JOURNAL OF BACTERIOLOGY, June 2005, p. 3878–3883 Vol. 187, No. 11
0021-9193/05/$08.00+0 doi:10.1128/JB.187.11.3878–3883.2005

Two Additional Components of the Accessory Sec System Mediating Export of the *Streptococcus gordonii* Platelet-Binding Protein GspB

Daisuke Takamatsu, Barbara A. Bensing, and Paul M. Sullam*

Department of Medicine, Veterans Affairs Medical Center, and the University of California, San Francisco, California

Received 7 December 2004/Accepted 16 February 2005

The *gspB-secY2A2* locus of *Streptococcus gordonii* strain M99 encodes the platelet-binding glycoprotein GspB, along with proteins that mediate its glycosylation and export. We have identified two additional components of the accessory Sec system (Asp4 and Asp5) encoded just downstream of *gtfB* in the *gspB-secY2A2* locus. These proteins are required for GspB export and for normal levels of platelet binding by M99. Asp4 and Asp5 may be functional homologues of SecE and SecG, respectively.

The attachment of bacteria to platelets is thought to be a major virulence determinant in the pathogenesis of infective endocarditis (8). Platelet binding by *Streptococcus gordonii* strain M99 is predominantly mediated by the cell surface protein GspB (3). Domains of this large protein (predicted molecular mass, 286 kDa) include a putative N-terminal signal peptide, two serine-rich regions (SRR1 and SRR2), a basic region between SRR1 and SRR2, and a C-terminal cell wall-anchoring domain (3). GspB is heavily glycosylated, primarily with glucose and glucosamine residues (1).

In recent studies, it has been shown that the two SRRs undergo glycosylation by at least four proteins (Gly, Nss, GtfA, and GtfB) that are encoded in a 13.6-kb region downstream of the 9.2-kb *gspB* structural gene (the *secY2A2* locus) (Fig. 1). GtfA and GtfB are essential for GspB glycosylation both in *S. gordonii* and in a heterologous host (*Escherichia coli*). Glycosylation mediated by GtfA and GtfB is important for the solubility of GspB (12). Although the precise functions of Gly and Nss have not been determined, these proteins affect the composition of the GspB-linked carbohydrate (11, 12).

In addition to the four proteins dedicated to GspB glycosylation, the *secY2A2* locus is known to encode at least five proteins (SecY2, Asp1, Asp2, Asp3, and SecA2) that selectively mediate the transport of GspB (Fig. 1) (3, 11). SecY2 shows strong similarity to the SecY transmembrane protein of the general protein secretion system (the canonical Sec system) and is likely to be a component of the translocase in the accessory Sec system. SecA2 is highly similar to SecA of the canonical Sec system and may have a multifaceted role in GspB export (3). Asp1, Asp2, and Asp3, in contrast, are not similar to any proteins of known function, and their precise roles in GspB export are still unclear.

To identify additional proteins that might be involved in GspB production or export, we analyzed the chromosomal region immediately downstream of the *gtfB* gene. M99 chromosomal DNA was digested with XbaI, treated with T4 DNA ligase under dilute conditions, and then used as a template for inverse PCR. A 3-kb product was obtained by amplification of the circularized chromosomal fragments using the 3′ *gtfA* reverse primer 1.8.4 and the 5′ *gtfB* forward primer 1.8.31 (Table 1). The PCR product was cloned into pBluescript SK− (Stratagene) and sequenced using the M13-40 forward and reverse universal sequencing primers and by primer walking.

The region downstream of *gtfB* contained two open reading frames (*orf5* and *orf6*) that were located on the same strand as the other genes in the *gspB-secY2A2* locus and one gene (*orf7*) that was located on the complementary strand (Fig. 1). Orf5 (60 amino acids [aa]) and Orf6 (73 aa) showed no significant similarities to proteins of known function (with the BLASTP program, available at http://www.ncbi.nlm.nih.gov/BLAST/). Orf7 (281 aa) showed significant similarity to a hypothetical protein of *Streptococcus pneumoniae* (72% identity and 86% similarity) (accession number, AAK74881). In the intergenic region between *orf6* and *orf7*, an inverted repeat sequence was identified 13 bp downstream of the *orf6* stop codon and 31 bp downstream of the *orf7* stop codon (Fig. 1). A stem-loop structure formed by this inverted repeat sequence could serve as a rho-independent terminator of transcription through the *secY2A2* operon, as well as through *orf7*.

