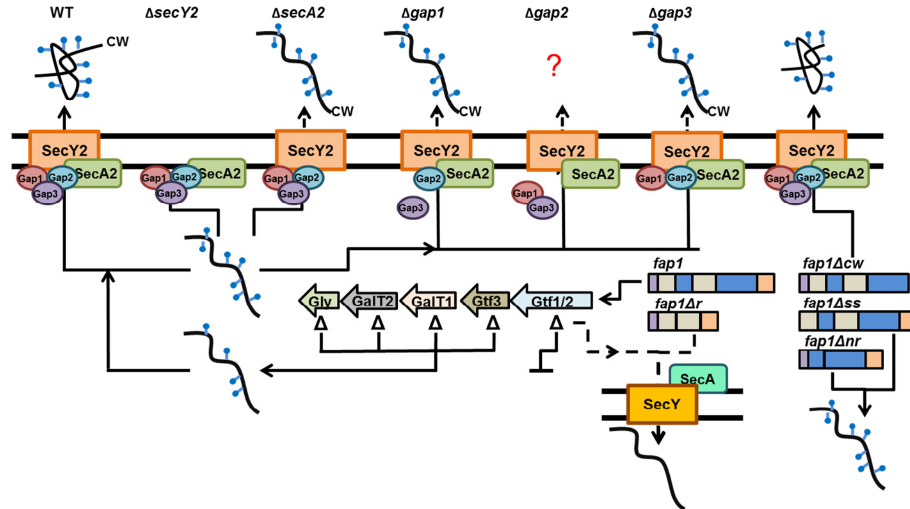

Figure 4. Model of glycosylation and export of SRRPs. Dashed lines represent rare events while solid lines represent frequent events. The domains of Fap1—signal sequence (SS), non-repeat regions (NR), repeat regions (R), and cell wall anchor domain (CW)—play a role in targeting export of Fap1 towards SecA2/SecY2. Loss of the initial glycosylation by Gtf1/Gtf2 prevents the majority of export of Fap1; a minor amount can be exported via SecA/SecY. Deletion of the other glycosyltransferases (*gtf3, galT1, galT2, gly*) results in an partially glycosylated Fap1 that can be exported at a lower efficiency. SecY2, SecA2, Gap1, and Gap3 are required for Fap1 export; a minor amount of a HMM Fap1 can be exported in the mutants. The role of Gap2 is unknown.

Although SecA2, SecY2, Gap1, and Gap3 are required for proper Fap1 export, the impact of Gap2 on Fap1 biogenesis and its function is unknown. In this study, we focused on elucidating the function of Gap2 by understanding the interactions between Gap2 and Gap1 and Gap3 and how these interactions affect Fap1 biogenesis.

CHAPTER 2

GAP2 PROMOTES THE FORMATION OF A STABLE PROTEIN COMPLEX REQUIRED FOR MATURE FAP1 BIOGENESIS

by

HALEY ECHLIN, FAN ZHU, YIRONG LI, ZHIXIANG PENG, TERESA RUIZ, GREGORY J. BEDWELL, PETER E. PREVELIGE, AND HUI WU

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ABSTRACT

Serine-rich repeat glycoproteins (SRRPs) are important bacterial adhesins conserved in streptococci and staphylococci. Fap1, a SRRP identified in *Streptococcus parasanguinis*, is the major constituent of bacterial fimbriae and is required for adhesion and biofilm formation. An eleven gene cluster is required for Fap1 glycosylation and secretion; however, the exact mechanism of Fap1 biogenesis remains a mystery. Two glycosylation-associated proteins within this cluster—Gap1 and Gap3—function together in Fap1 biogenesis. Here we report the role of the third glycosylation-associated protein, Gap2. A *gap2* mutant exhibited the same phenotype as the *gap1* and *gap3* mutants in terms of Fap1 biogenesis, fimbrial assembly, and bacterial adhesion—suggesting that the three proteins interact. Indeed, all three proteins interacted with each other independently and together to form a stable protein complex. Mechanistically, Gap2 protected Gap3 from degradation by ClpP protease and Gap2 required the presence of Gap1 for expression at wild-type level. Gap2 augmented Gap1's function of stabilizing Gap3; this function was conserved in Gap homologs from *Streptococcus agalactiae*. Our studies demonstrate that the three Gap proteins work in concert in Fap1 biogenesis and reveal a new function of Gap2. This insight will help us elucidate the molecular mechanism of SRRP biogenesis in this bacterium and in pathogenic species.

INTRODUCTION

Two of the most prevalent infectious diseases of humans are dental caries and inflammatory periodontal disease. Oral streptococci comprise a large proportion of oral bacterial species in dental plaque and are one of the first colonizers of the tooth surface (1-

3). As such, oral streptococci will encounter not only host oral epithelial cells, but also other microbial cells, of which there are over 500 species in the oral cavity, including the major periodontal pathogens—which often cannot colonize unless a layer of initial colonizers, such as oral streptococci, has developed first (4-8). Like other oral streptococci, *S. parasanguinis* has several colonization and adhesion factors; one of its adhesion factors is long peritrichous fimbriae (9). *S. parasanguinis* fimbriae are made of Fap1 (fimbriae-associated protein 1), a 200 kDa cell wall anchored serine-rich repeat glycoprotein (SRRP) (10). Fap1 is required for fimbrial formation, bacterial adhesion (1, 11), and biofilm formation (10, 12). Since the discovery of Fap1 (13, 14), Fap1-like SRRPs have been identified in many streptococci, staphylococci, and other gram-positive bacteria and have been implicated in bacterial interactions with hosts, adhesion, biofilm formation, and pathogenesis (10, 11, 15-20). They include GspB and Hsa of *S. gordonii* (21, 22), SraP of *S. sanguinis* (23), PsrP of *S. pneumoniae* (18), Srr-1 and Srr-2 of *S. agalactiae* (16, 17), SrpA of *S. cristatus* (24), SraP of *S. aureus* (10, 19), and FimS of *S. salivarius* (25).

The exact mechanism of SRRP biogenesis is not well understood. The chromosomal region dedicated to SRRP glycosylation and secretion is quite large and highly conserved. For Fap1, the cluster is separated into two regions: a core region that is conserved in every genome (*secY2*, *gap1-3*, *secA2*, and *gtf1-2*) and a variable region that includes several putative glycosyltransferases (*gly*, *nss*, *galT1*, and *galT2*) (10). *gtf1* and *gtf2* and genes from the *gly-gtf3-galT1*-galT2 locus mediate Fap1 glycosylation (13, 26-30); Fap1 is glycosylated in the cytoplasm with several monosaccharides, including glucose, N-acetyl glucosamine, N-acetyl galactosamine, and rhamnose (11, 29). The *secY2-gap1-gap2-gap3-secA2* locus is responsible for secretion of Fap1 (28, 29, 31). SecA2 and

SecY2 have homology to their counterparts in the canonical Sec pathway and are required for the export of mature Fap1 to the cell wall surface (28, 29). There is no known homology for the remainder of the locus—*gap1-gap2-gap3*—outside of the SRRP family. We have shown previously that both *gap1* and *gap3* mutants produce a similar immature Fap1 and that the interaction between Gap1 and Gap3 is required for Fap1 biogenesis, indicating that Gap1 and Gap3 are involved in mature Fap1 biogenesis (32-34). However, to date, the function of Gap2 is unknown.

In this study, we determined the role of Gap2 and found it is involved in Fap1 biogenesis by stabilizing Gap3 through interactions with Gap1 and Gap3; this study reveals an activity of Gap2 and its homolog that was previously unknown.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Primers, and DNA Manipulation

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *S. parasanguinis* strains were cultured as previously described (15). *S. parasanguinis* cell concentrations were determined by absorbance at 470 nm. Antibiotics were used at the following concentrations: 10 µg/ml erythromycin, 125 µg/ml kanamycin, and 250 µg/ml spectinomycin in Todd Hewitt (TH) broth or agar plates for *S. parasanguinis*; 300 µg/ml erythromycin, 50 µg/ml kanamycin, 50 µg/ml ampicillin, and 50 µg/ml spectinomycin in Luria-Bertani (LB) broth or agar plates for *E. coli*. Standard recombinant DNA techniques were used for DNA preparation and analyses (35). Plasmid DNA preparations were isolated with QIAprep Miniprep Kit (Qiagen). Primers used in this study are listed in Table 2. PCR was carried out with Taq DNA polymerase

(Promega) or KOD DNA polymerase (Novagen). PCR products were purified with QIAquick PCR Purification Kit (Qiagen). DNA digestion, ligation, and transformation were performed using standard methods. Competent cells for *S. parasanguinis* electroporation were prepared as described previously (36).

Western Blot Analysis

All *S. parasanguinis* strains were grown to OD_{470}=0.5-0.6 and centrifuged; cell pellets were subjected to amidase treatment to lyse the cells (28). Cell lysates were boiled in sample buffer (0.0625 M Tris, pH6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue) for 10 min before loading into 10% SDS-PAGE gels and subjected to western blotting analysis. Two monoclonal antibodies were used to detect Fap1—mAb E42, which is specific to the peptide backbone of Fap1, and mAb F51, which is specific for the mature Fap1 (11); mAbF51 only recognizes the 200 kDa mature Fap1, whereas mAb E42 recognizes both the 200 kDa mature Fap1 and the 470 kDa Fap1 precursor. Rabbit polyclonal antibodies against Gap1, Gap2, and Gap3 were custom produced using recombinant Gap1, Gap2, Gap3, or Gap1/2/3 complex as an antigen. Monoclonal antibody against Hsv (Novagen) was used to detect tagged proteins. Polyclonal antibody against FimA was used to standardize protein loading of *S. parasanguinis* proteins.

Construction of the Insertional gap2 *Mutant and* gap2/clpP *Double Mutant*

A *gap2* mutant was constructed by allelic replacement of *gap2* with a kanamycin resistant cassette, *aphA-3* (aminoglycoside phosphotransferase). A fragment containing the *gap2* gene and its flanking regions was amplified from *S. parasanguinis*

chromosomal DNA using Gap2+Flank-F/ Gap2+Flank-R. The PCR fragment was ligated into pGEM-T easy (Promega). A 850 bp region of *gap2* was deleted by inverse PCR using Gap2-StuI-F/ Gap2-StuI-R. The inverse PCR product was digested with *StuI* and ligated with a promoterless *aphA-3* kanamycin resistant cassette from pALH124 (37) to generate pGEM::Δ*gap2-aphA3*. Finally, the *gap2* insertion mutant was constructed by transformation of FW213 with pGEM::Δ*gap2-aphA3*, followed by selection of kanamycin resistant colonies. The in-frame insertion was further examined by DNA sequencing analyses. A Western blot analysis probed with antiserum against SecA2, a protein encoded by a gene downstream of *gap2*, was performed to confirm that the mutation was non-polar (data not shown). The *fap1* (1), *secY2* (28), *gap1* (34), *gap3* (32), and *clpP* (38) mutants were constructed in a similar method. For the *gap2/clpP* double mutant, a spectinomycin resistant cassette (Spec) was inserted into *clpP* in the *gap2* mutant. The pGEM::Δ*clpP-aphA-3* construct (38)was digested with *HindIII* to remove the kanamycin resistant cassette and then ligated in-frame with the spectinomycin resistance cassette amplified from pCG1 (39) to construct pGEM::Δ*clpP-spec*. The *gap2*/*clpP* double mutant was constructed by transformation of the *gap2* mutant with pGEM::Δ*clpP-spec*, followed by selection of kanamycin and spectinomycin resistant colonies. The in-frame insertion was further examined by DNA sequencing analyses.

Complementation of the gap1, gap2, *and* gap3 *Mutants*

The full-length *gap1*, *gap2*, and *gap3* genes were amplified from FW213 genomic DNA by PCR using primers Gap1-SalI-F/ Gap1-KpnI-R, Gap2-SalI-F/ Gap2-KpnI-R, and Gap3-SalI-F/ Gap3-KpnI-R, respectively (Table 2). The purified *gap1*, *gap2*, and

gap3 PCR products were digested with *SalI* and *KpnI* and then cloned into *E. coli*-*Streptococcus* shuttle vector pVPT-*gfp* (40) to generate corresponding complementation plasmids pVPT-*gap1*-*gfp*, pVPT-*gap2*-*gfp*, and pVPT-*gap3* (no *gfp*). The plasmid and its control vector pVPT-*gfp* were then transformed into the *gap1*, *gap2*, and *gap3* mutants via electroporation. The transformants were selected on TH agar plates containing kanamycin and erythromycin.

Modification of an E. coli-Streptococcus Shuttle Vector pIB184

A second *E. coli*-*Streptococcus* shuttle vector, pIB184 (41), was used in this study for better expression and genetic manipulation. To enhance the utility of this vector, pIB184 was modified by cloning in *gfp* and *hsv-his* tags within the multiple cloning site. The full-length *gfp* and *hsv-his* were amplified from pVPT-*gfp* and pET27b (Novagen) using primers GFP-XmaI-F/ GFP-SacI-R and HsvHis-Xmal-F/ HsvHis-SacI-R, respectively. The purified *gfp* and *hsv-his* PCR products were digested with *XmaI* and *SacI* and then ligated with the vector pIBI84 to create pIB184-*gfp* and pIB184-*hsv-his*.

Construction of Overexpression Strains in S. parasanguinis *FW213*

The full-length *gap3, gap2-gap3,* and *gap1-gap2-gap3* were amplified from FW213 genomic DNA by PCR using primers Gap3-BamHI-F/ Gap3-XmaI-R, Gap2-BamHI-F/ Gap3-XmaI-R, and Gap1-BamHI-F/ Gap3-XmaI-R, respectively (Table 2). The purified *gap3*, *gap2-gap3*, and *gap1-gap2-gap3* PCR products were digested with *BamHI* and *XmaI* and then cloned into *E. coli*-*Streptococcus* shuttle vector pIB184-*gfp* to generate pIB184-*gap3*-*gfp*, pIB184-*gap2-gap3*-*gfp*, and pIB184-*gap1-gap2-gap3*-*gfp*,

where Gap3 is tagged with GFP in all vectors. The plasmids were then transformed into the wild-type and *gap1* and *gap2* mutants via electroporation. pIB184-*gap2-hsv-his* and pIB184-*gap1-gap2-hsv-his* were created in the same fashion using pIB184-*hsv-his* and primer pairs Gap2-BamHI-F/ Gap2-XmaI-R and Gap1-BamHI-F/ Gap2-XmaI-R, respectively. The Gap homologs, Asp1-2-3, from *S. agalactiae* wild-type J48 were used to check for conservation of function. pIB184-*asp3-gfp*, pIB184-*asp2-gap3-gfp*, pIB184-*asp1-asp2-asp3-gfp*, pIB184-*asp2-hsv-his*, and pIB184-*asp1-asp2-hsv-his* were created in the same manner as above, using primers Asp3-BamHI-F/ Asp3-XmaI-R, Asp2-BamHI-F/ Asp3-XmaI-R, Asp1-BamHI-F/ Asp3-XmaI-R, Asp2-BamHI-F/ Asp2-XmaI-R, and Asp1-BamHI-F/ Asp2-XmaI-R, respectively (Table 2). The resulting plasmids were then transformed into FW213 and *gap1* and *gap2* mutants via electroporation. The transformants were selected on TH agar plates containing erythromycin (wild-type) or kanamycin and erythromycin (mutants).

Bacterial Adhesion Assay

Saliva-coated hydroxyapatite (SHA) was used as an *in vitro* tooth model to test the binding abilities of *S. parasanguinis* and the relevant derivatives as described previously (42). Briefly, [^{3}H]-thymidine-labeled bacteria of OD_{470}=1.0 in adhesion buffer (67 mM phosphate buffer, pH 6.0) were sonicated for 15 s at 85W using an ultrasonic cuphorn system (Heat Systems-Ultrasonics). 1 ml of sonicated bacteria (in triplicate) were added to 7 ml scintillation vials containing SHA and incubated for 1 h at 37°C with gentle shaking. The supernatant fluids were removed and the beads were washed 3 times with adhesion buffer. The amounts of unbound bacteria in the supernatant fluids and

bacteria bound to SHA were determined in a Beckman Coulter LS6500 Scintillation Counter (Beckman-Coulter) (1). Differences in SHA adhesion were analyzed via 2-tailed Student's t-test for two samples with equal variances.

