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Inhibition of Cell Surface Export of Group A Streptococcal Anchorless Surface Dehydrogenase Affects Bacterial Adherence and Antiphagocytic Properties

Grégory Boël, Hong Jin, and Vijay Pancholi*

Laboratory of Bacterial Pathogenesis, Public Health Research Institute at The International Center for Public Health, 225 Warren Street, Newark, New Jersey 07103-3535

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Surface dehydrogenase (SDH) is an anchorless, multifunctional protein displayed on the surfaces of group A *Streptococcus* (GAS) organisms. SDH is encoded by a single gene, *sdh* (*gap* or *plr*) that is essential for bacterial survival. Hence, the resulting nonfeasibility of creating a knockout mutant is a major limiting factor in studying its role in GAS pathogenesis. An insertion mutagenesis strategy was devised in which a nucleotide sequence encoding a hydrophobic tail of 12 amino acids (^{337}IVLVGLVMLLLS^{348}) was added at the 3′ end of the *sdh* gene, successfully creating a viable mutant strain (M1-SDH$_{HBtail}$). In this mutant strain, the SDH$_{HBtail}$ protein was not secreted in the medium but was retained in the cytoplasm and to some extent trapped within the cell wall. Hence, SDH$_{HBtail}$ was not displayed on the GAS surface. The mutant strain, M1-SDH$_{HBtail}$, grew at the same rate as the wild-type strain. The SDH$_{HBtail}$ protein displayed the same GAPDH activity as the wild-type SDH protein. Although the whole-cell extracts of the wild-type and mutant strains showed similar GAPDH activities, cell wall extracts of the mutant strain showed 5.5-fold less GAPDH activity than the wild-type strain. The mutant strain, M1-SDH$_{HBtail}$, bound significantly less human plasminogen, adhered poorly to human pharyngeal cells, and lost its innate antiphagocytic activity. These results indicate that the prevention of the cell surface export of SDH affects the virulence properties of GAS. The anchorless SDH protein, thus, is an important virulence factor.

Surface proteins of pathogens play a variety of roles in virulence. Group A *Streptococcus* (GAS) (*Streptococcus pyogenes*), a human pathogen that is responsible for a variety of mild to severe invasive diseases of the pharynx and skin, displays an array of multifunctional proteins on its surface (5, 9, 11). In gram-positive pathogens, including GAS, classical surface proteins with defined structural components, such as a signal sequence, a proline/glycine-rich cell wall-associated domain, a hydrophobic membrane domain, and a cell wall-sorting hexapeptide motif (LPXTGX), have been characterized most extensively (15, 30). More recently, complete genome sequence analyses of six different GAS serotypes have revealed that lipoproteins also contribute significantly to the surface layer of GAS and in disease pathogenesis (1, 2, 13, 29, 43).

In the last decade, after reports of the presence of the glycolytic enzymes glycerladehyde-3-phosphate dehydrogenase (GAPDH) or streptococcal surface dehydrogenase (SDH) (27, 33) and streptococcal surface enolase (SEN) (37) on the surface of GAS, there has been an increasing awareness about the presence of similar glycolytic enzymes on the surfaces of a variety of human pathogens (for a review, see reference 32). Since these surface-located metabolic enzymes are generally found in the cytoplasm and lack C-terminal hydrophobic tails and N-terminal signal sequences required for their export to the cell surface, they are called anchorless surface proteins (32).

In addition to innate glycolytic activities, both SDH and SEN perform a variety of functions (27, 31, 33, 34, 36). Whereas some of the notable functions of SDH are its ability to bind various mammalian proteins, including plasminogen (Plg) (27, 33), ADP ribosylation (34), and host cell-signaling regulation (36), the function of SEN seems to be to serve as a major Plg-binding protein on the surface of GAS (31, 37). Although SDH in comparison to SEN is a weak Plg-binding protein (33), the C-terminal lysine residues of both SDH and SEN seem to play a crucial role in Plg binding (37). Like many other GAPDH molecules (4, 17, 18, 44), the predictive three-dimensional structure of SDH has indicated that the SDH molecule possesses two domains (24). The N-terminal NAD-binding domain (amino acid residues [aa] 1 to 149) consists of a six-stranded parallel β-sheet flanked by helices. The C-terminal half of SDH contains the catalytic domain, which consists of a nine-stranded antiparallel sheet followed by three approximately parallel helices and a polypeptide chain that ends in a long helix. The region spanning amino acid residues 179 and 200, which forms an S-shaped antiparallel sheet, is also called the S-loop (18, 24, 44). Recently, we identified CD87/urokinase plasminogen activator receptor (uPAR) as the SDH-specific receptor on the surfaces of pharyngeal cells (24). The N-terminal domain of uPAR binds to the C-terminal alpha-helix and the flanking regions of the S-loop of the SDH molecule (24). This specific binding also plays a crucial role in GAS adherence to pharyngeal cells (24). Thus, the C-terminal portion of SDH is essential to perform a variety of biologically relevant functions. We also reported that ATP-producing glycolytic en-

* Corresponding author. Mailing address: Laboratory of Bacterial Pathogenesis, Public Health Research Institute at The International Center for Public Health, 225 Warren Street, Newark, NJ 07103-3535. Phone: (973) 854-3430. Fax: (973) 854-3431. E-mail: pancholi@phri.org.

zymes likely form a specialized complex on the surface of GAS (35). Although accumulated reports now indicate that anchorless proteins are present on the surfaces of many pathogens, including gram-positive bacteria, fungi, and parasites (32), our understanding of their biological functions and their roles in the pathogenesis of GAS and other bacterial pathogens is far from complete. Likewise, how they are exported to the cell surface is unknown.