Although Orf5 and Orf6 did not show significant primary sequence similarity to any well-characterized proteins when analyzed with the BLASTP program, their sizes and secondary-structure predictions are remarkably similar to those of SecE and SecG of *Bacillus subtilis*. These proteins consist of 59 and 76 amino acid residues that include one and two transmembrane segments (TMS), respectively (5, 13). Similarly, Orf5 and Orf6 are predicted to have one and two TMS (Fig. 2). In view of these similarities, we then compared the sequences of these proteins in greater detail using the ClustalW program. As shown in Fig. 2A, Orf5 of *S. gordonii* M99 was 17% identical (52% similar) to SecE of *B. subtilis* 168 and 13% identical (52% similar) to SecE of *S. gordonii* Challis. Orf6 of *S. gordonii* M99 was 15% identical (55% similar) to SecG of *B. subtilis* 168 and 16% identical (55% similar) to SecG of *S. gordonii* Challis (Fig. 2B). Moreover, the location of the TMS within the proteins was well conserved between Orf5 and SecE and between Orf6 and SecG (Fig. 2).

In the canonical Sec system, SecE and SecG, in conjunction with SecY, form a heterotrimeric complex (SecYEG) that constitutes a pathway for polypeptide movement (reviewed in ref-

* Corresponding author. Mailing address: Division of Infectious Diseases, VA Medical Center (111W), 4150 Clement Street, San Francisco, CA 94121. Phone: (415) 221-4810, ext. 2550. Fax: (415) 750-0502. E-mail: sullam@itsa.ucsf.edu.

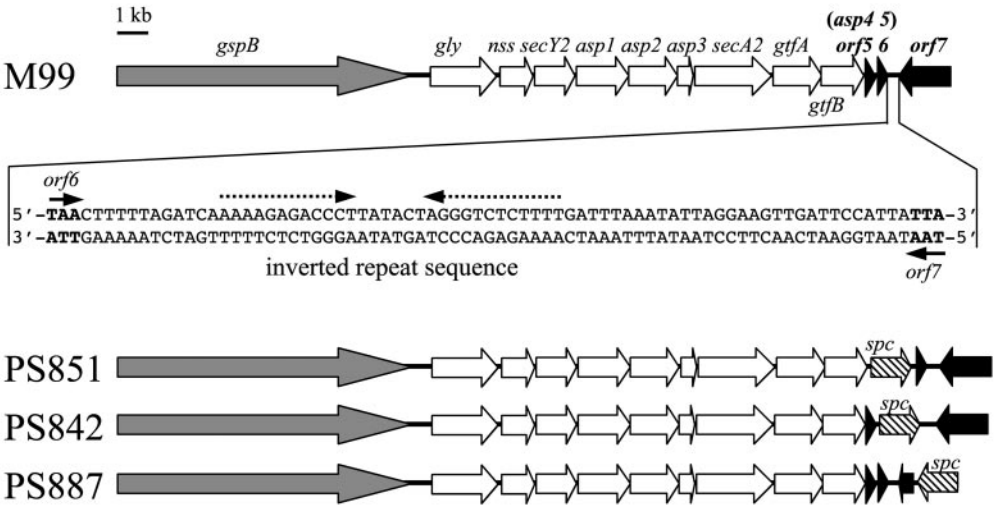

FIG. 1. Genetic map of the *gspB-secY2A2* locus in M99 and in the derived mutants. The nucleotide sequence between *orf6* and *orf7* is also shown. The dotted arrows above the sequence represent an inverted repeat. The bold sequences represent stop codons for *orf6* and *orf7*. Striped arrows, spectinomycin resistance gene (*spc*); gray arrows, *gspB*; black arrows, target genes for gene replacement; and open arrows, the other genes in this locus.