Transmission Electron Microscopy

S. parasanguinis cell cultures (5 ml) grown to OD_{470} = 0.4 were harvested by centrifugation. Cell pellets were washed twice with ice-cold PBS and resuspended in 100 µl PBS. 5 µl of the bacterial suspension was diluted in PBS and was applied to 400 mesh copper grids coated with a thin carbon film. The grids were first washed by several drops of PBS buffer. The samples were stained with a few drops of 2% phosphotungstic acid, pH 7.0 (PTA) over the grid surfaces. The excess liquid was wicked off and the grids were fast air dried. The grids were observed on a Tecnai 12 Philips electron microscope (FEI, Holland) equipped with a LaB6 cathode operated in point mode (Kimball) and a 2048 CCD camera (TVIPS, Germany). The microscope was run to obtain images that show Thon rings beyond 0.9 nm resolution in vitreous ice preparations (43). Images were recorded at an accelerating voltage of 100kV and nominal magnifications in the range of 40,000-70,000X under low dose conditions on either film (S0-163 Kodak) or the CCD camera. Images were converted to SPIDER format (44) and high-pass filter to remove the background.

in vitro *GST Pull-down Assays*

The GST pull-down protocol was developed to determine protein-protein interactions in solutions (45). Gap1- and Gap3-pGADT7 were constructed as described

(34). Gap2-pGADT7 was constructed by PCR amplification of *gap2* using primers Gap2-EcoRI-F/ Gap2-BamHI-R (Table 2) from FW213 chromosomal DNA, digestion with *EcoRI* and *BamHI*, and ligation into pGADT7. GST-Gap1, GST-Gap2, and GST-Gap3 fusion proteins were created by cloning of *EcoRI* and *XhoI* digested fragments from Gap1-, Gap2- and Gap3-pGADT7 into pGEX-5X-2, respectively. The GST fusion proteins were expressed and purified using glutathione Sepharose 4B beads. The same amounts of GST or GST fusion proteins (5 µg) immobilized on beads and estimated by SDS-PAGE analysis were re-suspended in NETN washing buffer (20 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1 mM EDTA, 0.5% NP40) and mixed with 5 µl of *in vitro* translated c-Myc -Gap1, -Gap2, and -Gap3 fusion protein products (34). The mixtures were reconstituted in a final volume of 200 µl with NETN binding buffer and incubated at 4°C overnight on a rotary shaker. The beads were washed three times with 600 µl of NETN washing buffer. The proteins bound to the beads were eluted by boiling in SDS loading buffer and subjected to Western blotting analyses using anti-c-Myc antibody (Invitrogen). The interaction between Gap1 and Gap3 was confirmed previously (34) and was used here as a control.

Analytical Ultracentrifugation

Sample Preparation

A fusion plasmid was constructed to express His-SUMO-tagged Gap1-2-3 by the same method used in the construction of His-SUMO- tagged Gap1-3 (38). Briefly, full-length *gap1-gap2-gap3* was amplified from genomic DNA of *S. parasanguinis* FW213 using Gap1-NotI-1F/Gap3-XhoI-R, digested by *NotI* and *XhoI*, and ligated into pET-

SUMO to construct the His-SUMO-Gap1-2-3 fusion protein. The constructed plasmid was verified by DNA sequence analysis and then transformed into *E. coli* BL21 (DE3). Gap1-3 and Gap1-2-3 were expressed and purified as described previously (26). Peak fractions from gel filtration were collected and used for ultracentrifugation. Concentrations of the proteins were determined by measuring sample absorbance at 280 nm using a Beckman DU-640 Spectrophotometer (International MI-SS, Inc. Corona CA). The sample proteins were diluted to desired concentrations with buffer G (26).

Sedimentation Equilibrium

Sedimentation equilibrium (SE) experiments were performed at 20°C using six-channel centerpieces in a Beckman Optima XL-A with absorption optics. Three concentrations (0.2 mg/ml, 0.4 mg/ml, and 0.9 mg/ml) were analyzed at two rotor speeds—17,000 rpm and 20,000 rpm—with detection by absorbance at 280 nm. All data sets from different protein concentrations and rotor speeds were fit to a single global model (global fits) to determine the stoichiometry and equilibrium constants. Model fittings of the SE data were performed by software HETEROANALYSIS (Biotechnology/Bioservices Center, University of Connecticut, Storrs, CT).

RESULTS

gap2 *Mutant Exhibits Same Phenotype as* gap1 *and* gap3 *Mutants*

Gap1 and Gap3 have been shown to be involved in Fap1 biogenesis (32, 34). However, there have been no reports on the function of the third glycosylation-associated protein, Gap2. In this study, we generated a Gap2 deficient mutant and examined its

phenotype. Fap1 production in the Gap2 deficient strain was similar to that in the strains deficient in Gap1, Gap3, and SecY2, where mature Fap1 (Fig. 1, Lane 1), recognized by F51, was undetectable and a larger band corresponding to an immature Fap1 (Lanes 3-6) was observed when probed by E42, a peptide specific antibody. The wild-type phenotype was restored upon complementation (Lanes 7-9); the empty vectors could not restore the wild-type phenotype (Lanes 10-12). This result demonstrates that Gap2, like Gap1 and Gap3, is required for the production of mature Fap1 (32, 34).

Since Fap1 is required for assembly of *S. parasanguinis* fimbriae (1), the cell surface structure of *S. parasanguinis* variants was examined using transmission electron microscopy. In the *gap2* mutant (Fig. 2C), fimbriae were no longer detected as they are in the wild-type FW213 strain (Fig. 2A). However, the Gap2 deficiency had no effect on a smaller fibril (indicated by the white arrows in Fig. 2B-D), which has been identified previously as BapA1 (46). This fimbriae phenotype is comparable to that of the *gap1* (Fig. 2B) and *gap3* (Fig. 2D) mutants. Furthermore, the Gap2 deficiency decreased bacterial adherence to SHA (Fig. 3). This phenotype was similar to that observed in strains deficient in Gap1 or Gap3. For all three strains, complementation nearly restored adhesion levels to that of the wild-type (Fig. 3). These results indicate that Gap2 functions in concert with Gap1 and Gap3 in Fap1 biogenesis, with a subsequent effect on fimbriae biogenesis and adhesion level.

Gap1, Gap2, and Gap3 Interact with Each Other to Form a Complex

Because not only do Gap1, Gap2, and Gap3 deficient strains share a similar phenotype (Fig. 1-3), but also the interaction between Gap1 and Gap3 is required for

biogenesis of Fap1 (34, 38), it is likely that Gap2 interacts with Gap1 and Gap3 as well. To determine this, we coexpressed all three proteins in *E. coli*, with Gap1 tagged with GST, and performed GST-pull down assays. Gap2 and Gap3 were invariably pulled down with GST-Gap1 (Fig. 4A). GST itself did not pull down Gap2 and Gap3 (data not shown). To address whether Gap2 could interact with Gap1 and Gap3 independently, we expressed each protein tagged with GST individually and incubated them with *in vitro* translated c-Myc fusion proteins. Upon GST pull-down assays, GST-tagged Gap2 pulled down Gap1 and Gap3, and Gap2 was pulled down by GST-tagged Gap1 and Gap3 (Fig. 4B). This result indicates that Gap2 can interact with both Gap1 and Gap3 directly. The interaction between Gap1 and Gap3 was used as positive assay controls. In negative controls, Gap1, Gap2, nor Gap3 interacted with GST alone, indicating that the interaction between Gap2 and Gap1, and Gap3 was specific.

Analytical ultracentrifugation sedimentation equilibrium experiments were performed to further characterize the interaction among Gap1, Gap2, and Gap3. Sedimentation equilibrium (SE) data show that the Gap1/3 complex fits a single species model well (Fig. 5A), suggesting that the binding between Gap1 and Gap3 was tight. The binding of Gap2 to the already formed Gap1/3 complex fits a heterodimer model ("A+B<>AB," where A represents Gap1/3 and B represents Gap2; K_d of 4.4E-07 M) (Fig. 5B), suggesting that Gap2 binds to Gap1/3 to form a Gap1/2/3 complex in a reversible manner. The experimental data fit the models regardless of the concentration (0.2 mg/ml, 0.4 mg/ml, and 0.9 mg/ml; Fig. 5) or speed [17,000 rpm (Fig. 5) and 20,000 rpm (data not shown)] used.

Gap2 Is Increased with Gap1 Overexpression

Previously, we have shown that Gap1 is required for the stability of Gap3 (38). In this study, we demonstrated that Gap2 interacted with both Gap1 and Gap3. In order to determine how Gap2 affects or is affected by Gap1, protein levels of Gap1 and Gap2 were determined in wild-type and *gap* mutant variants (Fig. 6A). A *gap2* mutant had no effect on the amount of Gap1 (Lane 3). On the other hand, in the absence of Gap1 (Lane 2), Gap2 was decreased compared to the wild-type (Lane 1). The *gap1* complemented strain restored the wild-type phenotype (Lane 5); expression of Gap1-GFP was observed as a band slightly above 75 kDa when probing with the Gap1 antibody. The negative vector had no effect on the decreased amount of Gap2 (Lane 8). This result suggests that Gap1 expression increases the amount of Gap2. To confirm this, we compared expression of Gap2 in a strain that overexpressed Gap2 alone to a strain that overexpressed both Gap1 and Gap2 (Fig. 6B). Gap2 expression was greatly increased when both Gap1 and Gap2 (Lanes 2, 4, and 6) were overexpressed compared to overexpression of Gap2 alone (Lanes 1, 3, and 5); expression of Gap2-HH was observed as a band about 65 kDa when probing with the Gap2 antibody. This phenotype was observed in wild-type strain, as well as *gap1* and *gap2* mutants. RT-PCR analysis of *gap2* transcription demonstrates no difference between wild-type and the *gap1* mutant, indicating that the effect of Gap1 on Gap2 occurs on the post-transcriptional level (Figure S1A). Together, these data demonstrate that the amount of Gap2 is modulated by Gap1.

Gap2 Expression Results in Increased Gap3

To determine the association between Gap2 and Gap3, we examined the effect of Gap2 deficiency on Gap3 (Fig. 6A). In the absence of Gap2 (Lane 3), Gap3 was decreased compared to wild-type. Further, in the *gap2* complement (Lane 6), the amount of Gap3 was restored to wild-type level; expression of Gap2-GFP was observed as a stronger band compared to a non-specific band present at 75 kDa when probing with the Gap2 antibody. The negative vector had no effect on the decreased the amount of Gap3 (Lane 9). However, the *gap3* mutant had no effect on the amount of Gap2 (Lane 4). This result suggested that Gap2 expression increases the amount of Gap3. To confirm this, we overexpressed Gap2 and determined its impact on Gap3 (Fig. 7A). Overexpression of Gap2 in the wild-type strain (Lane 4) indeed increased Gap3. In the *gap2* mutant (Lane 6) background, overexpression of Gap2 did not quite restore the amount of Gap3 to the wild-type level. However, this could be due to the reduced amount of Gap2 in the mutant strain compared to the wild-type strain. RT-PCR analysis of *gap3* transcription demonstrates no difference between wild-type and the *gap2* mutant, indicating that the effect of Gap2 on Gap3 occurs on the post-transcriptional level (Figure S1A). These data demonstrate that Gap2 modulates Gap3 amount.

Gap2 Modulates Gap3 Amount Independently of Gap1

Gap2 deficiency resulted in a diminished amount of Gap3 and overexpression of Gap2 led to a greater Gap3 amount. However, from these data, we cannot determine whether Gap2 functions independently of Gap1; in the absence of Gap1, native Gap3 was no longer detected, even when Gap2 was overexpressed (Fig. 7A, Lanes 2 and 5). To

determine if Gap2 can affect Gap3 independently of Gap1, strains were created that overexpressed Gap3 alone, Gap2 and Gap3, or Gap1, Gap2, and Gap3 in wild-type and in *gap1* and *gap2* mutants (Fig. 7B); expression of Gap3-GFP was observed as a band slightly below 50 kDa when probing with the Gap3 antibody. Again, when Gap2 was overexpressed, both native and overexpressed Gap3 was increased (Lane 2), compared to the strain overexpressing Gap3 alone (Lane 1). Moreover, Gap3 was increased even further when both Gap1 and Gap2 were overexpressed along with Gap3 (Lane 3). This phenomenon was not limited to the wild-type as it also occurred in the *gap1* (Lanes 4-6) and *gap2* (Lanes 7-9) mutant strains, albeit the overall levels were lower compared to the wild-type. RT-PCR analysis of *gap3* transcription demonstrates no difference between overexpressing strains, indicating that the effect of Gap1 and Gap2 on Gap3 occurs on the post-transcriptional level (Fig. S1B). These data demonstrate that increasing Gap2 expression can increase the amount of overexpressed Gap3 in the absence of Gap1, suggesting that Gap2 augments Gap1's function in stabilizing Gap3.

Gap Homologs from S. agalactiae *Displayed Same Conserved Functions as Gap Proteins*

Gap1, Gap2, and Gap3 are highly conserved in SRRP-containing gram-positive bacteria. We have previously shown that the Gap1 homolog from *S. agalactiae* stabilizes the Gap3 homolog, much like Gap1 acts as a chaperone for Gap3 (38). To determine if the relationship between Gap2 and Gap1, and Gap3 is conserved, we expressed Gap homolog from *S. agalactiae* (Asp1, Asp2, and Asp3) in *S. parasanguinis* (Fig. 8A). In *S. parasanguinis* wild-type, Asp2 was detected when both Asp1 and Asp2 were expressed (Lane 2), but was undetectable when expressed alone (Lane 1). This result suggests that

the amount of Asp2 is increased in the presence of Asp1, much like the Gap proteins in *S. parasanguinis* (Fig. 6B). This phenomenon was also observed in the absence of Gap1 (Fig. 8A, Lanes 3 and 4), further demonstrating that Asp1 can increase the Asp2 amount. To determine if the function of Gap2 is conserved, we expressed Gap homologs (Asp1, Asp2, and Asp3) from *S. agalactiae* in *S. parasanguinis* strains lacking Gap2 (*gap2* mutant). In these strains, Asp3 was expressed by itself, with Asp2, or with Asp1 and Asp2 (Fig. 8B). When Asp2 was expressed along with Asp3 (Lane 2), the amount of Asp3 increased compared to Asp3 expressed alone (Lane1); when Asp1 was expressed with Asp2 and Asp3 (Lane 3), the amount of Asp3 was even greater. Because this trend is similar to the one observed in the *S. parasanguinis* homologs (Fig. 7B), this result indicates that Asp2 can function in a similar manner as Gap2. Together, these data suggest that the relationship among the Gap proteins is conserved.

Gap2 Prevents Gap3 Degradation by ClpP Protease

Proteases are often involved in the degradation of misfolded proteins. Previously, the protease ClpP was shown to be responsible for the degradation of Gap3 in the absence of Gap1, a specific chaperone of Gap3 (38). Here, we wanted to determine if Gap2 protected Gap3 in a similar fashion. We constructed a *clpP* mutant and a *gap2/clpP* double mutant to examine the ability of Gap2 to shield Gap3 from degradation by ClpP (Fig. 9). No difference in Gap3 was observed between wild-type (Lane 1) and the *clpP* mutant (Lane 2). In the absence of both ClpP and Gap2 (Lane 4), the amount of Gap3 was increased compared to the *gap2* single mutant (Lane 3), nearly restoring it to wild-

type level. This result suggests that Gap2, similarly to Gap1, protects Gap3 from degradation by ClpP.

DISCUSSION

Biogenesis of SRRPs is mediated by glycosylation and accessory secretory loci, which are highly conserved in many streptococci and staphylococci (10). In *S. parasanguinis,* an eleven gene cluster including glycosyltransferase genes and genes involved in protein secretion have been identified for Fap1 biosynthesis. Accessory secretion components—containing SecA2 and SecY2, and glycosylation associated proteins, Gap1, Gap2, and Gap3 (10, 28, 29)—are implicated in Fap1 secretion and maturation. The exact role of Gap1, Gap2, and Gap3 in Fap1 biogenesis remains unknown. We have shown previously that Gap1 and Gap3 are required for production of mature Fap1, formation of fimbriae, and adhesion to SHA (32, 34). In this study, we have determined the function of Gap2. Similar to Gap1 and Gap3, Gap2 was necessary for mature Fap1 biogenesis, with direct effects on fimbriae production and adhesion to an *in vitro* tooth surface model (Fig. 1-3). Because all three of the *gap* mutants shared a similar phenotype, it is likely they interact and work in concert to complete Fap1 biogenesis. Indeed, we show here that Gap1, Gap2, and Gap3 interact to form a complex (Fig. 4). The formation of a protein complex by Gap homologs has been demonstrated in *S. gordonii* as well (47); however, the details of the interactions were not characterized. Through ultracentrifugation, we determined that Gap2 could interact with an already formed Gap1/3 complex in a reversible manner. While Gap1 and Gap3 bind tightly to

each other, Gap2 has a lower binding affinity toward the Gap1/3 complex, suggesting Gap2 may have regulatory activity toward the Gap1/3 complex (Fig. 5).

Based on the data obtained from the current study (summarized in Fig. 10), we can expand our previous model of Fap1 biogenesis. In this model, Gap1 binds to Gap3 (38) (Fig. 5A). This is then followed by binding of Gap2, which can further stabilize Gap3 and is, itself, stabilized by Gap1 (Fig. 5B, 6, and 7). Such binding and stabilization was also observed for Gap homologs from *S. agalactiae* (Fig. 8), suggesting that this new function of Gap2 is conserved among SRRP-containing gram-positive bacteria. Further, the current study indicates that Gap2, protects Gap3 from degradation by ClpP (Fig. 9). Similarly, we have previously shown that the protease ClpP is responsible for the degradation of Gap3 in the absence of Gap1, which acted as a specific chaperone of Gap3 (38). As to how ClpP gains access to the Gap3 protein remains to be determined.