To evaluate the role of virulence factors, the genetic approach to create a knockout mutant that can be tested in various in vitro and/or in vivo disease model systems as a distinct attenuated or nonpathogenic phenotype is highly desirable. However, the knockout mutagenesis approach is not achievable for a gene that is essential for microbial survival. In certain organisms, such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Saccharomyces cerevisiae*, more than one GAPDH and enolase isoenzymes are present; each of these isoenzymes is encoded by a distinct gene or the organism is equipped with the Entner-Doudoroff alternate metabolic pathway (7, 20–22, 28, 46). Hence, it has been possible to create mutants defective in GAPDH, enolase, and other enzymes of glycolysis (19, 22, 23, 28). In GAS, glucose is metabolized solely by the glycolytic pathway, as GAS lacks the tricarbolic-acetic acid (TCA)-metabolic cycle and the Entner-Doudoroff alternative metabolic pathway (1, 2, 13, 29, 43). The multifunctional SDH is encoded by a single essential gene (33, 45). The gene knockout approach to prevent SDH expression in GAS, therefore, is not feasible. The site-directed mutagenesis strategies that allow alteration in the specific functional domains without disturbing the conformational structure responsible for the catalytic function have been successfully employed to create Plg-binding enolase- and SDH/Plr-specific deletion mutants (3, 12, 45). Since the putative transport system responsible for exporting the anchorless metabolic surface enzymes is not known, it has not been possible to create a GAS mutant in which the anchorless enzyme is selectively retained in the cytoplasm and not expressed on the surface.

Since the SDH molecule does not possess the N-terminal signal sequence and the C-terminal hydrophobic tail, we hypothesized that the introduction of only a hydrophobic tail at the C-terminal end may result in its prevention of export to the surface and the SDH may be retained in the cytoplasm and/or in the membrane. Accordingly, in the present investigation, we altered the *sdh* gene by introducing a nucleotide sequence encoding a de novo C-terminal hydrophobic tail. The resulting mutated SDH protein remained largely restricted to the cytoplasm. This mutant strain behaved in a manner similar to that of the wild-type strain in terms of growth characteristics and GAPDH enzyme activity, but it bound significantly less Plg, adhered poorly to human pharyngeal cells, and lost the typical antiphagocytic activity associated with the wild-type strain. Our results also suggest that SDH may have a role in the regulation of the expression of a certain virulence factor-related gene(s), possibly at the transcriptional level.

MATERIALS AND METHODS

Bacterial strains, vectors, growth conditions, and DNA techniques. *S. pyogenes* wild-type strain M1-SF370 (M1-WT; ATCC 700294; American Type Culture Collection, Manassas, VA) was grown in Todd-Hewitt broth (Difco Laboratories) supplemented with 0.5% yeast extract or on proteose peptone-3 blood agar plates supplemented with spectinomycin (up to 500 μg/ml), when required. *E. coli* strain XL1-Blue was used for the cloning experiments and grown in Luria-Bertani (LB) broth or on LB agar plates. Vector pFW5 containing the spectinomycin resistance gene (*aad-9*) was used to transform the M1-SF370 strain (12, 39).

Reagents. Unless otherwise stated, all chemicals were procured from Sigma (St. Louis, MO).

Human pharyngeal cell lines. Detroit 562 (ATCC CCL138) pharyngeal cells were grown to confluence and maintained in a CO_2 incubator as described previously (36).

Determination of the sequence of the hydrophobic tail. The prediction of hydrophobicity of a putative hydrophobic tail was determined based on the dense alignment surface (DAS) score as described at the website http://www.sbc.su.se/~miklos/DAS/. The amino acid sequence of a hydrophobic tail to be inserted at the C-terminal end of SDH was based on the translated amino acid sequence of the *epf* gene in the M1 genome sequence database (13) and was edited using the DAS method to get an optimum DAS score (≥3.0), one indicative of a typical transmembrane location (8).