erences 6 and 14). To investigate the possibility that Orf5 and Orf6 might be involved in GspB export by the accessory Sec system, we generated isogenic mutants of M99 in which either *orf5* or *orf6* was replaced with a spectinomycin resistance gene (*spc*) cassette. We also made an *orf7* gene replacement mutant to assess any potential role of Orf7 in GspB expression and export. The *spc* gene was first amplified from pS326 using primers T3S and SpcBamHI (Table 1), and the PCR product was digested with PstI and BamHI. The upstream regions of *orf5*, *orf6*, and *orf7* were amplified by PCR with primer pairs Orf5KO1/Orf5+6KO3, Orf6KO1/Orf5+6KO3, and Orf7KO1/Orf7KO2 (Table 1), respectively. The downstream regions of *orf5*, *orf6*, and *orf7* were amplified by PCR with primer pairs Orf5KO2/Orf5+6KO4, Orf6KO2/Orf5+6KO4, and Orf7KO4/Orf7KO5 (Table 1), respectively. The upstream PCR products were digested with EcoRI and BamHI. The downstream PCR products were digested with PstI and HindIII. These fragments were cloned into the multiple cloning site of pUC19 to construct recombinant plasmids containing the *spc* gene flanked by the upstream and downstream segments of *orf5*, *orf6*, and *orf7* (pORF5K, pORF6K, and pORF7K, respectively) (Table 2). The resultant plasmids were propagated in *E. coli* strain DH5α, linearized with the proper restriction enzymes, and then introduced into *S. gordonii* strain M99 by natural transformation, as

TABLE 1. Primers used for this study

Primer	Sequence (5′-3′)[a]	Restriction enzyme[b]
1.8.4	TCCTCTTCGCTCCCCTTGCCGTAAATATCC	
1.8.31	AATCAAGTGCCAGTGCCCCC	
Orf5KO1	TATTGGATCCTTAGCCATGCTGACCTCCTA	BamHI
Orf5KO2	TTCGCTGCAGGCTTGATGATCTTGATTGGC	PstI
Orf6KO1	TTTCGGATCCATTTTATTTCCTCAAGGCTC	BamHI
Orf6KO2	TGATCTGCAGTATTCTTCTCTTTTTAACCC	PstI
Orf5+6KO3	TTAAGAATTCAAGCGGGACAACTACCTACG	EcoRI
Orf5+6KO4	TGGTAGCCATAACAGCGCTC	
Orf7KO1	GTTTGAATTCTTTTGAGTTGTAGTTTCGGT	EcoRI
Orf7KO2	CAATGGATCCTGAATGGTTAGTGGGACATT	BamHI
Orf7KO4	AGTTCTGCAGCTGGAGGTGAAGTTGACTTG	PstI
Orf7KO5	GAACAAGCTTCCATTATTAGCCGATTTCAT	HindIII
T3S	GAAATTAACCCTCACTAAAG	
SpcBamHI	CAGTGGATCCTAATACGACTCACTATAGGG	BamHI
Orf5C1	GACACCATGGCTAAAAAAGATTTATTTCAT	NcoI
Orf5C2	TATTTCTAGATTATTTCCTCAAGGCTCCAA	XbaI
Orf6C1	AAGACCATGGAAAAATTATTACTTATTCTG	NcoI
Orf6C2	AAAATCTAGAAAAAAGTTAGGCTGTTTGAA	XbaI
SecEF	TGAGCCATGGAATTTCTTAAGGACACTTTT	NcoI
SecER	AGTCTCTAGACGACTTAAAAGAGATTCAAC	XbaI
SecGF	GATACCATGGATAATCTATTATTAATGATT	NcoI
SecGR	CAAATCTAGAGCCCATTTTATCTACTAGAT	XbaI

[a] Underlined sequences are the restriction sites.
[b] Restriction enzymes listed are those that can digest the underlined sequences.