Since Gap2 works in concert with Gap1 to stabilize Gap3—the putative key scaffolding protein required for the formation of the Fap1 biosynthetic protein complex—we believe the function of Gap2 is to ensure Gap3 activity, which promotes Fap1 biogenesis. A similar proposition has been made in *S. gordonii*, in which Asp2 interacts with the Asp1, Asp3, and SecA2 complex for optimal export of GspB (47). Gap2 can interact with the Gap1/3 complex, which then interacts with SecA2 and SecY2 to aid in Fap1 secretion (31). However, the precise biochemical function of this Gap complex in the conversion of an immature form of Fap1 to the mature form remains to be elucidated. Recent work in *S. gordonii* indicates that Asp2 is required for export of GspB as well as the conversion to the final glycoform of GspB, where mutants of Asp2 resulted in altered

GspB glycoforms that had increased GlcNAc content (48). Our previous study also suggested that the Gap1 deficiency altered glycosyl composition of Fap1 (34).

Although these data provide insights into the function of the accessory secretion components, the question regarding details of biochemical activity of the complex still remains unanswered. It is possible that by binding to the Gap1/3 complex, Gap2 is brought within an appropriate distance to monitor glycosylation status of Fap1 to ensure export of a correctly folded Fap1- possibly suggesting a role for Gap2 as a glycoside hydrolase, an important activity in quality control of glycoproteins in eukaryotes (49, 50). This activity is often associated with removal of sugar residues and typically function through the Ser-Asp-His catalytic triads identified in the Gap2 homolog (48). Indeed, analysis of the Gap2 sequence with the Phyre fold predication program predicted Gap2 is a hydrolase (51). In *S. gordonii*, Asp2 alone does not exhibit detectable enzymatic activity against a panel of hydrolase substrates—suggesting that the catalytic activity requires additional cofactors (48). Alternatively, Gap2 may also bind to Fap1, bringing Gap3 within proximity of Fap1, therefore modulating Fap1 maturation. Indeed, in *S. gordonii*, Asp2, along with Asp3, is capable of binding the unglycosylated serine-rich repeat domains of GspB, and these interactions are required for optimal GspB export (52). Along the lines of this alternative, Gap2 may possess some sort of regulatory function, which may then become a means of controlling Fap1 fimbrial assembly and fine tune bacterial adhesion levels.

In this study, we identify the necessity of Gap2 for mature Fap1 biogenesis, fimbriae production, and adhesion to the *in vitro* tooth surface model and demonstrate that Gap2 forms a complex with Gap1/3 and is required for full amount of Gap3.

However, whether and how Gap2 acts as a regulatory protein for Fap1 biogenesis remains to be determined.

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TABLES

Table 1. Bacterial strains and plasmids used in this study

Strain, plasmid	Relevant characteristics	Reference, source
Strains		
S. parasanguinis		
FW213	Wild-type	(9)
fap1^{-}	*fap1* insertion mutant, Kan^{R}	(1)
secY2^{-}	*secY2* insertion mutant, Kan^{R}	(28)
gap1^{-}	*gap1* insertion mutant, Kan^{R}	(34)
gap2^{-}	*gap2* insertion mutant, Kan^{R}	This study
gap3^{-}	*gap3* insertion mutant, Kan^{R}	(32)
gap1^{-}/pVPT	*gap1*$^{-}$ containing pVPT-*gfp*, vector control strain, Erm^{R} Kan^{R}	(38)
gap2^{-}/pVPT	*gap2*$^{-}$ containing pVPT-*gfp*, vector control strain, Erm^{R} Kan^{R}	This study
gap3^{-}/pVPT	*gap3*$^{-}$ containing pVPT-*gfp*, vector control strain, Erm^{R} Kan^{R}	This study
gap1^{-}/pVPT-*gap1*	*gap1*$^{-}$ containing pVPT-*gap1-gfp* plasmid, Erm^{R} Kan^{R}	(38)
gap2^{-}/pVPT-*gap2*	*gap2*$^{-}$ containing pVPT-*gap2-gfp* plasmid, Erm^{R} Kan^{R}	This study
gap3^{-}/pVPT-*gap3*	*gap3*$^{-}$ containing pVPT-*gap3-gfp* plasmid, Erm^{R} Kan^{R}	This study
FW213/pIB184-*gap3*	FW213 containing pIB184-*gap3-gfp* plasmid, Erm^{R}	This study
gap1^{-}/pIB184-*gap3*	*gap1*$^{-}$ containing pIB184-*gap3-gfp* plasmid, Erm^{R} Kan^{R}	This study
gap2^{-}/pIB184-*gap3*	*gap2*$^{-}$ containing pIB184-*gap3-gfp* plasmid, Erm^{R} Kan^{R}	This study
FW213/pIB184-*gap2-3*	FW213 containing pIB184-*gap2-gap3-gfp* plasmid, Erm^{R}	This study
gap1^{-}/pIB184-*gap2-3*	*gap1*$^{-}$ containing pIB184-*gap2-gap3-gfp* plasmid, Erm^{R} Kan^{R}	This study
gap2^{-}/pIB184-*gap2-3*	*gap2*$^{-}$ containing pIB184-*gap2-gap3-gfp* plasmid, Erm^{R} Kan^{R}	This study
FW213/pIB184-*gap1-2-3*	FW213 containing pIB184-*gap1-gap2-gap3-gfp* plasmid, Erm^{R}	This study
gap1^{-}/pIB184-*gap1-2-3*	*gap1*$^{-}$ containing pIB184-*gap1-gap2-gap3-gfp* plasmid, Erm^{R} Kan^{R}	This study
gap2^{-}/pIB184-*gap1-2-3*	*gap2*$^{-}$ containing pIB184-*gap1-gap2-gap3-gfp* plasmid, Erm^{R} Kan^{R}	This study
FW213/pIB184-*gap2*	FW213 containing pIB184-*gap2-hsv-his* plasmid, Erm^{R}	This study
gap1^{-}/pIB184-*gap2*	*gap1*$^{-}$ containing pIB184-*gap2-hsv-his* plasmid, Erm^{R} Kan^{R}	This study
gap2^{-}/pIB184-*gap2*	*gap2*$^{-}$ containing pIB184-*gap2-hsv-his* plasmid, Erm^{R} Kan^{R}	This study
FW213/pIB184-*gap1-2*	FW213 containing pIB184-*gap1-gap2-hsv-his* plasmid, Erm^{R}	This study
gap1^{-}/pIB184-*gap1-2*	*gap1*$^{-}$ containing pIB184-*gap1-gap2-hsv-his* plasmid, Erm^{R} Kan^{R}	This study
gap2^{-}/pIB184-*gap1-2*	*gap2*$^{-}$ containing pIB184-*gap1-gap2-hsv-his* plasmid, Erm^{R} Kan^{R}	This study
gap2^{-}/pIB184-*asp3*	*gap2*$^{-}$ containing pIB184-*asp3-gfp* plasmid, Erm^{R} Kan^{R}	This study
gap2^{-}/pIB184-*asp2-3*	*gap2*$^{-}$ containing pIB184-*asp2-asp3-gfp* plasmid, Erm^{R} Kan^{R}	This study
gap2^{-}/pIB184-*asp1-2-3*	*gap2*$^{-}$ containing pIB184-asp1-*asp2-asp3-gfp* plasmid, Erm^{R} Kan^{R}	This study
FW213/pIB184-*asp2*	FW213 containing pIB184-*asp2-hsv-his* plasmid, Erm^{R}	This study
gap1^{-}/pIB184-*asp2*	*gap1*$^{-}$ containing pIB184-*asp2-hsv-his* plasmid, Erm^{R} Kan^{R}	This study
FW213/pIB184-*asp1-2*	FW213 containing pIB184-*asp1-asp2-hsv-his* plasmid, Erm^{R}	This study
gap1^{-}/pIB184-*asp1-2*	*gap1*$^{-}$ containing pIB184-*asp1-asp2-hsv-his* plasmid, Erm^{R} Kan^{R}	This study
S. agalactiae		
J48	Wild-type	(17)
E. coli		
Top10	Host strain for cloning	Invitrogen
BL21	Host strain for protein expression	Invitrogen
Plasmids		
pVPT-*gfp*	*E. coli and S. parasanguinis* shuttle vector. Erm^{R}	(40)
pVPT-Gap1-*gfp*	*gap1* from FW213 cloned into pVPT-*gfp*. Erm^{R}	This study
pVPT-Gap2-*gfp*	*gap2* from FW213 cloned into pVPT-*gfp*. Erm^{R}	This study
pVPT-Gap3-*gfp*	*gap3* from FW213 cloned into pVPT-*gfp*. Erm^{R}	This study
pIB184	*E. coli and S. parasanguinis* shuttle vector, Erm^{R}	(41)
pIB184-*gfp*	*E. coli and S. parasanguinis* shuttle vector with *gfp* tag, Erm^{R}	This study
pIB184-*hsv-his*	*E. coli and S. parasanguinis* shuttle vector with *hsv-his* tag, Erm^{R}	This study
pIB184-Gap3-*gfp*	*gap3* from FW213 cloned into pIB184-*gfp*. Erm^{R}	This study
pIB184-Gap2-3-*gfp*	*gap2* and *gap3* from FW213 cloned into pIB184- *gfp*. Erm^{R}	This study
pIB184-Gap1-2-3-*gfp*	*gap1, gap2* , and *gap3* from FW213 cloned into pIB184 *gfp*. Erm^{R}	This study

pIB184-Gap2-*hsv-his*	*gap2* from FW213 cloned into pIB184-*hsv-his*. Erm^{R}	This study
pIB184-Gap1-2-*hsv-his*	*gap1* and *gap2* from FW213 cloned into pIB184-*hsv-his*. Erm^{R}	This study
pIB184-Asp3-*gfp*	*asp3* from J48 cloned into pIB184-*gfp*. Erm^{R}	This study
pIB184-Asp2-3-*gfp*	*asp2* and *asp3* from J48 cloned into pIB184- *gfp*. Erm^{R}	This study
pIB184-Asp1-2-3-*gfp*	*asp1, asp2* , and *asp3* from J48 cloned into pIB184-*gfp*. Erm^{R}	This study
pIB184-Asp2-*hsv-his*	*asp2* from J48 cloned into pIB184-*hsv-his*. Erm^{R}	This study
pIB184-Asp1-2-*hsv-his*	*asp1* and *asp2* from J48 cloned into pIB184-*hsv-his*. Erm^{R}	This study
pGEX-GST-Gap1	pGEX-GST vector containing *gap1* gene from FW213. Amp^{R}	(34)
pGEX-GST-Gap2	pGEX-GST vector containing *gap2* gene from FW213. Amp^{R}	(34)
pGEX-GST-Gap3	pGEX-GST vector containing *gap3* gene from FW213. Amp^{R}	This study
pET-His-SUMO-Gap1-3	pET-His-SUMO vector containing *gap1* and *gap3* genes. Kan^{R}	(38)
pET-His-SUMO-Gap1-2-3	pET-His-SUMO vector containing *gap1, gap2* , and *gap3* genes. Kan^{R}	This study
pGEM::Δ*gap2-aphA3*	pGEM vector containing *gap2* with *aphA-3* insertion. Kan^{R}	This study
pGEM::Δ*clpP-aphA-3*	pGEM vector containing *clpP* with *aphA-3* insertion. Kan^{R}	(38)
pGEM::Δ*clpP-spec*	pGEM vector containing *clpP* with *spec* insertion. Spec^{R}	This study

Table 2. Primers used in this study

Primers	**Sequences**
Gap1-SalI-F	ATACGCGTCGACATGTTTTATTTTGTACCTTC
Gap1-KpnI-R	CGGGGTACCTTTCTTTTTTAGCATACCTTTCC
Gap2-SalI-F	ATACGCGTCGACATGAAGATTTTACAATTGGC
Gap2-KpnI-R	CGCGGTACCTCTTCCAAACTGATCTTCTAG
Gap3-SalI-F	ACTCGCGTCGACATGACTAAACAGTTAATTTCTG
Gap3-KpnI-R	CGCGGTACCAATATATTCTATTAAATTTTTCACC
Gap2+Flank-F	ATACGCGTCGACATGAAG ATTTTACAAATTGGCCG
Gap2+Flank-R	CGGGGTACCTCTTCCAAACTGATCTTC TAG
Gap2-StuI-F	GCAGAGGCCTACAAGTGCTGATATGCTACTG
Gap2-StuI-R	GCAGAGGCCTCTTTGCTCCGTATTGACTAC
Spec-HindIII-F	CGGCCGCAAGCTTGTGAGGAGGATATATTTGAA
Spec-HindIII-R	CGGGCGCCGCAAGCTTTTATAATTTTTTTAATCTG
Gap1-BamHI-F	CCGGCGCCGGATCCGGATGTTTTATTTTGTACCTTCTTGG
Gap2-BamHI-F	GAGCGGATCCGGATGAAGATTTTACAAATTGGCCG
Gap2-XmaI-R	CCGCTGCCCGGGTCTTCCAAACTGATCTTCTA
Gap3-BamHI-F	GCGGCCTCGCGGATCCGAATGACTAAACAGTTAATTTCTG
Gap3-XmaI-R	GGCTCGCCGCGGTCCCGGGAATATATTCTATTAAATTTTTCACCAAATC
GFP-XmaI-F	GACGCCCGGGATGAGTAAAGGAGAAGAACTTTTCACTG
GFP-SacI-R	GCCGCGAGCTCCTATTTGTATAGTTCATCCATGCC
HsvHis-XmaI-F	ATATAACCCGGGAGCCAGCCAGAACTCGC
HsvHis-SacI-R	TATTGAGCTCTCAGTGGTGGTGGTGGTGGTGC
Asp1-BamHI-F	GGCGCGCGGATCCGGATGTTTTATTTTATTCCTTCGTGG
Asp2-BamHI-F	CGCCGCCGGCGGATCCGGATGGAAAAATTAAAAATTTTGCAG
Asp2-XmaI-R	GATCCCCGGGACCACTAAACACTCTCCCAAAAT
Asp3-BamHI-F	GCCGATCGGATCCGGATGATTTTGGGAGAGTGTTTAG
Asp3-XmaI-R	GCGGCCGGATGCCCGGGCGATTTTTTATCCTTAGAAAATGCTATCAACG
Gap2-EcoRI-F	GACGAATTCATGAAGATTTTACAATTGGC
Gap2-BamHI-R	TGTGGATCCTCTTCCAAACTGATCTTCTAG
Gap1-NotI-1F	AAGGAAAAAAGCGGCCGCATGTTTTATTTTGTACCTTCTTGG
Gap3-XhoI-R	ACCGCTCGAGTTAAATATATTCTATTAAATTTTTC

FIGURES

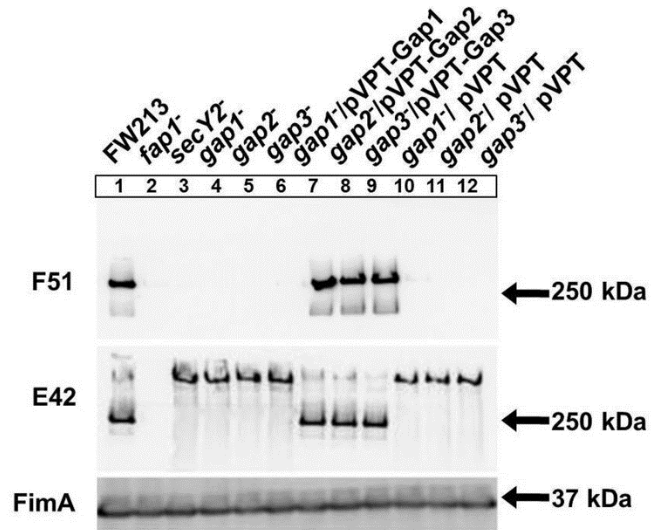

Figure 1. Biogenesis of mature Fap1 requires Gap2, as well as Gap1 and Gap3. Western blot analysis of Fap1 present in *S. parasanguinis* cell lysates. Strains used include FW213 wild-type, insertional mutants of *fap1*, *secY2*, *gap1*, *gap2*, and *gap3*, and complemented strains of the *gap1*, *gap2*, and *gap3* mutants with the full gene in pVPT or with the empty vector. Antibodies used include F51 (specific to mature Fap1), E42 (specific to the polypeptide Fap1), and FimA (loading control).

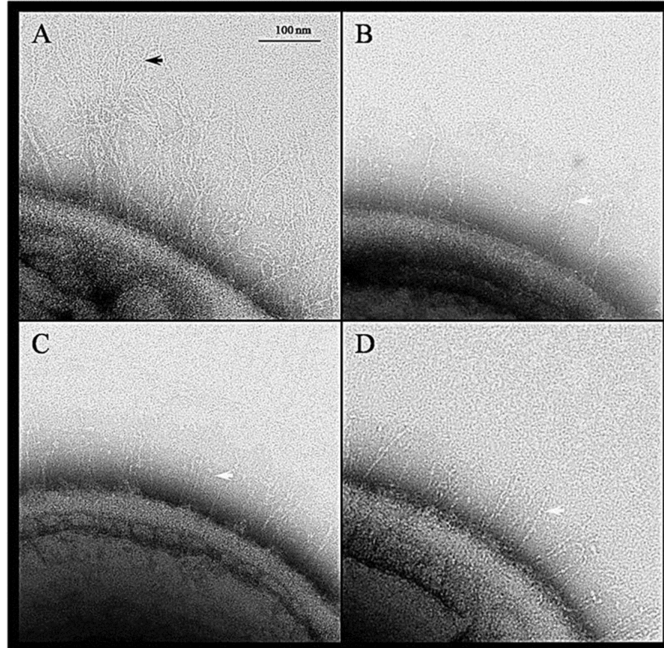

Figure 2. Gap2 is necessary for production of wild-type fimbriae. Transmission electron micrographs of *S. parasanguinis* bacteria wild-type strain and mutants: **(A)** FW213, **(B)** *gap1* mutant, **(C)** *gap2* mutant, **(D)** *gap3* mutant. Black arrow points to the long fimbriae. White arrows point to the short fibrils. Scale bar=100nm.

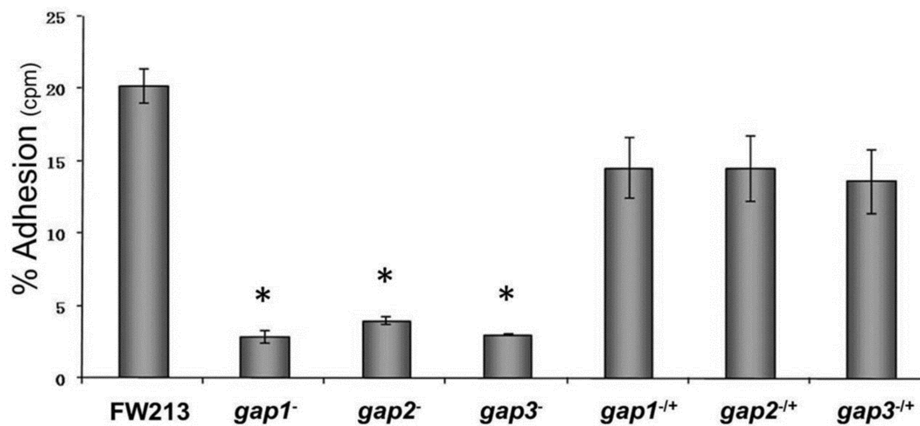

Figure 3. Gap2, like Gap1 and Gap3, is required for *S. parasanguinis* adhesion to SHA. *in vitro* adhesion of *S. parasanguinis* FW213 and its derivatives to saliva-coated hydroxyapatite (SHA). The data were obtained from two independent experiments in

three replicates and are presented as means ± standard deviation. *gap1*$^{-}$, *gap2*,$^{-}$ and *gap3*$^{-}$ are the insertional mutants of *gap* genes; *gap1*$^{-/+}$, *gap2*$^{-/+}$, and *gap3*$^{-/+}$ are the complemented mutant strains. (*) indicates that the level of adhesion was significantly lower than that observed for FW213 (P < 0.003).

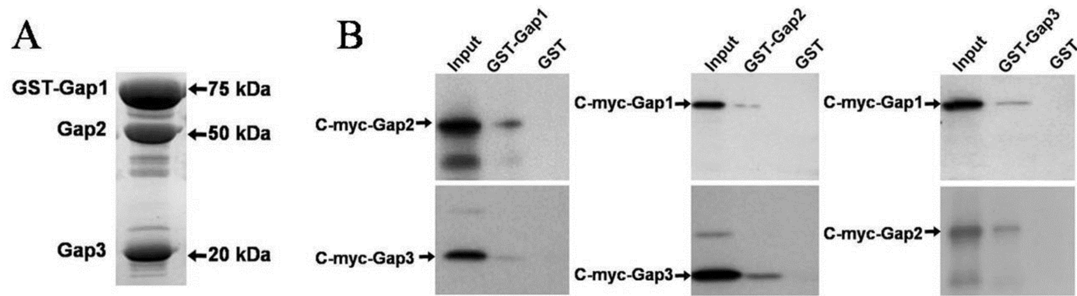

Figure 4. Gap2 interacts with Gap1 and Gap3 individually and together to form a complex. *in vitro* GST pull-down assays to detect interaction among Gap1, Gap2 and Gap3. **(A)** SDS-PAGE analysis of *E. coli* cell lysates expressing GST-Gap1, Gap2, and Gap3. Gap2 and Gap3 are invariably pulled down by GST-Gap1. **(B)** Western blot analysis of GST pull-down assay between Gap1, Gap2, and Gap3. Antibody against c-myc was used.

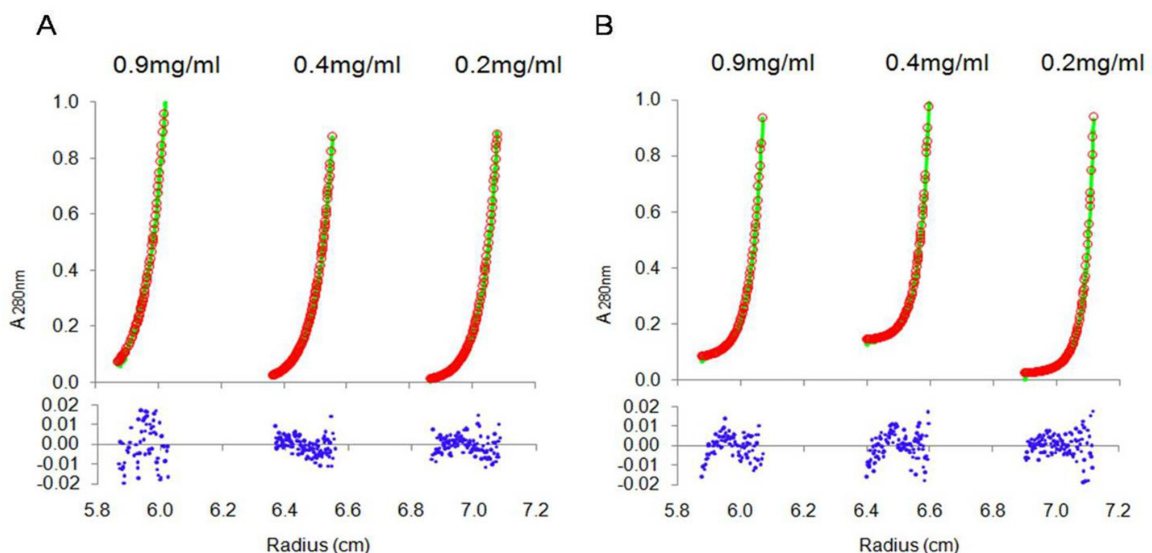

Figure 5. Gap2 binds to the Gap1/3 complex in a reversible manner. Sedimentation equilibrium analysis of protein complex Gap1/3 **(A)** and Gap1/2/3 **(B)**. Three concentrations (0.2 mg/ml, 0.4 mg/ml, and 0.9 mg/ml) were analyzed at 17,000 rpm with detection by absorbance at 280 nm. **(A)** Sedimentation equilibrium data from Gap1/3 were well fit to a single species model. RMSD=0.00736. **(B)** Sedimentation equilibrium data from Gap1/2/3 were well fit to a heterodimer model which consists of monomers Gap2 and Gap1/3, with a K_d of 4.4E-07 M. RMSD=0.00643. *The green curves are the calculated sample based on the model fitting; the red circles are the experimental data points of Gap1/3 or Gap1/2/3 concentration distribution along the radius; the blue dots are the residuals, which represent the difference between the sample and the model values. All residuals were randomly distributed.*

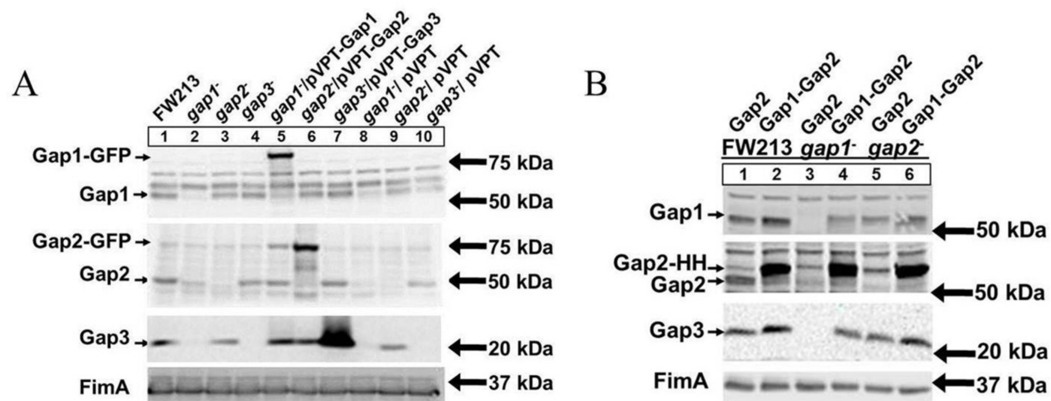

Figure 6. Gap2 amount is increased with overexpression of Gap1. Western blot analysis of Gap1, Gap2, and Gap3 in *S. parasanguinis* cell lysates. **(A)** Strains used include FW213 wild-type, insertional mutants of *gap1*, *gap2*, and *gap3*, and complemented strains of the *gap1*, *gap2*, and *gap3* mutants with the full gene in pVPT or with the empty vector. In the *gap1* and *gap2* complement strains, Gap1 and Gap2 are tagged with GFP.

(B) Strains used include FW213 wild-type, *gap1* mutant, and *gap2* mutant overexpressing Gap2 alone or Gap1 and Gap2 in the pIB184-*hsv-his* vector, where Gap2 is tagged with Hsv-His (abbreviated as HH) in all strains. Polyclonal antibodies against Gap1, Gap2, and Gap3 and FimA (loading control) were used.

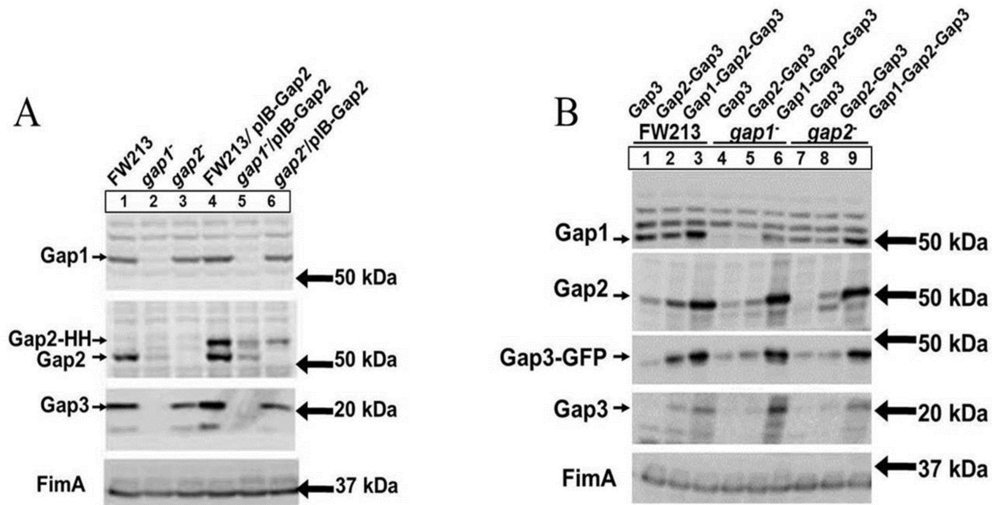

Figure 7. Overexpression of Gap2 increases Gap3 amount; addition of Gap1 to overexpressed Gap2 results in an even greater amount of Gap3. Western blot analysis of Gap1, Gap2, and Gap3 in *S. parasanguinis* cell lysates. **(A)** Strains used include FW213 wild-type, *gap1* mutant, and *gap2* mutant and FW213, *gap1*mutant, and *gap2* mutant overexpressing Gap2 in the pIB184-*hsv-his* vector (tagged protein is abbreviated with HH). **(B)** Strains used include FW213 wild-type, *gap1* mutant, and *gap2* mutant overexpressing Gap3 alone, Gap2 and Gap3, or Gap1, Gap2, and Gap3 in the pIB184-*gfp* vector, where Gap3 is tagged with GFP in all strains. Polyclonal antibodies against Gap1, Gap2, Gap3, and FimA (loading control) were used.

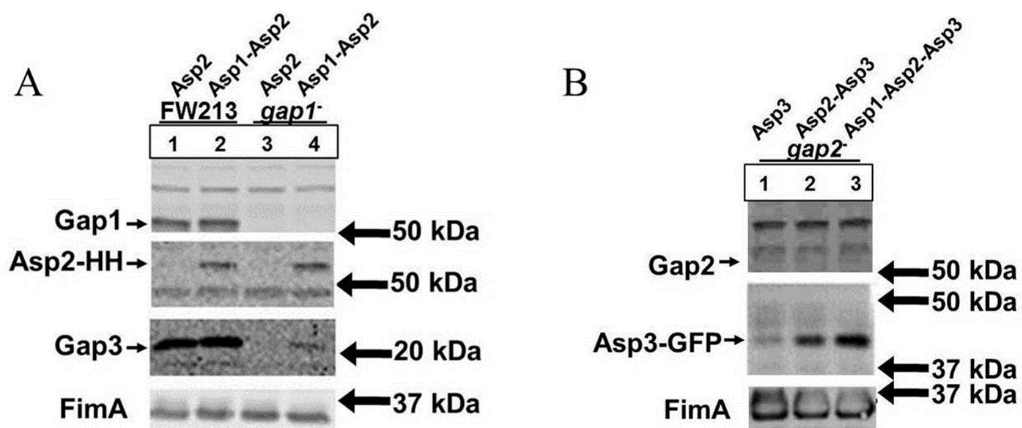

Figure 8. Gap homologs from *S. agalactiae* display same conserved functions as Gap proteins. Western blot analysis of Gap1, Gap2, and the Gap homologs in *S. parasanguinis* cell lysates. **(A)** To check conservation of function, the Gap homologs from *S. agalactiae* J48- Asp1-2- were transformed into *S. parasanguinis* wild-type *and gap1* mutant. Strains included wild-type and *gap1* mutant overexpressing Asp2 alone or Asp1 and Asp2 in the pIB184-*hsv-his* vector, where Asp2 is tagged with Hsv-His (abbreviated as HH) in all strains. **(B)** Gap homologs- Asp1-2-3- were transformed into *S. parasanguinis gap2* mutant. Strains included *gap2* mutant overexpressing Asp3 alone, Asp2 and Asp3, or Asp1, Asp2, and Asp3 in the pIB184-*gfp* vector, where Asp3 is tagged with GFP in all strains. Polyclonal antibodies against Gap1, Gap2, and Gap3 were used. Monoclonal antibodies against Hsv **(A)** and GFP **(B)** were used to detect Asp2 and Asp3, respectively. Antibody against FimA was used as a loading control.

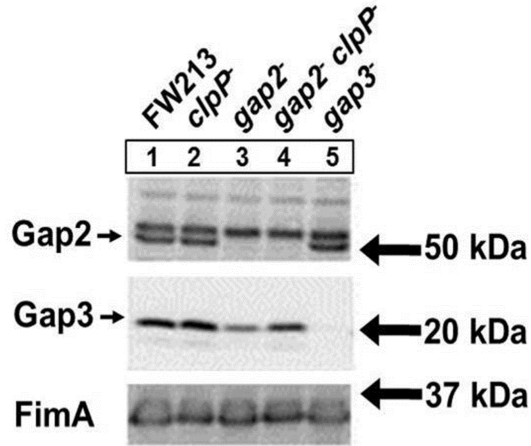

Figure 9. ClpP deficiency in the *gap2* mutant restores the amount of Gap3 nearly to wild-type level. Western blot analysis of Gap2 and Gap3 in *S. parasanguinis* cell lysates. Strains used include FW213 wild-type, *clpP* mutant, *gap2* mutant, *gap2/clpP* double mutant, and *gap3* mutant. Polyclonal antibodies against Gap2, Gap3, and FimA (loading control) were used.

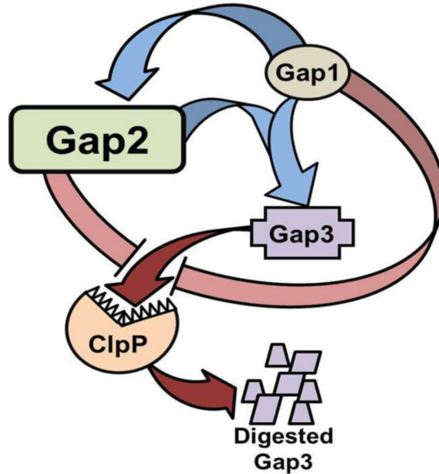

Figure 10. Model representation of Gap interactions. Gap2 is stabilized by Gap1 and augments Gap1's ability to stabilize Gap3 (indicated by blue arrows). Gap2 inhibits (pink arrows) Gap3 degradation by ClpP (red arrows), similar to Gap1.