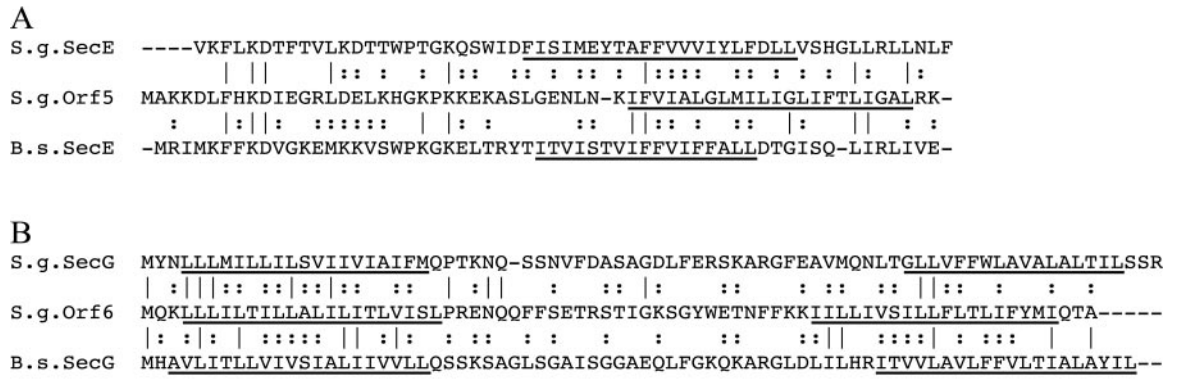

FIG. 2. Alignments of the deduced amino acid sequences of Orf5 and Orf6 of *S. gordonii* strain M99 with SecE and SecG of other gram-positive species. (A) Alignment of Orf5 with SecE of *B. subtilis* 168 (accession number, CAB11876) and with SecE of *S. gordonii* Challis. (B) Alignment of Orf6 with SecG of *B. subtilis* 168 (CAB15368) and with SecG of *S. gordonii* Challis. The SecE and SecG sequences of *B. subtilis* 168 were obtained from GenBank. The SecE and SecG sequences of *S. gordonii* Challis were retrieved from the genome sequence data for this strain (recently available from TIGR at http://www.tigr.org) by performing a BLAST search with the SecE (AAK76075) and SecG (AAK75095) sequences of *S. pneumoniae* TIGR4. The sequences were aligned using ClustalW (http://www.ddbj.nig.ac.jp/search/clustalw-e.html). Identities and similarities between proteins were calculated on the basis of the alignments. Dashes indicate gaps in the aligned sequences. Bars and colons between two sequences indicate identical amino acid residues and conservative changes, respectively. Underlined sequences represent TMS predicted by using TMpred (http://www.ch.embnet.org/software/TMPRED_form.html). S.g., *S. gordonii*; B.s., *B. subtilis*.

described previously (3). Recombination at the expected site in the *S. gordonii* chromosome was confirmed by Southern hybridization analysis of DNA isolated from the spectinomycin-resistant transformants. The genetic organizations of the *gspB-secY2A2* locus of M99 and of the *orf5*, *orf6*, and *orf7* mutant strains (PS851, PS842, and PS887, respectively) are shown in Fig. 1.

We first examined the expression and export of GspB in these strains. M99 and the derivative strains were grown in Todd-Hewitt broth (THB; Difco Laboratories) at 37°C for 16 h and then diluted 20-fold in fresh THB. After 3 to 4 h of incubation at 37°C, the cells were harvested and the cell wall and protoplast proteins were extracted, as described previously (3). Proteins were separated by electrophoresis through a 3 to 8% polyacrylamide gradient gel and then subjected to Western blot analysis using a polyclonal anti-GspB serum (3, 11).

Immunoblotting of these extracts revealed that the cell wall of the parent strain M99 contained two forms of GspB, both with apparent molecular masses of greater than 250 kDa (Fig. 3A, lane 3). The major protein (mature GspB) migrated well ahead of the minor protein. The minor protein migrated at the same apparent molecular mass as that of the nonexported protoplast form of GspB (pre-GspB) (Fig. 3A, lanes 3 and 4). In view of its higher molecular mass, we also refer to this minor protein in the cell wall as pre-GspB. Neither pre-GspB nor mature GspB was detected in the cell wall or in protoplasts of the *gspB* mutant PS436 (Fig. 3A, lanes 1 and 2).

We then assessed the effect of *orf5*, *orf6*, and *orf7* disruption

TABLE 2. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
Strains		
S. gordonii		
M99	Parent strain	9
PS436	M99 *gspB*::pVA891 via pM995′Bint; Erm^r	3
PS851	M99 Δ*orf5*::*spc* Spc^r	This study
PS842	M99 Δ*orf6*::*spc* Spc^r	This study
PS887	M99 Δ*orf7*::*spc* Spc^r	This study
E. coli DH5α	Host for cloning vectors	7
Plasmids		
pBluescript SK−	ColE1 replication origin, *lacZ′*, MCS, Amp^r	Stratagene
pUC19	ColE1 replication origin, *lacZ′*, MCS, Amp^r	15
pS326	*E. coli* vector with MCS; Spc^r	11
pORF5K	pUC19 carrying *spc* flanked by upstream and downstream fragments of *orf5*; Amp^r Spc^r	This study
pORF6K	pUC19 carrying *spc* flanked by upstream and downstream fragments of *orf6*; Amp^r Spc^r	This study
pORF7K	pUC19 carrying *spc* flanked by upstream and downstream fragments of *orf7*; Amp^r Spc^r	This study
pMSP3545	pAMβ1 and ColE1 replication origins, *nisRK PnisA*, MCS, Erm^r	4
pORF5C	pMSP3545 carrying intact *orf5*; Erm^r	This study
pORF6C	pMSP3545 carrying intact *orf6*; Erm^r	This study
pSecE	pMSP3545 carrying intact M99 *secE*; Erm^r	This study
pSecG	pMSP3545 carrying intact M99 *secG*; Erm^r	This study

[a] Spc^r, spectinomycin resistant; Amp^r, ampicillin resistant; Erm^r, erythromycin resistant; MCS, multiple cloning site.