SUPPLEMENTAL MATERIALS

MATERIALS AND METHODS

RT-PCR to evaluate gene expression

To examine expression of *gap2* and *gap3*, total RNA was extracted from *S. parasanguinis* wild-type, *gap* mutants, and Gap3 overexpressing strains. Standard protocol for ZR RNA MiniPrep (Zymo Research) was followed, with the exception of the lysing step in which 1.5 ml of pelleted cells (OD_{470}= 0.6) were vortexed in 1 mL of TRIzol® Reagent (Invitrogen) and Lysing Matrix B (MP Bio) for 5 minutes on high. Following extraction, RNA was treated with RQ1 DNase (Promega) and then subjected to reverse transcription using M-MLV (Promega) standard protocol and Random Primers (Promega) for cDNA synthesis. cDNA was subsequently used as a template for PCR amplification using a *gap2* primer set (Gap2-1191-F, Gap2-1584-R) and a *gap3* primer set (Gap3-1-F, Gap3-534-R). Another primer set (Gap3-1-F, GFP-101-R) was used to detect only Gap3 expressed by the plasmid in the overexpressing strains. Expression of *fimA* was also detected by RT-PCR with FimA-1-F and FimA-465-R, serving as a control.

TABLES

Table S1. Primers used in this study

Primers	Sequences
Gap2-1191-F	GATCGTCGACCCGACGGCTGTAATTGTAGGTAAG
Gap2-1584-R	GGCGCCGGGATCCTCTTCCAAACTGATCTTCTAGAAT
Gap3-1-F	GCGCCGGCCATGGATGACTAAACAGTTAATTTCTG
Gap3-534-R	GCGGCGCCGGCGGATCCAATATATTCTATTAAATTTTTCACC
GFP-101-F	TCACCCTCTCCACTGACAGAAAATTTGTG
FimA-1-F	GGCATGAAAAAAATCGCTTCTGTCCTCGCCC
FimA-465-R	GGCAATGTTTTTAGCGTAGAGGATCCCG

FIGURES

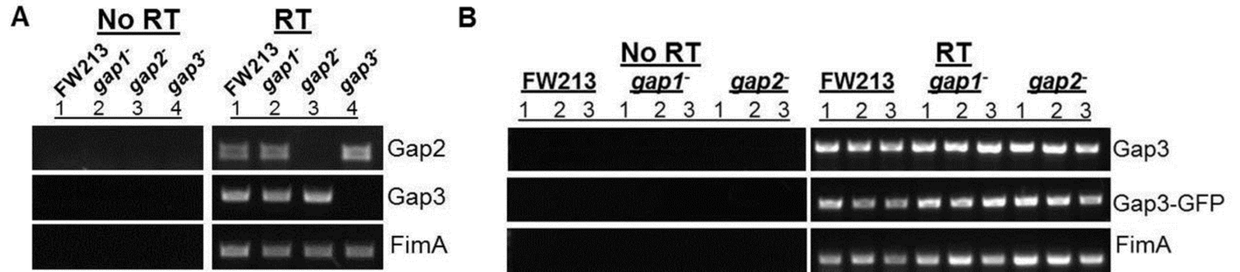

Figure S1. No difference is observed in transcription of *gap2* and *gap3* among strains. RT-PCR analysis in wild-type, *gap* mutants, and Gap3 overexpressing strains. **(A)** Strains include FW213 wild-type and insertional mutants of *gap1*, *gap2*, and *gap3*. Transcription levels of *gap2* and *gap3* were determined. **(B)** Strains include FW213 wild-type, *gap1* mutant, and *gap2* mutant overexpressing Gap3 alone (lanes 1, 4, and 7), Gap2 and Gap3 (lanes 2, 5, and 8), or Gap1, Gap2, and Gap3 (lanes 3, 6, and 9) in the pIB184-*gfp* vector, where Gap3 is tagged with GFP in all strains. Transcription levels of *gap3* and *gap3* transcribed from the plasmid (*gap3*-*gfp*) were determined. Transcription of FimA was used a control.

CHAPTER 3

CLPE SPECIFICALLY TARGETS GAP3 FOR DEGRADATION BY CLPP PROTEASE

by

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ABSTRACT

Serine-rich repeat glycoproteins (SRRPs) are important bacterial adhesins conserved in streptococci and staphylococci that play a role in adhesion, biofilm formation, and pathogenesis. Fap1, a SRRP identified in oral *Streptococcus parasanguinis*, is the major constituent of bacterial fimbriae and is required for adhesion to the tooth surface. Although the exact mechanism of Fap1 biogenesis remains a mystery, an eleven gene cluster surrounding Fap1 is required for its glycosylation and export. Three glycosylation-associated proteins within this cluster (Gap1, Gap2, and Gap3) function together in Fap1 biogenesis. In particular, Gap1 stabilizes Gap3 through prevention of degradation by ClpP protease; Gap2 augments Gap1's function of stabilizing Gap3. Here, we investigated whether Gap3 is targeted for degradation in a specific manner through Clp ATPase selection. Four distinct Clp ATPases were identified in *S. parasanguinis.* Deletion of only one of these four ATPases (*clpE*) restored Gap3 levels in the *gap1* and *gap2* mutant strains, suggesting selectivity of ClpE for the degradation of Gap3. Moreover, Gap1 protected Gap2 from degradation by ClpP; however, this degradation is not as selective as that of Gap3 since loss of both *clpC* and *clpE* restored Gap2 levels. Although the Clp proteolytic complex has an effect on Gap2 and Gap3 stability, there is no apparent effect on Fap1 biogenesis if any of the three Gap proteins are absent—indicating that all three Gap proteins are required for mature Fap1 biogenesis; thus, the Gap proteins play a direct role in the Fap1 biogenesis, while the Clp complex has an accessory function.

INTRODUCTION

Bacterial adhesion to a multitude of different substrates is often the first step required for pathogens to cause disease. Thus, understanding the mechanisms behind production and maintenance of these adhesins is fundamental to understanding bacterial pathogenesis. One adhesin is the family of serine-rich repeat glycoproteins (SRRPs), which are conserved in many Gram-positive bacteria including streptococci, staphylococci, and lactobacilli (1). The SRRP (Fap1) identified in *Streptococcus parasanguinis* plays a role in fimbrial formation, bacterial adhesion, and biofilm formation (1-4). Through adhesion to the tooth surface, *S. parasanguinis* act as a necessary platform for oral pathogens to colonize the oral cavity. Other SRRPs are directly involved in the adhesion of pathogens—including GspB and Hsa of *Streptococcus gordonii* (5-7)*,* PsrP of *Streptococcus pneumoniae* (8), Srr-1 and Srr-2 of *Streptococcus agalactiae* (9, 10), SraP of *Staphylococcus aureus* (11)—and of other important commensals—including SraP of *Streptococcus sanguinis* (12), SrpA of *Streptococcus cristatus* (13), and FimS of *Streptococcus salivarius* (14).

Although SRRPs play such an important role, the exact mechanism of SRRP biogenesis is not well understood. The chromosomal region dedicated to SRRP glycosylation and export is quite large and highly conserved. For Fap1, the cluster is separated into two regions: a conserved core region (*secY2*, *gap1-3*, *secA2*, and *gtf1-2*) and a variable region (*gly*, *nss*, *galT1*, and *galT2*) (1). While *gtf1* and *gtf2* and genes from the *gly-gtf3-galT1*-galT2 locus mediate Fap1 glycosylation, the *secY2-gap1-gap2-gap3-secA2* locus is associated with export of Fap1. SecA2 and SecY2 have homology to their counterparts in the canonical Sec pathway and are required for the export of mature Fap1 to

the cell wall surface (15, 16). There is no known homology for the remainder of the locus (*gap1-gap2-gap3*) outside of the SRRP family. Gap1, Gap2, and Gap3 are required for production of mature Fap1, where loss of any of these proteins results in an immature Fap1, with direct effects on fimbrial formation and adhesion (17). The three Gap proteins interact with each other to form a complex, where Gap1 and Gap3 bind tightly and Gap2 binds in a reversible manner. Gap1 is required for stabilization of both Gap2 and Gap3; Gap2 augment's Gap1 function of stabilizing Gap3. In the absence of Gap1 or Gap2, Gap3 is degraded by ClpP (17, 18).

Clp degradation is important for several cellular processes. One of which is general maintenance through proteolytic removal of misfolded or aggregated proteins. The Clp protease can also be upregulated in response to environmental factors—including oxidation, DNA damage, starvation, and antimicrobials—and in regulation of key cellular processes—including cell cycle, development, and adaptation (19-21). Moreover, Clp proteases can play a role in controlling virulence factors, such as the Isd system in *Staphylococcus aureus* (22) or listeriolysin O in *Listeria monocytogenes* (23). Because Clp proteases have such a wide range of substrates, it is vital that cells regulate substrate specificity. The Clp protease is unable to degrade proteins by itself and requires complexing with an ATPase, which is responsible for providing energy for degradation and for unfolding and translocating the substrates into the proteolytic chamber. ATPases also are responsible for substrate recognition specificity. ATPases can bind substrates directly or bind to adaptor proteins which recognize the substrate—a process which can extend and regulate ATPase binding range (24-26). The number and types of Clp ATPases vary by

species, but those with the recognition tripepetide (e.g. ClpA, ClpC, and ClpE of *E. coli*) will bind the Clp protease to control substrate degradation (25).

Because Gap3 is degraded by ClpP in the absence of Gap1 or Gap2, we hypothesized that Gap3 is selectively targeted by a Clp ATPase for degradation. In this study, we identified four distinct Clp ATPases in *S. parasanguinis*. Deletion of only one of these Clp ATPases (*clpE*) restored Gap3 levels, suggesting that specifically ClpE plays a role in targeting Gap3 for degradation by ClpP. The interactions between these proteins may play an important role in the formation of the stable Gap1/2/3 complex, with direct effect on Fap1 biogenesis. Understanding these interactions will give us insights into bacterial pathogenesis and potential targets for drug development.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Primers, and DNA Manipulation

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *S. parasanguinis* strains were cultured as previously described (27). *E. coli* and *S. parasanguinis* cell concentrations were determined by absorbance at 600 nm and 470 nm, respectively. Antibiotics were used at the following concentrations: 300 µg/ml erythromycin, 50 µg/ml kanamycin, and 50 µg/ml spectinomycin in Luria-Bertani (LB) broth or agar plates for *E. coli*; 10 µg/ml erythromycin, 125 µg/ml kanamycin, and 250 µg/ml spectinomycin in Todd Hewitt (TH) broth or agar plates for *S. parasanguinis* (17). Competent cells for *S. parasanguinis* electroporation were prepared as described previously (28). Standard recombinant DNA techniques were used for DNA preparation and analyses, DNA digestion, ligation, and transformation (29). PCR was carried out with

Taq DNA polymerase (Promega) or KOD DNA polymerase (Novagen). Primers used in this study are listed in Table 2. PCR products were purified with QIAquick PCR Purification Kit (Qiagen). Plasmid DNA preparations were isolated with QIAprep Miniprep Kit (Qiagen).

Western Blot Analysis

All *S. parasanguinis* strains were grown to OD_{470}=0.6-0.7 and centrifuged; cell pellets were subjected to amidase treatment to lyse the cells (30). Samples were boiled in loading buffer (0.0625 M Tris, pH6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue) for 10 min before loading into 10% SDS-PAGE gels and subjected to western blot analysis. Two monoclonal antibodies were used to detect Fap1- mAb E42, which is specific to the peptide backbone of Fap1, and mAb F51, which is specific to the mature Fap1 (3); mAb F51 only recognizes the 200 kDa mature Fap1, whereas mAb E42 recognizes both the 200 kDa mature Fap1 and the 470 kDa Fap1 precursor identified in the *gap* mutants (17). Polyclonal antibodies against Gap1, Gap2, and Gap3 were used to detect each Gap protein individually (17). Polyclonal antibody against FimA was used to standardize protein loading of *S. parasanguinis* lysates.

Construction of the clp *Mutants and the* gap1clp *and* gap2clp *Double Mutants*

The *clp* mutants (including *clpC*, *clpE*, *clpL*, and *clpX*) were constructed by allelic replacement of the respective gene with a kanamycin resistance cassette, *aphA-3* (aminoglycoside phosphotransferase). A fragment containing the *clp* gene and its flanking regions was amplified from *S. parasanguinis* chromosomal DNA using

Clp(C/E/L/X)+Flank-F/Clp(C/E/L/X)+Flank-R. The PCR fragment was ligated into pGEM-T easy (Promega). The entire coding region of the *clpC, clpE,* and *clpL* and 1137 bp region of *clpX* were deleted by inverse PCR using ClpC-PstI-F/ClpC-PstI-R, ClpE-SalI-F/ClpE-SalI-R, ClpL-HindIII-F/ClpL-HindIII-R, and ClpX-HindIII-F/ClpX-HindIII-R, respectively (Table 2). The inverse PCR product was digested with *PstI, SalI,* or *HindIII* and ligated with a promoterless *aphA-3* kanamycin resistance cassette from pALH124 (31) to generate pGEM::Δ*clp(C/E/L/X)-aphA3*. Finally, the *clp* insertion mutants were constructed by transformation of FW213 with pGEM::Δ*clp(C/E/L/X)-aphA3*, followed by selection of kanamycin resistant colonies. The in-frame insertion was further examined by DNA sequencing analyses. The *gap1* (32), *gap2* (17), *gap3* (33), and *clpP* (18) mutants were constructed in a similar method. For the double mutants of *gap1* or *gap2* and the *clp* genes (including *clpC*, *clpE*, *clpL*, and *clpX*), a spectinomycin resistant cassette (Spec) was inserted into the *clp* genes in the *gap1* or *gap2* mutant background. The pGEM::Δ*clp(C/E/L/X)-aphA3* construct was digested with *PstI, SalI,* or *HindIII* to remove the kanamycin resistance cassette and then ligated in-frame with the spectinomycin resistance cassette amplified from pCG1 (34) to construct pGEM::Δ*clp(C/E/L/X)-spec*. The *clp* double mutants were constructed by transformation of the *gap1* or *gap2* mutant with pGEM::Δ*clp(C/E/L/X)-spec*, followed by selection of kanamycin and spectinomycin resistant colonies. The in-frame insertion was further examined by DNA sequencing analyses. The *gap1clpP* and *gap2clpP* double mutants were constructed in a similar method (17).

Complementation of the gap1clpE *and* gap2clpE *Double Mutants*

The full-length *clpE* was amplified from FW213 genomic DNA by PCR using primers ClpE-SalI-F/ClpE-KpnI-R (Table 2). The purified *clpE* PCR products were digested with *SalI* and *KpnI* and then cloned into *E. coli*-*Streptococcus* shuttle vector pVPT-*gfp* (35) to generate corresponding complementation plasmid pVPT-*clpE*-*gfp*. The control vector pVPT-*gfp* was transformed into FW213 and the *gap1*, *gap2*, *gap1clpE*, and *gap2clpE* mutants via electroporation to represent controls. pVPT-*clpE*-*gfp* was transformed into the *gap1clpE* and *gap2clpE* double mutants to act as complemented strains. The transformants were selected on TH agar plates containing kanamycin and erythromycin.

Protein Expression under Nutrient Starvation

To determine if ClpP and ClpE play a role in Fap1 biogenesis under nutrient starvation, protein levels of Fap1 were detected using E42 and F51 monoclonal antibodies in FW213 and *clpP* and *clpE* mutants. Strains were grown overnight in 5 mL THB +/- kanamycin. Overnight cultures were diluted 1:25 into 10 mL THB (no kanamycin). 1 mL of bacterial culture was taken at OD_{470}= 0.2, 0.6, 1.3, 2.4 (overnight-nutrient starved). Samples were diluted back to OD_{470}=0.2 and run on 10% SDS-PAGE for western blot analysis.

RESULTS

Four AAA+ Clp ATPases Identified in S. parasanguinis *FW213*

Because ClpP degrades Gap3 in the absence of Gap1 and Gap2 and because ClpP must associate with an ATPase, which can provide substrate specificity, it is likely that Gap3 is targeted for degradation by a specific ATPase. The number and type of Clp ATPases can vary among bacterial species. For *S. parasanguinis*, four distinct Clp ATPases were identified in the FW213 genome, each localized to a different part of the chromosome. All four Clp ATPases contained AAA+ domains (2 for ClpC, ClpE, and ClpL; one for ClpX) and ATP-binding motifs (Walker A and B, sensor-1 and -2) characteristic of enzymes with ATPase activity. All four also contained a C-terminal ClpB D2-small domain, which likely plays a role in oligimerization into a ring- or cylinder-shaped oligomers or in substrate binding (36, 37) (Fig. 1A). Like ClpX from *E. coli*, the second AAA+ domains of ClpC and ClpE and the single AAA+ domain of ClpX contain the conserved tripeptide ([LIV]-G-[FL]) essential for ClpP recognition (38). ClpL domain does not have this conserved tripeptide, which suggests that it may not interact with ClpP (25, 39); however, ClpL may still play a role in protein stabilization without interaction with ClpP.

Only Double Mutants of clpE *Restore Gap3 Levels*

To determine if any of the four Clp ATPases play a role in targeting Gap3 for degradation, we generated double mutants of each of the *clp* genes- *clpC*, *clpE*, *clpL*, and *clpX*- in the *gap1* or *gap2* mutant background and compared levels of Gap3 to those in FW213 wild-type and the *clpP* double mutants. In the *gap1clpC*, *gap1clpL,* and *gap1clpX*

double mutants (Fig. 2A, Lanes 4, 6, 7) Gap3 was not detected, similar to the *gap1* single mutants (Lane 2). In contrast, Gap3 was partially restored in the *gap1clpE* double mutant (Lane 5 versus 1), but not to the extent as the *gap1clpP* double mutant (Lane 3). Similarly, Gap3 was partially restored in the *gap2clpE* double mutant (Fig. 2B, Lane 5 versus 1)—similar to the *gap2clpP* double mutant—but not in the *gap2clpC*, *gap2clpL*, and *gap2clpX* double mutants (Fig. 2B, Lanes 4, 6, 7). The *clpP*, *clpC*, *clpE*, and *clpX* single mutants (Fig. 2A, 2B, Lanes 8, 9, 10, 12) demonstrated similar Gap3 levels (when adjusted for loading amount). Gap3 in the *clpL* single mutant (Lane 11) is reduced compared to the wild-type. However, this may be due primarily to the reduced level of Gap1 observed. These data suggest that Gap3 is recognized specifically by ClpE—and not by the other three ATPases—for degradation by ClpP.

Gap1 Also Protects Gap2 from Degradation by ClpP

Gap1 not only stabilizes Gap3, but Gap2 as well (17). Degradation of Gap3 by the protease ClpP is inhibited by the presence of Gap1 (18). However, it is not known if Gap1 can protect Gap2 in a similar manner—that is, by preventing degradation by ClpP. Here, we analyzed Gap2 levels in the *gap1clpP* double mutant compared to the *gap1* mutant. Upon deletion of *clpP* in the *gap1* mutant background (Fig. 2A, Lane3), Gap2 was restored to wild-type levels (Lane 1), which was more than in the *gap1* single mutant (Lane 2). No difference in Gap2 was observed between wild-type (Lane 1) and the *clpP* mutant (Lane 8). This result indicates that, like Gap3, Gap2 can be protected by Gap1 from degradation of ClpP.

gap1clpE *and* gap1clpC *Double Mutants Both Restore Gap2 Levels*

Because Gap3 is restored specifically in the *clpE* double mutant and because the presence of Gap1 protects both Gap3 and Gap2, we next wanted to determine if any of the four Clp ATPases play a role in targeting Gap2 for degradation by ClpP in the absence of Gap1. We analyzed Gap2 levels in the *clp* mutants in the *gap1* mutant background in comparison to those observed in wild-type and the *gap1* mutant. Gap2 in the *gap1clpL* and *gap1clpX* double mutants (Fig. 2A, Lanes 6, 7) resembled that observed in the *gap1* single mutant (Lane 2). In the *gap1clpC* and *gap1clpE* double mutants, it appears that Gap2 was partially restored (Lanes 4, 5 versus Lane 1). However, the Gap2 antibody affinity is low and the results were unclear. To adjust for the low affinity, we probed the *gap1clpC* and *gap1clpE* double mutants with a higher antibody concentration (Fig. 3) to more closely examine the effects of the loss of ClpC and ClpE in the *gap1* mutant on Gap2 levels. Here, Gap2 was partially restored in both the *gap1clpC* and *gap1clpE* double mutants (Lanes 4, 5), where Gap2 was detected in a greater amount than in the *gap1* mutant (Lane 2), but not quite to the amount in wild-type (Lane 1) or the *gap2clpP* double mutant (Lane 3). The *clpP*, *clpC*, and *clpE* single mutants (Fig. 2A, Lanes 8, 9, 10) demonstrated Gap2 levels similar to the wild-type. Gap2 in the *clpL* and *clpX* single mutants (Lane 11, 12) was reduced compared to the wild-type. However, this may be due to the reduced level of Gap1 observed.

gap1 *and* gap2 *Mutant Phenotypes Restored Upon ClpE Complementation*

To confirm that the phenotype observed in the *clpE* double mutants is genuine and principally due to the loss of ClpE, we generated complement strains in the *clpE*

double mutants that express a GFP-tagged ClpE on a shuttle vector. As a control, wild-type, *gap1* mutant, *gap2* mutant, and the two *clpE* double mutants were also transformed with the empty shuttle vector solely expressing GFP. Levels of Gap2 and Gap3 were compared by western blot analysis (Fig. 4). As seen previously, Gap3 was restored when *clpE* is deleted in the *gap1* mutant (Lane 3) compared to the *gap1* single mutant (Lane 2). Interestingly, the level of Gap3 is fully restored to that of the wild-type in the double mutant strain containing the shuttle vector (Fig. 4, Lane 3), whereas Gap3 was only partially restored in the double mutant strain alone (Fig. 2A, Lane 5). Similarly, Gap2 was restored to wild-type levels in the *gap1clpE* double mutant harboring the shuttle vector (Fig. 4, Lane 3). This restoration was eradicated upon complementation of the *gap1clpE* double mutant with *clpE-gfp*, where Gap2 and Gap3 levels were similar to those seen in the *gap1* single mutant, indicating that ClpE is responsible for the phenotype of Gap2 and Gap3 restoration in the *clpE* double mutants. Comparably, Gap3 was increased in the *gap2clpE* double mutant (Lane 7) related to the *gap2* mutant (Lane 6), as observed previously. Complementation of *clpE* (Lane 8) reduces the amount of Gap3 detected to levels lower than that in the *gap2* mutant (Lane 6). Together these data suggest that ClpE indeed targets Gap2 and Gap3 for degradation in the absence of Gap1 or Gap2.

clp *Double Mutants Have No Effect on Biogenesis of Mature Fap1*

Because all three Gap proteins are required for mature Fap1 biogenesis (17, 27, 33) and because Gap2 and Gap3 can be degraded through the actions of ClpP/ ClpE (and ClpC for Gap2) in the absence of Gap1 or Gap2, we next tested if the Clp proteins play a

role in directly regulating Fap1 biogenesis. We detected Fap1 levels in the *gap1* and *gap2* single mutants and the *clpP, clpC,* and *clpE* single and double mutants using two distinct antibodies—E42, which recognizes the polypeptide backbone of Fap1, and F51, which detects mature Fap1. In all double mutants (Fig. 5A, 5B, Lanes 3, 4, 5), an immature form of Fap1 was observed similar to that in the *gap1* and *gap2* single mutants (Lane 2), which was only recognized by E42. The *clp* single mutants (Lanes 6, 7, 8) had a similar phenotype as wild-type, where mature Fap1 could be detected by both E42 and F51. These results suggest that the Clp proteins may not play a direct role in mature Fap1 biogenesis. More importantly, these data indicate that all three Gap proteins are required for Fap1 biogenesis. In the *clp* double mutants, in which Gap2 or Gap3 is restored but Gap1 or Gap2 is absent, Fap1 is detected solely as the immature form by E42 and not detected by F51—a phenotype similar to the single *gap* mutants. Because the Clp complex has direct effects on the Gap proteins, it likely plays an accessory role in regulating Fap1 biogenesis under non-optimal conditions.

ClpP and ClpE Do Not Play a Role in Fap1 Biogenesis under Nutrient Starvation

The above data suggest the Clp proteins do not play a direct role in regulating Fap1 biogenesis. However, this experiment was performed under normal logarithmic nutrient available conditions, which may not provide enough stress to induce Clp protein upregulation—which plays an important role in regulating many factors in response to stress—and thereby impact the production of a mature Fap1 through regulation of the Gap complex. Since Fap1 is such a large glycosylated protein, it is likely the cell would modify its production in nutrient limiting conditions; ClpP may be one method of

regulation. It is currently unknown what role ClpP plays in *S. parasanguinis*. In other species, ClpP and its ATPase can play a role in adapting to nutrient limiting conditions (40, 41) and in modulating adhesins (42). To test if the Clp proteins regulate Fap1 biogenesis under nutrient limiting conditions, we subjected wild-type and *clpP* and *clpE* mutants to a condition where nutrient limitation increased over time (over increasing bacterial growth as measured by OD_{470}). Mature Fap1, as detected by both E42 and F51, was observed in all three strains (Fig. 6). The production of mature Fap1 was constant over time in that no immature form was detected regardless if ClpP and ClpE were present or not. This result indicates that ClpP and ClpE do not play a role in regulation of production of mature Fap1 under nutrient limiting conditions. Moreover, the cell produced a consistent amount of mature Fap1 over time (when adjusting for total cells as determined by FimA levels), suggesting that biogenesis of Fap1 is not regulated in these conditions. However, the media used in these experiments was nutrient rich and may not represent true nutrient limiting conditions; this concern can be addressed by using true limiting media in future studies. Indeed, the total amount of mature Fap1 and FimA were reduced overall in the *clpP* mutant, which suggests that ClpP may play some sort of role in regulating production of Fap1, and possibly FimA.

DISCUSSION

Understanding the biogenesis of SRRPs will provide us with a better comprehension of bacterial adhesion and pathogenesis. SRRP biogenesis consists of two parts: glycosylation and export (1). The export of SRRPs to the cell surface is controlled by a conserved accessory secretion locus, which consists of the export system itself

(SecA2 and SecY2) and of accessory secretion proteins (Asp). The exact role of these accessory secretion proteins in the biogenesis of SRRPs is not known. For Fap1, the Asp homologs (Gap1, Gap2, and Gap3) are required for production of mature Fap1, as deletion of any of these three proteins result in an immature form of Fap1. Moreover, these three Gap proteins form a complex, in which Gap1 binds Gap3 tightly and Gap2 binds the Gap1/ Gap3 complex in a reversible manner (17). Within this complex, Gap1 stabilizes both Gap2 and Gap3 and Gap2 further stabilizes Gap3. Without Gap1 or Gap2 present, Gap3 is degraded by ClpP (17, 18).

In this study, we investigated how Gap3 is degraded by ClpP and why Gap2 is unstable in the absence of Gap1. ClpP requires pairing with an ATPase for functionality and for selectivity. *S. parasanguinis* contains four distinct Clp ATPases (Fig. 1), three of which are known to bind ClpP (25, 38, 43). Of these four ATPases, only one was found to restore Gap3- ClpE (Fig. 2). This suggests that ClpE specifically targets Gap3 for degradation by ClpP. Gap2 is also degraded by ClpP in the absence of Gap1 (Fig. 2), but this degradation seems to be less selective, as deletion of both ClpC and ClpE can restore Gap2 (Fig. 3). Targeting these two Gap proteins by the ATPase for degradation by the protease may be a way to regulate Fap1 biogenesis. Indeed, in several other species, specific ATPases degrade particular proteins to regulate virulence, adhesion, and biofilm formation (25, 44-46). For example, in *L. monocytogenes*, while ClpC plays a role in dissemination to hepatocytes and virulence factor expression (45), ClpE affects cell division and survival at higher temperatures (47). *S. pneumoniae* contains the same four orthologs of ATPases as *S. parasanguinis*. In *S. pneumoniae*, ClpC is involved in pneumolysin release and the adaptation to diverse stress conditions in a strain dependent

manner (48). ClpL affects the expression of virulence genes and bacterial adherence (42, 49). ClpX appears to be essential (50)—although we were able to delete the N-terminal region of ClpX in this study. ClpE affects adhesion to host cells and metabolism factors (51). Thus, it is likely that ClpE in *S. parasanguinis* can target specific proteins for degradation, one of which appears to be Gap3 as shown in this study.

If ClpE specifically targets Gap3 for degradation by ClpP, how does in recognize Gap3 and how does Gap1 and Gap2 protect Gap3 from being recognized? ATPases can either recognize and bind a protein directly or can bind via an intermediary adaptor protein. For example, ClpXP in *E. coli* can degrade substrates independently of adaptors, but the adaptor protein greatly enhances the proteolytic activity (21). In other cases, the adaptor recognizes the substrate and then delivers it to the ATPase/protease complex (24, 52). Different ATPases have different preferences for binding short degradation signal (called tags or degrons) that can be found on the N terminus, C terminus, or sometimes in the internal region of a target protein (19, 37, 52). Five distinct classes of peptide tags have been identified in *E. coli* that signal the target protein to be degraded by the ClpXP complex (43). Another type of signal is the N-end rule degron, where substrates have several aromatic residues that destabilize the N-terminus (53, 54). It is currently unknown if Gap3 has a degron that signals it for degradation. There are three to four amino acids conserved is Gap3 homologs that follow the N-end rule; these may act as the target for ClpE binding. In some cases, a protein with a degradation signal only becomes committed to the proteolytic pathway when that tag becomes exposed due to changes in its interaction partners (54). This could explain why Gap3 can be degraded in the absence

of its interaction partners, Gap1 and Gap2; in other words, Gap1 and Gap2 hide the degradation signal of Gap3 when they bind to it.

Because Gap1 and Gap2 protect Gap3 from degradation, it may be possible that Gap3 is the primary mediator or the scaffolding protein necessary for Fap1 biogenesis and the main purpose of Gap1 and Gap2 is to ensure Gap3 stability and activity (17). In contrast, our current data suggest that all three Gap proteins play a role in Fap1 biogenesis directly. If the sole role of Gap1 and Gap2 were to provide protection for Gap3, then deletion of ClpP or ClpE—which are responsible for Gap3 degradation-would restore Fap1 to wild-type phenotype. However, in all double mutants—where Gap3 is no longer degraded, but Gap1 or Gap2 is absent—mature Fap1 was still not produced. In fact, the Fap1 detected in the *clp* double mutants resembled that of the single *gap1* or *gap2* mutants (Fig. 5). This suggests that all three Gap proteins have distinct functions in Fap1 biogenesis and that degradation of Gap2 and Gap3 by Clp protease may be a way to regulate production of mature Fap1, and thereby modulate fimbriae formation and adhesion. Interestingly, Gap3 targeted solely by ClpE while Gap2 is targeted by both ClpE and ClpC, suggesting that Gap2 targeting is less selective. Mechanistically, this may be due to the signal sequence found on the Gap proteins; Gap3 may have a signal that is specific towards ClpE, while the sequence of Gap2 can be recognized by both ClpE and ClpC. This difference in selectivity may be another level of regulation of the Gap complex (and thus Fap1 biogenesis) where ClpE and ClpC may be upregulated under different environmental conditions. For example, ClpC could be induced under low stress conditions and, thereby, degradation of Gap2 would be increased and mature Fap1 biogenesis would be slightly decreased. On the other hand,

ClpE could be induced under high stress conditions and degradation of both Gap2 and Gap3 would ensue—thereby, limiting two of the Gap proteins required for Fap1 biogenesis and, therefore, having more impact on mature Fap1 production than ClpC regulation. Thus, the Clp protease likely regulates Fap1 biogenesis under varying stress conditions. Although there was no immature Fap1 detected in the *clpP* mutant, the total amount of mature Fap1 was reduced overall in the *clpP* mutant, suggesting that ClpP plays some role in regulating production of Fap1 (Fig. 6). Further experiments will need to be performed to test other stress conditions that could induce ClpP expression and alter Fap1 processing via regulation of the Gap complex.

In this study, we provided insights into the regulation of the Gap complex through ClpE targeting and ClpP degradation. Moreover, we demonstrated the necessity of all three Gap proteins for Fap1 biogenesis. However, the exact biochemical function of the Gap complex and if the Clp proteins can regulate the formation of this Gap complex to have an effect of Fap1 biogenesis remains to be elucidated.