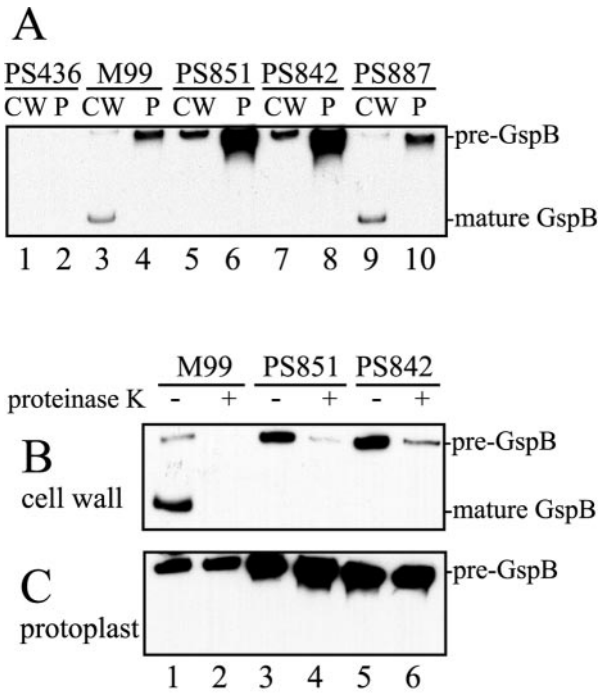

FIG. 3. Effect of *orf5*, *orf6*, and *orf7* disruption on the export and expression of GspB. (A) GspB expression and export by PS436 (*gspB*::pM995′Bint), M99, PS851 (Δ*orf5*::*spc*), PS842 (Δ*orf6*::*spc*), and PS887 (Δ*orf7*::*spc*). (B and C) Effect of proteinase K treatment of M99, PS851, and PS842. Bacterial cells suspended in DPBS were incubated for 1 h at 37°C with (+) or without (−) proteinase K. Cells were recovered by washing, and protoplasts were generated as described in the text. Proteins were separated by electrophoresis through a 3 to 8% polyacrylamide gradient gel and then subjected to Western blot analysis using a polyclonal anti-GspB serum. All proteins shown in this figure migrated above the highest standard (250 kDa). Each lane contains cell wall proteins extracted from bacteria in 75 μl of a broth culture or protoplast proteins extracted from bacteria in 50 μl of a broth culture. CW, cell wall proteins; P, protoplast proteins.

on GspB expression. Little or no mature GspB was detected in the cell wall extracts of PS851 (Δ*orf5*::*spc*) (Fig. 3A, lane 5). Mature GspB was also undetectable in the cell wall of PS842 (Δ*orf6*::*spc*) (Fig. 3A, lane 7). Instead, these mutants contained increased amounts of pre-GspB in the protoplasts (Fig. 3A, lanes 6 and 8). In addition, pre-GspB was more evident in the cell walls of the mutant strains (Fig. 3A, lanes 5 and 7). In contrast to PS851 and PS842, strain PS887 (Δ*orf7*::*spc*) had levels of GspB expression and export unaltered from those of strain M99 (Fig. 3A, lanes 9 and 10).

To confirm that pre-GspB detected in the protoplasts was localized intracellularly, strains M99, PS851, and PS842 were incubated at 37°C for 1 h in Dulbecco phosphate-buffered saline (DPBS) containing 1 mg/ml of proteinase K (Sigma) or in DPBS containing protease inhibitors (Complete Mini [Roche] and 2 μg/ml of aprotinin [Sigma]). The cells were then washed three times with DPBS containing the protease inhibitors, and the cell wall and protoplast proteins were extracted and examined by immunoblotting with the anti-GspB serum as described above.

After proteinase K treatment, neither mature GspB nor pre-GspB was detected among the cell wall proteins of M99 (Fig. 3B, lane 2). In PS851 and PS842, proteinase K treatment drastically reduced the amount of pre-GspB detected in the cell wall (Fig. 3B, lanes 4 and 6). In contrast, exposure of M99 and the derivatives to proteinase K did not affect the detection of pre-GspB in the protoplasts (Fig. 3C), indicating that pre-GspB in the protoplasts was retained intracellularly. These data, in conjunction with the marked accumulation of pre-GspB in the protoplasts of PS851 and PS842, suggest that Orf5 and Orf6, but not Orf7, are directly or indirectly involved in the efficient export of GspB.

It is noteworthy that minor amounts of pre-GspB, but not mature GspB, were also present in the cell walls of strains carrying mutations in each of the accessory secretory genes (*secY2*, *asp1*, *asp2*, *asp3*, and *secA2*) (references 3 and 11 and our unpublished data). In addition, a similar phenomenon has been observed in the *S. gordonii* strain Challis, where Hsa (a GspB homologue) is exported more efficiently and has a lower apparent molecular mass when the accessory Sec system is intact (2). Although not explicitly described in our previous reports, the combined findings indicate that the accessory Sec system is required not only for the efficient export but also for the proper processing of GspB and Hsa. Because mature GspB has never been observed in the protoplasts of export-deficient strains (Fig. 3 and data not shown), GspB export and maturation appear to be coupled. However, the precise structural differences between pre-GspB and mature GspB, whether these include differences in glycosylation or in the polypeptide core, have not been defined. Moreover, the components that are directly responsible for the processing of GspB have not been identified.

We performed complementation analyses of the *orf5* and *orf6* mutants to confirm that the above mutations were not polar. The *orf5* and *orf6* coding regions were amplified by PCR using the primer pairs Orf5C1/Orf5C2 and Orf6C1/Orf6C2 (Table 1), respectively. The amplified fragments were digested with NcoI and XbaI and cloned into pMSP3545 (Table 2), which placed these genes under the control of a nisin-inducible promoter. PS851 and PS842 were then transformed with pMSP3545 (as a control) or with the resultant recombinant plasmids pORF5C and pORF6C (Table 2). Each mutant carrying pMSP3545 with or without the *orf5* or *orf6* coding region was grown in THB at 37°C for 16 h and then diluted 10-fold with THB containing 60 μg/ml of erythromycin and 250 ng/ml of nisin (Sigma). After incubation at 37°C for 3 to 4 h, the cell wall proteins of the transformants were extracted and analyzed by Western blotting for GspB. As an internal control for protein content, we also evaluated these preparations for the presence of another cell wall protein (GspA). Western blot analysis with anti-recombinant GspA serum (11) revealed comparable amounts of this protein in these samples, confirming that equivalent amounts of total protein were loaded in the gels (data not shown).

As expected, normal GspB export was not restored by introducing the pMSP3545 vector alone into the mutant strains (Fig. 4, lanes 3 and 7). In contrast, the expression of Orf5 and Orf6 in *trans* restored the export of mature GspB in the *orf5* and *orf6* mutant strains, respectively (Fig. 4, lanes 4 and 9). The results confirmed that the mutations in these strains were not polar. Intriguingly, when Orf6 was overexpressed in the *orf5* mutant, the export of GspB was partially complemented (Fig. 4, lane 5). Conversely, partial complementation was not readily

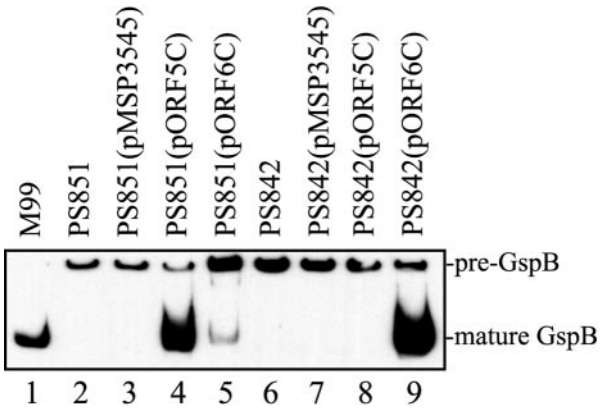

FIG. 4. Complementation analysis of the *orf5* and *orf6* mutant strains (PS851 and PS842, respectively). Protein expression was induced with nisin, as described in the text. Extracted cell wall proteins were separated by electrophoresis through a 3 to 8% polyacrylamide gradient gel and then subjected to Western blot analysis using the anti-GspB serum. Each lane contains proteins extracted from 200 μl of broth culture.

apparent in PS842 complemented by *orf5* (Fig. 4, lane 8). An extremely small amount of mature GspB was seen when this blot was overexposed. These data suggest that Orf5 and Orf6 may have somewhat redundant functions.