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TABLES

*Table 1.*Bacterial strains and plasmids used in this study

Strain, plasmid	Relevant characteristics	Reference, source
Strains		
S. parasanguinis		
FW213	Wild-type	Fives-Taylor *et al.* (1985)
gap1^{--}	*gap1* insertion mutant, Kan^{R}	Li *et al*. (2008)
gap2^{--}	*gap2* insertion mutant, Kan^{R}	This study
gap3^{--}	*gap3* insertion mutant, Kan^{R}	Peng *et al.* (2008)
clpP^{--}	clpP insertion mutant, Kan^{R}	
clpC^{--}	clpC insertion mutant, Kan^{R}	This study
clpE^{--}	clpE insertion mutant, Kan^{R}	This study
clpL^{--}	clpL insertion mutant, Kan^{R}	This study
clpX^{--}	clpX insertion mutant, Kan^{R}	This study
gap1^{--} clpP^{--}	*gap1* insertion mutant, Kan^{R}; *clpP* insertion mutant, Spec^{R}	
gap1^{--} clpC^{--}	*gap1* insertion mutant, Kan^{R}; *clpC* insertion mutant, Spec^{R}	This study
gap1^{--} clpE^{--}	*gap1* insertion mutant, Kan^{R}; *clpE* insertion mutant, Spec^{R}	This study
gap1^{--} clpL^{--}	*gap1* insertion mutant, Kan^{R}; *clpL* insertion mutant, Spec^{R}	This study
gap1^{--} clpX^{--}	*gap1* insertion mutant, Kan^{R}; *clpX* insertion mutant, Spec^{R}	This study
gap2^{--} clpP^{--}	*gap2* insertion mutant, Kan^{R}; *clpP* insertion mutant, Spec^{R}	Echlin et al. (2013)
gap2^{--} clpC^{--}	*gap2* insertion mutant, Kan^{R}; *clpC* insertion mutant, Spec^{R}	This study
gap2^{--} clpE^{--}	*gap2* insertion mutant, Kan^{R}; *clpE* insertion mutant, Spec^{R}	This study
gap2^{--} clpL^{--}	*gap2* insertion mutant, Kan^{R}; *clpL* insertion mutant, Spec^{R}	This study
gap2^{--} clpX^{--}	*gap2* insertion mutant, Kan^{R}; *clpX* insertion mutant, Spec^{R}	This study
FW213/*gfp*	FW213 containing pVPT-*gfp*, vector control strain, Erm^{R}	
gap1^{--}/ *gfp*	*gap1*$^{--}$ containing pVPT-*gfp*, vector control strain, Erm^{R}	Zhou *et al.* (2012)
gap2^{--}/ *gfp*	*gap2*$^{--}$ containing pVPT-*gfp*, vector control strain, Erm^{R}	Echlin et al. (2013)
gap1^{--} clpE^{--}/ *gfp*	*gap1*$^{--}$ clpE^{--} containing pVPT-*gfp*, vector control strain,	This study
gap2^{--} clpE^{--}/ *gfp*	*gap2*$^{--}$ clpE^{--} containing pVPT-*gfp*, vector control strain,	This study
gap1^{--} clpE^{--}/ *clpE-gfp*	*gap1*$^{--}$ clpE^{--} containing pVPT-clpE-*gfp*. Erm^{R} Kan^{R} Spec^{R}	This study
gap2^{--} clpE^{--}/ *clpE-gfp*	*gap2*$^{--}$ clpE^{--} containing pVPT-clpE-*gfp*. Erm^{R} Kan^{R} Spec^{R}	This study
E. coli		
Top10	Host strain for cloning	Invitrogen
Plasmids		
pVPT-*gfp*	*E. coli and S. parasanguinis* shuttle vector. Erm^{R}	Zhou *et al.* (2008a)
pVPT-*clpE-gfp*	*clpE* from FW213 cloned into pVPT-*gfp*. Erm^{R}	This study
pGEM::Δ*clpC-aphA-3*	pGEM vector containing *clpC* with *aphA-3* insertion. Kan^{R}	This study
pGEM::Δ*clpC-spec*	pGEM vector containing *clpC* with s*pec* insertion. Spec^{R}	This study
pGEM::Δ*clpE-aphA-3*	pGEM vector containing *clpE* with *aphA-3* insertion. Kan^{R}	This study
pGEM::Δ*clpE-spec*	pGEM vector containing *clpE* with s*pec* insertion. Spec^{R}	This study
pGEM::Δ*clpL-aphA-3*	pGEM vector containing *clpL* with *aphA-3* insertion. Kan^{R}	This study
pGEM::Δ*clpL-spec*	pGEM vector containing *clpL* with s*pec* insertion. Spec^{R}	This study

pGEM::Δ*clpX-aphA-3*	pGEM vector containing *clpX* with *aphA-3* insertion. Kan^R	This study
pGEM::Δ*clpX-spec*	pGEM vector containing *clpX* with *spec* insertion. $Spec^R$	This study

*Table 2.*Primers used in this study

Primers	**Sequences**
ClpC+Flank-F	GATCCCGCGGCAGACCATTCGGAAAAAGAAGGTGTTGAAAGTCCG
ClpC+Flank-R	GATCCATATGGGGCTGTTGCTCCCAGATTTGAGGTC
ClpE+Flank-F	CAGATCCCGCGGAAAATGAGCTTGAGACCACCCCAAAC
ClpE+Flank-R	GGATCGACACATATGCCGACACCGGTTGGTCCGACAAAGAG
ClpL+Flank-F	GACTCCGCGGGAAAGAGAGTTTCAAAAAGCGC
ClpL+Flank-R	CGCGGCGCCATATGTTATTCTGCTTCTTTATCCGTAT
ClpX+Flank-F	GCATTTCAAAGAAACAACCA
ClpX+Flank-R	GAGAAAACGATAAAGGTGTC
ClpC-PstI-F	GATCCTGCAGGGAACCTTGCGTACAGTTGGAGCAA
ClpC-PstI-R	GCGCCTGCAGCTAGTCGGTATTTCCTTTTCTATC
ClpE-SalI-F	GATCGTCGACCGCTACATCCAAGATCGCTTCCTG
ClpE-SalI-R	GGCGCGTCGACACCTCACAGTAGGTTTTTGATGG
ClpL-HindIII-F	GGCGCCAAGCTTACCAAACAAGAAGAAGCTGCTG
ClpL-HindIII-R	GGCGCCAAGCTTATTGTATACCTCTAATTTAC
ClpX-HindIII-F	TAGCAAGCTTATTACAAAAGAAGCAGTAGAC
ClpX-HindIII-R	TAGCAAGCTTTGAACAATAAACCATCATATC
ClpE-SalI-F	GGCGGCGGTCGACATGTATATGCTTTGTCAAAATTG
ClpE-KpnI-R	GATCGGTACCTGCTTCTGACTTCTTTTCGGCCGTTT

FIGURES

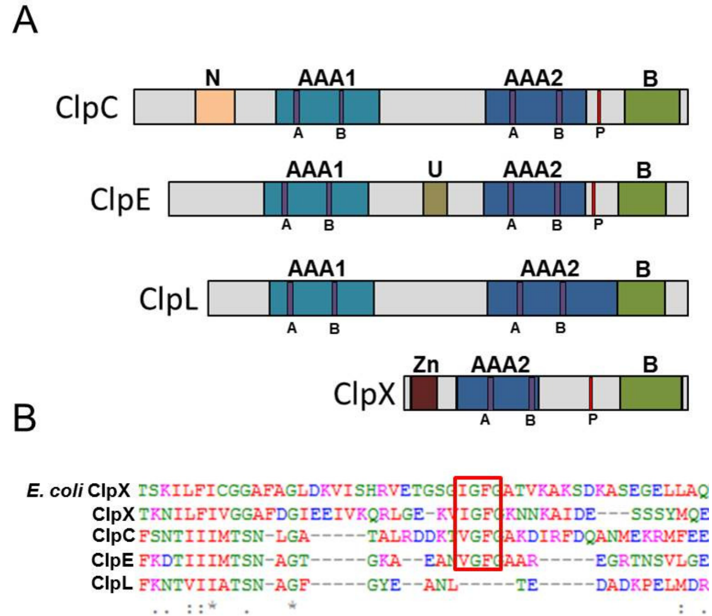

Figure 1. Four Distinct ATPases were identified in *S. parasanguinis* FW213 genome. **(A)** Schematic depicting domains identified in each ATPase. All four ATPases contain characteristic AAA+domains with Walker A (A) and Walker B (B) motifs; ClpC, ClpE, and ClpL contain two, while ClpX contains one. Other functional domains include the P domain required for binding to ClpP, the Zn binding domain involved in dimerization, the N domain proposed to be involved in protein binding, the UVR (U) domain which is homologous to the interaction domain between the nucleotide excision repair proteins, and the ClpB (B) subunit which likely plays a role in oligimerization. **(B)** Clustal Omega alignment of the P tripeptide (red box) of the four ATPases from *S. parasanguinis* in comparison with ClpX from *E. coli*, which is known to bind to ClpP. Notable, ClpL lacks the P tripeptide.

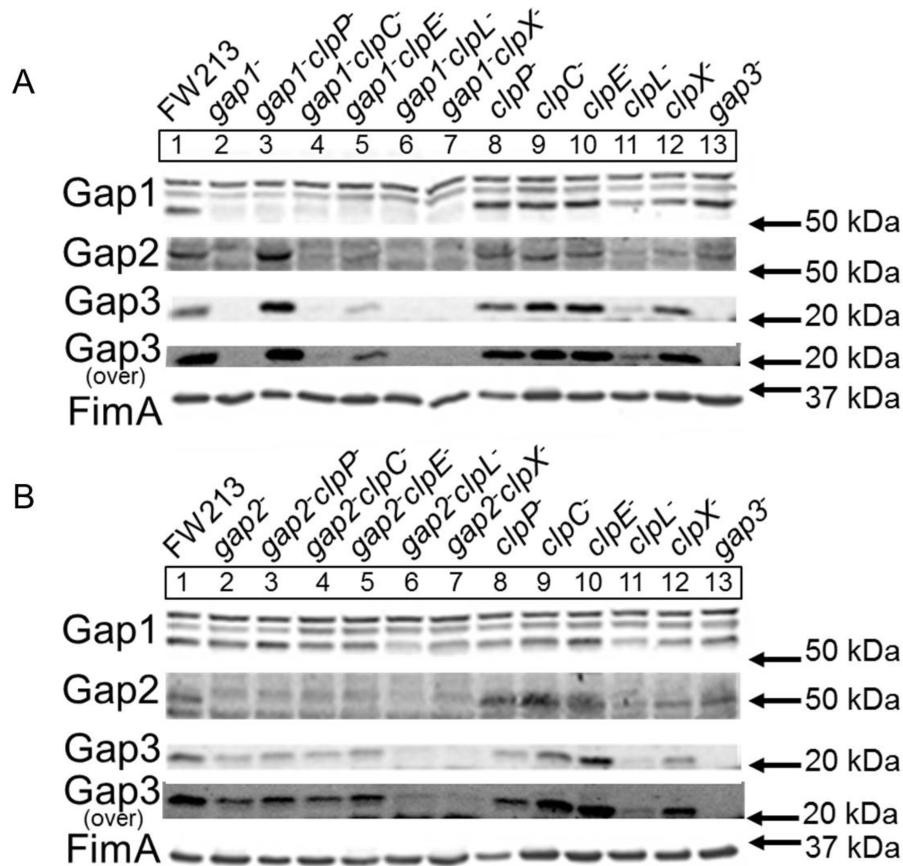

Figure 2. ClpE deficiency in the *gap1* or *gap2* mutant background restores Gap3. Western blot analysis of Gap1, Gap2, and Gap3 in *S. parasanguinis* cell lysates. **(A)** Strains used include FW213 wild-type and insertional mutants of *gap1, gap3, clpP*, *clpC*, *clpE*, *clpL*, and *clpX* in FW213 background and of *clpP*, *clpC*, *clpE*, *clpL*, and *clpX* in the *gap1* mutant background. **(B)** Strains used include FW213 wild-type and insertional mutants of *gap2, gap3, clpP*, *clpC*, *clpE*, *clpL*, and *clpX* in the FW213 background and of *clpP*, *clpC*, *clpE*, *clpL*, and *clpX* in the *gap2* mutant background. Polyclonal antibodies against Gap1, Gap2, Gap3, and FimA (loading control) were used.

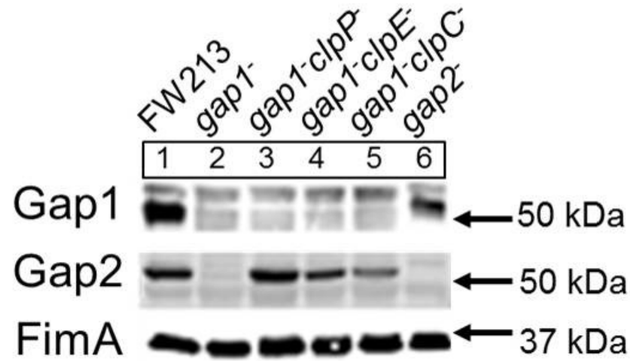

Figure 3. Deficiency of ClpC or ClpE in the *gap1* mutant background restores Gap2. Western blot analysis of Gap1 and Gap2 in *S. parasanguinis* cell lysates. Strains used include FW213 wild-type and insertional mutants of *gap1* and *gap2* and of *clpP*, *clpC*, and *clpE* in the *gap1* mutant background. Polyclonal antibodies against Gap1, Gap2, and FimA (loading control) were used.

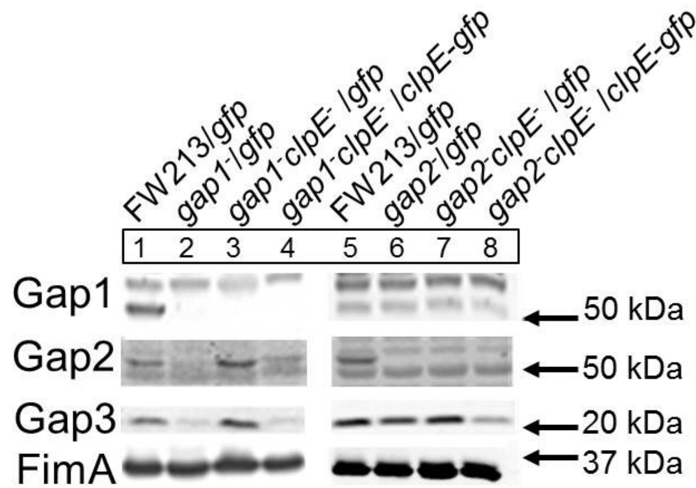

Figure 4. ClpE phenotype can be complemented back to wild-type. Western blot analysis of Gap1, Gap2, and Gap3 in *S. parasanguinis* cell lysates. Strains harboring the empty vector pVPT-*gfp* included FW213 wild-type and insertional mutants of *gap1* and *gap2* and of *clpE* in the *gap1* and *gap2* mutant background. Strains harboring the

complementation vector pVPT-*clpE-gfp* included insertional mutants of *clpE* in the *gap1* and *gap2* mutant background. Polyclonal antibodies against Gap1, Gap2, Gap3, and FimA (loading control) were used.

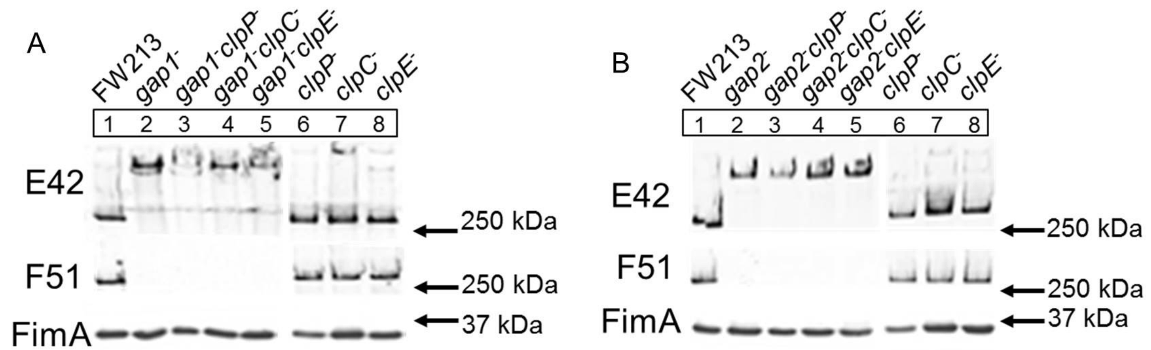

Figure 5. Restoration of Gap2 or Gap3 in the *gap1* or *gap2* mutant background does not restore Fap1 biogenesis. Western blot analysis of Fap1 in *S. parasanguinis* cell lysates. **(A)** Strains used include FW213 wild-type and insertional mutants of *gap1*, *clpP*, and *clpE* in FW213 background and of *clpP*, *clpC*, and *clpE* in the *gap1* mutant background. **(B)** Strains used include FW213 wild-type and insertional mutants of *gap2*, *clpP*, and *clpE* in FW213 background and of *clpP*, *clpC*, and *clpE* in the *gap2* mutant background. Monoclonal antibodies against Fap1—F51 (specific to mature Fap1), E42 (specific to the polypeptide Fap1)—and polyclonal antibody against FimA (loading control) were used.

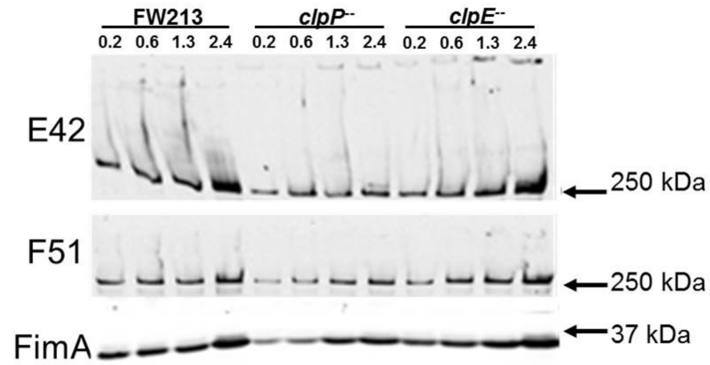

Figure 6. ClpP and ClpE do not regulate Fap1 biogenesis under nutrient limiting conditions. Western blot analysis of Fap1 in *S. parasanguinis* cell lysates at OD_{470}= 0.2, 0.6, 1.3, and 2.4. Strains used include FW213 wild-type and insertional mutants of *clpP* and *clpE.* Monoclonal antibodies against Fap1—F51 (specific to mature Fap1), E42 (specific to the polypeptide Fap1)—and polyclonal antibody against FimA (loading control) were used.

CHAPTER 4

CONCLUSION

SIGNIFICANCE

Streptococci species can be found in a wide array of environments, including numerous surfaces on or within the human body. These bacteria possess a multitude of adhesins on their cell surfaces that facilitate adherence to a wide range of substrates, allowing streptococci to interact with diverse environments (1, 2). Their ability to bind to such a great number of substrates contributes, in part, to their high success rate in colonization of the oral and epithelial surfaces of humans. One type of adhesin is the family of serine-rich repeat proteins (SRRPs), which are conserved in Gram-positive bacteria (3). The first identified SRRP was Fap1, which is required for fimbrial formation, bacterial adhesion, and biofilm formation of *Streptococcus parasanguinis* (2-5). *S. parasanguinis* adheres to the tooth surface and plays an important role in the formation of dental plaque by acting as a necessary platform for oral pathogens to attach and form dynamic and complex oral biofilms (6). Several other SRRPs have been identified and have implications in adhesion, biofilm formation, interaction with hosts, and pathogenesis (3, 4, 7-12); these include GspB and Hsa of *S. gordonii* (13, 14), SraP of *S. sanguinis* (15), PsrP of *S. pneumoniae* (10), Srr-1 and Srr-2 of *S. agalactiae* (8, 9), SrpA of *S. cristatus* (16), SraP of *S. aureus* (3, 11), and FimS of *S. salivarius* (17).