To investigate whether disruption of *orf5* or *orf6* affects the export of proteins other than GspB, cell wall proteins and proteins secreted into the culture supernatants were extracted from cultures of M99, PS851, and PS842, as described previously (3). Proteins were separated by electrophoresis through a 3 to 8% polyacrylamide gradient gel and stained with SYPRO Ruby protein gel stain (Molecular Probes). No apparent differences in the numbers or relative intensities of protein bands were seen (data not shown), indicating that Orf5 and Orf6 specifically mediate the export of GspB.

We also examined whether overexpression of the canonical SecE and SecG proteins could enhance GspB export by the accessory Sec system in the *orf5* and *orf6* mutants, respectively. The *secE* and *secG* coding regions were amplified from M99 genomic DNA with primer pairs SecEF/SecER and SecGF/SecGR (Table 1), respectively, which were designed using the Challis *secE* and *secG* sequences. The amplified fragments were cloned into pMSP3545, and the resultant plasmids, pSecE and pSecG (Table 2), were introduced into PS851 and PS842, respectively. After the induction of SecE and SecG expression with nisin, as described above, the cell wall proteins of the transformants were extracted and analyzed for GspB by Western blotting. The induction of SecE or SecG expression had no effect on GspB export in the *orf5* and *orf6* mutants, respectively (data not shown). Although we have no direct evidence that SecE and SecG can function when expressed in *trans* in *S. gordonii*, it is likely that these genes were expressed. The *orf5* and *orf6* genes cloned into the same expression vector readily complemented the respective mutations, as described above, demonstrating that similarly sized genes can be expressed from this plasmid. In addition, no frameshift mutation or nonsense mutation was seen in the cloned *secE* and *secG* genes by sequencing. Therefore, these findings imply that Orf5 and Orf6 are not interchangeable functional homologues of SecE and SecG.

We then assessed the ability of the *orf5* and *orf6* mutants to bind human platelets in vitro, as described previously (3). PS851 and PS842 showed significant reductions in levels of binding to platelet monolayers (69.2% ± 16.9% and 69.0% ± 15.3% reduction, respectively; $P < 0.0001$) compared with that of M99. The reduced levels of platelet binding observed with these mutants were comparable to those seen with strain PS436, in which *gspB* has been disrupted (61.1% ± 21.3% reduction in platelet binding compared with that of M99) (Fig. 5A).

To investigate whether Orf5 and Orf6 expression in *trans* restored the platelet-binding phenotype in the *orf5* and *orf6* mutants, we examined the platelet-binding abilities of the com-

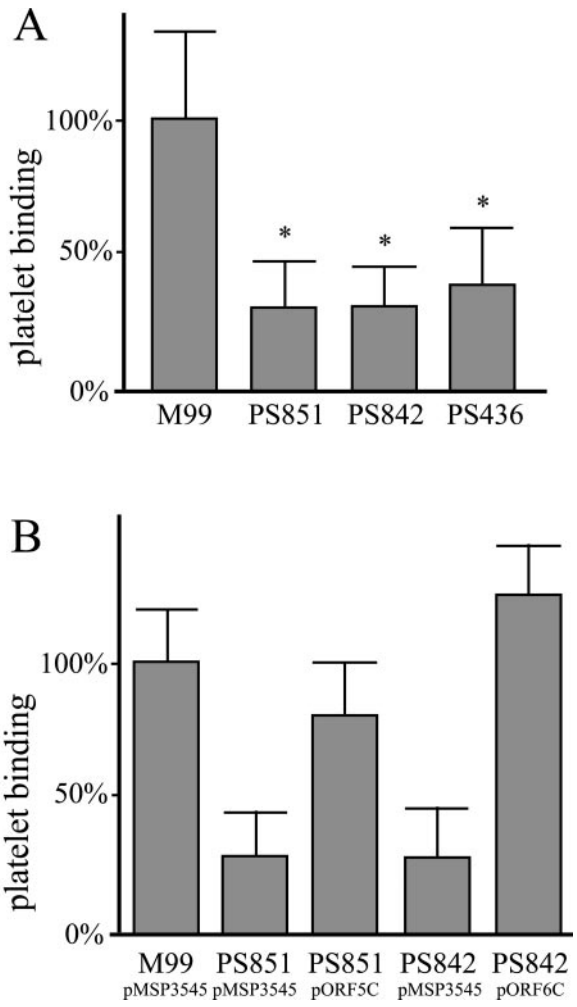

FIG. 5. Platelet binding by M99 and the derivative strains. (A) Platelet binding by strains M99, PS851 (Δ*orf5*::*spc*), PS842 (Δ*orf6*::*spc*), and PS436 (*gspB*::pM995′Bint). Data were collected from two experiments ($n = 8$) with platelets from two different human donors. For each strain, binding is expressed as a percentage of the binding levels achieved by the parent strain M99 (mean ± standard deviation). Differences in platelet binding were compared by the unpaired *t* test with the Welch modification. Asterisks indicate values that are significantly different ($P < 0.0001$) from that for M99. (B) Platelet binding by strains M99 (pMSP3545), PS851 (pMSP3545), PS851 (pORF5C), PS842 (pMSP3545), and PS842 (pORF6C). Data were collected from two experiments ($n = 12$) with platelets from two different human donors. For each strain, binding is expressed as a percentage of the binding levels achieved by M99 (pMSP3545) (mean ± standard deviation).

plemented strains. M99 and the *orf5* and *orf6* mutant strains carrying pMSP3545, pORF5C, or pORF6C were grown in THB containing 60 μg/ml of erythromycin at 37°C for 12 h and then diluted 10-fold with THB containing 60 μg/ml of erythromycin. After incubation at 37°C for 2 h, cultures were induced by the addition of nisin to a final concentration of 250 ng/ml. The cultures were incubated at 37°C for 9 h, and the bacterial cells were tested for platelet binding. As shown in Fig. 5B, platelet binding was not enhanced by introducing the pMSP3545 vector alone into the mutant strains. In contrast, the expression of Orf5 and Orf6 in *trans* restored platelet binding by the *orf5* and *orf6* mutant strains. The combined results demonstrate that Orf5 and Orf6 are needed for normal levels of platelet binding. Moreover, they indicate that platelet binding is predominantly mediated by mature GspB. Although PS851 and PS842 express pre-GspB on the cell surface, these strains bound platelets no better than PS436, which lacks GspB entirely.

Our findings indicate that Orf5 and Orf6 are components of the accessory Sec system that contribute to the selective export of GspB. In contrast, Orf7 appears to have no function in GspB expression or translocation. The presence of the inverted repeat sequence between *orf6* and *orf7*, which may serve as a rho-independent terminator, suggests that *orf6* is the 3′-end gene of the *secY2A2* operon. In view of their role in export, Orf5 and Orf6 have been renamed Asp4 and Asp5 (for *a*ccessory *s*ecretory *p*rotein). As described in previous studies, several other strains of gram-positive bacteria, including *S. gordonii* strain Challis, *S. pneumoniae* strain TIGR4, *Streptococcus agalactiae* strains NEM316 and 2603V/R, *Staphylococcus aureus* strains COL, NCTC8325, N315, Mu50, and MW2, and *Staphylococcus epidermidis* strain ATCC 12228, possess homologues of the *gspB-secY2A2* locus. The homologous loci encode a GspB-like protein and proteins predicted to be involved in export and glycosylation (3, 10, 11). Inspection of genome sequence data deposited in GenBank, or available from The Institute for Genomic Research (TIGR) or the University of Oklahoma (http://www.genome.ou.edu), revealed that *S. gordonii* Challis and *S. pneumoniae* TIGR4 have *asp4* and *asp5* homologues but that *S. agalactiae* strains have homologues of *asp4*, but not *asp5*, in the loci. No *asp4* or *asp5* homologues are apparent downstream of the *gtfB* homologues in *S. aureus* and *S. epidermidis* strains. These observations indicate that the requirement of Asp4 and Asp5 for the export of GspB homologues may be different among gram-positive organisms. We are currently pursuing experiments to elucidate more precisely the role of Asp4 and Asp5 in the accessory Sec system.

Nucleotide sequence accession number. The nucleotide sequence determined in this study has been deposited in the DDBJ, EMBL, and GenBank databases under accession number AY028381.

This work was supported by grants R01 AI041513 and R01 AI057433 from the National Institutes of Health, by the Department of Veterans Affairs, and by the American Heart Association.

The pMSP3545 shuttle vector was a gift from Gary M. Dunny. We thank Ian Siboo and Julie Higashi for their helpful scientific and editorial suggestions.

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