Although SRRPs play such an important role, the exact mechanism of SRRP biogenesis remains to be elucidated. SRRP biogenesis can be broken into two principal steps: glycosylation and export. To date, much has been learned concerning both the glycosylation and the export of SRRPs; the genes in the conserved loci surrounding the SRRP control both steps. The number and type of glycosyltransferases varies by species, but the conserved GtfA/GtfB complex is required for the initial glycosylation step, which is necessary for export of the SRRPs. The other glycosylation steps alter the glycosylation pattern of the SRRP; these sugar modifications are not required for SRRP export. For example, Fap1 of *S. parasanguinis* is glycosylated by GtfA/GtfB homologs through the addition of GlcNAc; this is followed by further sugar modification by Gtf3 (glucose), GalT1 (GlcNAc and glucose), and GalT2 and Gly (18-21). In *S. gordonii*, GspB is glycosylated by the GtfA/GtfB complex, followed by modification by Gly and Nss (13, 22). Differential glycosylation may mediate dynamic bacterial interactions with their encountered environments.

The five conserved accessory secretion components (*secA2-secY2-asp1-asp2-asp3*) are required for export of SRRPs (23-30). SecA2 and SecY2 have homology to their counterparts in the canonical Sec pathway and are believed to form the secretion apparatus (24, 25). There is no known genetic homology for the three Asp proteins outside of the SRRP family. Structurally, Asp1 shares homology with glycosyltransferases (27), Asp2 has some homology with a hydrolase (31), and Asp3 is predicted to possess a carbohydrate-binding domain—which may have affinity for the glycosylated SRRP precursor (27). However, to date, no studies have delineated the true purpose of these three proteins. Asp1 and Asp3 homologs (Gap1 and Gap3) in *S.*

parasanguinis are required for production of a mature Fap1, where *gap1* and *gap3* mutants produce a similar high molecular mass (HMM) form of Fap1. Moreover, the interaction between Gap1 and Gap3 is required for Fap1 biogenesis, further indicating that Gap1 and Gap3 are involved in mature Fap1 biogenesis (27, 28, 32). However, the impact and function of Gap2 in Fap1 biogenesis is unknown. In this study, to elucidate the function of Gap2, we analyzed the interactions between Gap2 and Gap1/Gap3 and how these interactions affect Fap1 biogenesis.

EFECT OF GAP2 ON FAP1 BIOGENESIS

Although current reports indicate that Gap1 and Gap3 are involved in Fap1 biogenesis, little is known about the role that Gap2 plays in Fap1 biogenesis. Deletion of *gap2* results in production of a HMM form of Fap1 (7, 27, 28, 31)—similar to that seen in the *gap1* and *gap3* mutants. Moreover, export of this HMM Fap1 is greatly reduced in the *gap2* mutant (Introduction, Fig. 3). Because export of Fap1 is minimal in the *gap2* mutant, there is a direct effect observed on fimbriae biogenesis and adhesion (31). Thus, loss of *gap2*, similar to loss of *gap1* and *gap3*, results in production of a HMM Fap1; export of this Fap1 is greatly reduced. Similarly, GspB and SraP from *S. gordonii* and *S. aureus*, respectively, fail to export when *asp2* is inactivated (26, 29). Taken together, these studies solidify the concept that Gap2, along with Gap1 and Gap3, is required for mature Fap1 biogenesis; without these three proteins, an immature form of Fap1 is generated, export of which is greatly reduced. Because Gap2 shares a similar phenotype as Gap1 and Gap3, it is likely that Gap2 interacts with Gap1 and Gap3.

GAP2 INTERACTIONS WITH GAP1 AND GAP3

In *S. parasanguinis*, Gap1 and Gap3 form a tight complex; interaction between Gap1 and Gap3 is required for Fap1 biogenesis (27). Gap2 can interact with this complex in a reversible manner (31). Because Gap2 forms a complex with Gap1/3, it is likely that the three proteins can affect one another within the complex. Indeed, Gap1 stabilizes Gap3 by preventing degradation by ClpP protease (33). Gap2 augments stabilization of Gap3 by preventing degradation by ClpP as well (31). Mechanistically, Gap2 and Gap1 protect Gap3 from being targeted for degradation via the ClpE ATPase—most likely via binding of the ATPase recognition site on Gap3 (34). Like Gap3, Gap2 is protected by Gap1 from degradation by ClpP protease (31, 34). Gap2 can be targeted for degradation by either ClpC or ClpE ATPase (34). Similarly, in *S. gordonii*, a complex is formed between Asp1, Asp2, and Asp3, where Asp3 can interact with the other two Asp proteins (35). Asp2 can stabilize Asp3 and can be stabilized by Asp1 (31, 33); however, whether the Clp proteolytic complex can degrade Asp2 and Asp3 is unclear.

Together, these studies demonstrate that Gap2 can interact with Gap1/3 to form a complex in which Gap2 and Gap3 are stabilized by Gap1 and in which Gap2 can enhance Gap3 stability. In the absence of Gap1 and Gap2, Gap3 is targeted by ClpE for degradation by ClpP and, in the absence of Gap1, Gap2 is targeted by ClpC or ClpE for degradation (Fig. 1). Under normal conditions, Gap1 protects Gap2 and Gap3 from degradation and, together, the Gap proteins form a stable complex necessary for Fap1 biogenesis. In non-optimal environments, the Clp protease may be upregulated. Due to the increase in Clp concentration, Gap2/Gap3 could become more easily targeted for degradation; thus, the Clp protease would have an indirect impact on Fap1 biogenesis.

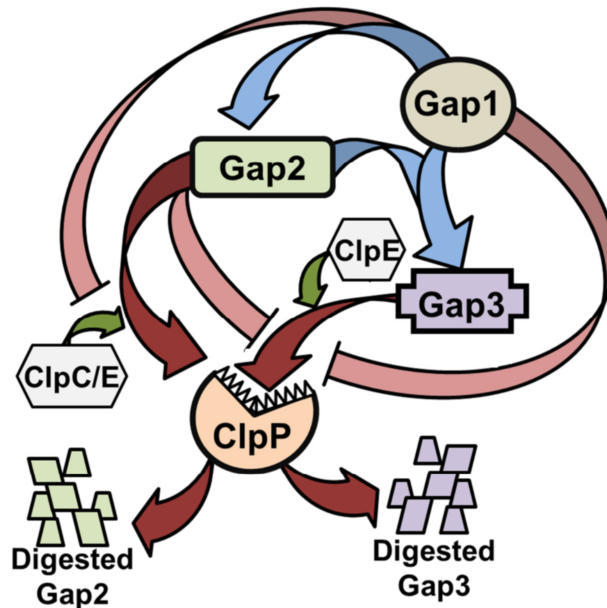

Figure 1. Model of Gap2 interactions with Gap1 and Gap3. Gap2 interacts with Gap1/3 to form a complex. Gap2, along with Gap3, is stabilized by Gap1 and can enhance Gap3 stability (blue arrows). In the absence of Gap1 and Gap2, Gap3 is targeted by ClpE (green arrow) for degradation by ClpP (red arrow). In the absence of Gap1, ClpC or ClpE target (green arrow) Gap2 for degradation by ClpP (red arrow). Gap1 and Gap2 can prevent targeting and degradation events (pink arrows). [From "Gap2 promotes the formation of a stable protein complex required for mature fap1 biogenesis" by H. Echlin, et al, 2013, *Journal of Bacteriology*, 195, p. 2166. Adapted with permission].

ROLE OF GAP2 IN EXPORT OF FAP1

Although interaction between Gap2 and Gap1/3 is required for Fap1 biogenesis, the following questions still remain: 1) does Gap2 play a role in glycosylation of Fap1, export, or both? 2) what is the exact function of Gap2 in Fap1 biogenesis? Gap1—and to some extent Gap3—interacts with SecA2, suggesting the Gap complex may play a role in export of Fap1 (23). It is currently unknown if Gap2 interacts with SecA2; however, a

recent study indicates that Asp2 from *S. gordonii* interacts with SecA2 (36). Moreover, the three *gap* mutants share a similar Fap1 phenotype as the *secA2* and *secY2* mutants (Introduction, Fig. 3). Thus, Gap2, along with Gap1 and Gap3, likely plays a role in export of Fap1 and, therein, would localize to the membrane. Subcellular localization experiments were performed to test this hypothesis. In wild-type *S. parasanguinis*, Gap1, Gap2, and Gap3 localized to both the membrane and cytoplasm fractions (Fig. 2). In the *gap1* and *gap3* mutants, Gap2 is detected only in the membrane fraction, suggesting that Gap2 is localized to the membrane in the absence of Gap1 and Gap3. If *gap2* is deleted, there is a shift from cytoplasmic to membrane for the other two Gap proteins, which likely associate with the membrane through interaction with SecA2.

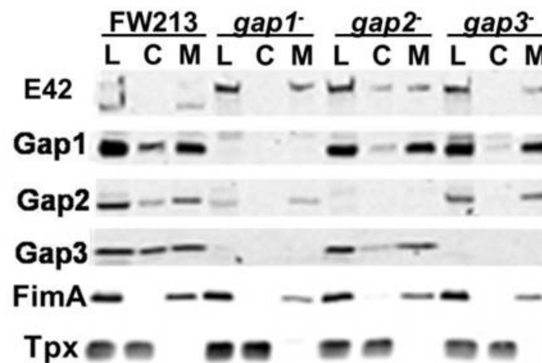

Figure 2. Cellular localization of wild-type and *gap* mutants of *S. parasanguinis*. Western blot analysis of whole cell lysate (L), cytoplasm fraction (C), and membrane fraction (M) of wild-type FW213 and *gap1, gap2,* and *gap3* mutant strains. Monoclonal antibody against Fap1 polypeptide backbone (E42) and polyclonal antibodies against Gap1, Gap2, Gap3, FimA, and Tpx were used. Fractionation controls included FimA (a lipoprotein associated with the membrane) and Tpx (a cytosolic protein).

Taken together, these data suggest that Gap2 localizes to the membrane and interacts with Gap1/3 (which is brought near the membrane via interaction with SecA2) to form the Gap1/2/3 complex. By forming a complex with Gap1/3, Gap2 may stimulate interruption of an interaction between Gap1/3 and SecA2, whereby the Gap1/2/3 complex becomes more cytoplasmic. The dynamic interaction between Gap2 and Gap1/3 likely regulates the cycling of the complex between the cytoplasm and the membrane.

MODEL FOR GAP2 INTERACTIONS

From our current knowledge, we established a working model that demonstrates how the three Gap proteins form a complex that can cycle between the cytoplasm and the membrane (Fig. 3). In this model, Gap1 and Gap3 interact with each other in the cytoplasm to form a tight complex that can then interact with SecA2 (Step I). Gap1/3 interaction with SecA2 brings the complex within proximity of Gap2, which is localized to the membrane. Gap2 interacts with Gap1/3 to form the Gap1/2/3 complex—thereby interrupting the interaction between Gap1/3 and SecA2—and is released into the cytoplasm (Step II). In the cytoplasm, the Gap1/2/3 complex recruits some unknown factor or interacts directly with Fap1 as proposed previously (36). Then, the Gap1/2/3 complex translocates back to the membrane (Step III). Here, Gap2 separates from Gap1/3 (perhaps when relinquishing its cargo) and, thereby, mature Fap1 is exported; Gap1/3 then can bind SecA2 again, providing the stage for another round of the cycle (Step IV).

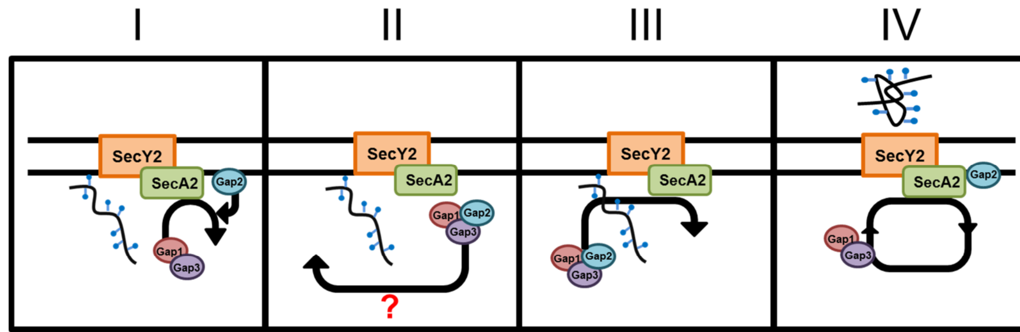

Figure 3. Model of interactions among accessory secretion components in *S. parasanguinis* wild-type. The sequence of events (I-IV) follows horizontally.

In this model, all three Gap proteins are required for biogenesis of a mature Fap1. In the absence of Gap1, Gap3 becomes accessible for degradation by the Clp protease. Gap2 remains membrane localized since interaction with Gap1/3 is required to release it into the cytoplasm; Gap2 is degraded by ClpP to some extent. In the absence of Gap2, the Gap1/3 complex interacts with SecA2. However, without Gap2, Gap1/3 remains bound to SecA2; over time, Gap1/3 can dissociate from SecA2. In the absence of Gap3, Gap1 alone can interact with SecA2 (23). Because Gap2 likely has a higher affinity for the Gap1/3 complex, Gap2 interaction with Gap1 is limited. Because interaction between Gap2 and Gap1 is rare, Gap1 remains bound to SecA2. In all of these circumstances, when any of the Gap proteins is absent, Fap1 is not processed and exported properly.

FUNCTION OF GAP2

Although these data indicate that Gap2 localizes to the membrane and can transit between the membrane and the cytoplasm upon interaction with Gap1/3, they do not explain how this cycling plays a role in Fap1 biogenesis. One possibility is that the

Gap1/2/3 complex interacts with Fap1 to traffic it toward the membrane. Indeed, Gap3 can bind Fap1 (unpublished data); the binding affinity between Gap1 or Gap2 and Fap1 is unknown. However, it is unlikely that the role of the complex is to actually traffic Fap1 to the membrane. Fap1 is targeted for export by its own signal sequence at the N terminus (37). Moreover, Fap1 localizes to the membrane even in the absence of the Gap1/2/3 complex (Fig. 2). Indeed, a similar proposal has suggested that binding of GspB to SecA2 is mediated by the signal peptide and can occur in the absence of Asp1/2/3 (38).

Alternatively, the Gap proteins may be cycling to recruit some other proteins to the membrane—perhaps glycosyltransferases. Indeed, the HMM Fap1 produced in the *gap1* mutant has altered glycosylation patterns (27), suggesting that the Gap proteins affect Fap1 glycosylation. The Gap proteins may play a direct role in Fap1 glycosylation or may play a role in processing and export of Fap1, with the ultimate effect on glycosylation. Either way, the Gap1/2/3 complex likely functions after partial glycosylation of Fap1. The HMM Fap1 produced in the *gap* mutants can be detected by D10, an antibody specific to Fap1 with a glucose-glucose modification, which is an intermediary step in the glycosylation of Fap1. This HMM form of Fap1 is larger than the HMM form of Fap1 found in the *gtf1, gtf2, galT1,* and *galT2* mutants (Introduction, Fig. 3), further indicating that the Gap1/2/3 complex functions after glycosylation. Thus, the effect the Gap proteins have on Fap1 glycosylation would occur in the later stages of Fap1 modification. One clue to help us elucidate the role the Gap proteins play is to better understand the HMM form of Fap1 that is observed in the *gap* mutants and how this form differs from the mature Fap1.

CONCLUSION

Serine-rich repeat proteins offer bacteria adhesion to a wide variety of substrates and are found among several groups of Gram-positive bacteria. Several proteins are involved in both the glycosylation and export of SRRPs. Only the initial glycosylation step is required for export of the SRRP; the remaining glycosylation steps alter the sugar modifications on SRRP but are not required for its export. The five proteins (SecA2, SecY2, Asp1, Asp2, and Asp3) required for SRRP export are highly conserved. These five proteins may also play a role in regulation of the production and export of the mature SRRP. For example, the three Asp homologs (Gap) from *S. parasanguinis* have a dynamic relationship of stabilization and translocation that may act as a way to regulate Fap1 export. In particular, Gap2 forms a complex with Gap1/3 in a reversible manner; this may prevent continuous Fap1 export. Although the precise function of each of the Gap proteins remains unknown, it is likely that each Gap protein is dependent on the other for full functionality, perhaps through conformational changes upon interaction or through modification. Understanding the dynamics and function of this complex will afford us with a greater insight into the biogenesis of SRRPs, which can then provide us a drug target to alter the adhesion of pathogens and thereby prevent disease.

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