Construction of insertion and allelic-exchange sdh_{HBtail} mutations in GAS. To create streptococcal mutant strains expressing SDH with a hydrophobic tail at the C-terminal end, the *sdh* gene (0.92-kb DNA fragment) and a 1.118-kb DNA fragment corresponding to the downstream region of the *sdh* gene in the M1-SF370 chromosome were PCR amplified using primer pairs SDH-SalI-F-/SDH-BamHI-R (5′-ACG*CGTCGAC*ATGGTAGTTAAAGT TGGTATTAACGG-3′ and 5′-CGC*GGATCC*TTATTTAGCAATTTTTGCGAAGTACTCAAGAGTA CG-3′) and SDH_{DWN}-PstI-F/SDH_{DWN}-NdeI-R (5′-TTTT*CTGCAGC*TTGGTT TATGCTG AGTTCTTTTTCG-3 and 5′-GGGAATTC*CATATG*TATTGGAA AATGCTATCGTGCG-3′), respectively. The PCR-amplified products were cloned in the multiple cloning sites located upstream and downstream of the spectinomycin resistance gene, *aad-9*, in the suicide plasmid pFW5 (2.7 kb) to construct pFW5-sdh. To insert a DNA sequence encoding the putative hydrophobic tail at the 3′ end of the *sdh* gene in the pFW5-sdh vector, a QuickChange II site-directed mutagenesis kit was used according to the manufacturer's instructions (Stratagene) with the plasmid pFW5-sdh as the template and a pair of complementary primers, SDH_{HBtail}-F (5′-GTACTTCGCAAAAATTGCTAAA ATTGTTCTTGTTGGCTTAGTTATGCTTCTTCTTTCTTAATAGGATCC TCGAGCTCTAG-3′) and SDH_{HBtail}-R (5′-CTAGAGCTCGAGGATCCTA TTAAGAAAGAAGAAGCATAACTAAGCCAACAAGAACAATTTTAGCAA TTTTTGCGAAGTAC-3′). The resulting plasmid, pFW5-sdh_{HBtail}, was introduced into strain M1-WT by electroporation, using a Gene Pulser II electroporator (Bio-Rad) as described previously (12, 29). The mutant strain thus obtained was called M1-SDH_{HBtail}. The confirmation of mutations and insertions at various stages of the experiments was obtained by one or more methods of PCR, DNA sequencing, and Southern hybridization. Using a similar strategy, a mutant strain lacking the M1 protein ($M1_{Demm1}$) was created. The primers used to create this mutant were emm1-F (5′-ACGC*GTCGAC*TAGGTCAAAAAGGTGGC-3′), emm1-R (5′-CGC *GGATCC*GCATTCTCTAAT CTCGCTT-3′), $emm1_{DWN}$-F (5′-AA*CTGCAG*GA CTTGGACGCATCACGTGAA-3′), and $emm1_{DWN}$-R (5′-GATTC*CATATG*CTG TCTCTTAGTTTCCTTCATTGGTGC-3′). The italicized sequences in these primers represent SalI, BamHI, PstI and NdeI restriction sites, respectively.

Cell fractionation and whole-cell extraction. M1-WT and M1-SDH_{HBtail} (mutant) strains were grown to late log phase/early stationary phase (optical density at 600 nm [OD_{600}], 0.8), and bacteria were harvested by centrifugation. The culture supernatants from these strains were saved, and the proteins therein were precipitated with 25% trichloroacetic acid (TCA). The resulting precipitates were separated by centrifugation (20,000 × *g* for 30 min at 4°C), washed twice with acetone, and suspended in 1/50 of the original volume of 50 mM Tris-HCl, pH 7.0. The harvested bacteria were subjected to mutanolysin treatment in a cell wall-extracting buffer (50 mM Tris-HCl, pH 7.0, 30% raffinose) containing 250 U/ml of mutanolysin (Sigma) as described previously to achieve nearly complete wall extraction (33). The resulting protoplasts were lysed in a hypotonic buffer, followed by repeated freezing and thawing. Bacterial membrane and cytoplasmic fractions were separated from the lysed protoplasts by ultracentrifugation (100,000 × *g* for 45 min at 4°C) as described previously (33). Whole-cell extracts from these two strains were obtained using the same procedures described above except that the cell wall-extracting buffer was used without 30% raffinose.

SDH and SDH_{HBtail} purification. Clear whole lysates obtained as described above from 500 ml THY broth culture with each of the M1-WT and M1-SDH_{HBtail} strains were dialyzed against 0.05 M NH_4HCO_3 and precipitated at 40%, 60%, and 80% ammonium sulfate saturation. The protein precipitates obtained from 60% to 80% ammonium sulfate saturation, which contained the major amount of SDH and SDH_{HBtail}, were purified by Hi-trap Blue-HP column (1 ml) chromatography (Pharmacia) after equilibration with a starting buffer (50

mM Tris-HCl, pH 7.4). Proteins other than SDH and SDH_{HBtail} were eluted first by the five-column volume of starting buffers containing 0.5 M NaCl followed by the five-column volume of the same buffer containing 1.0 M NaCl. The remaining column-bound proteins were eluted with the five-column volume of the starting buffer containing 5 mM NAD. The NAD-eluted fractions (1 ml each) contained only SDH or SDH_{HBtail}, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. The pooled fractions containing SDH or SDH_{HBtail} were dialyzed against the starting buffer and stored at −70°C until further use.

Preparation of monospecific anti-SDH antibody. Anti-SDH specific immunoglobulin G (IgG) was purified from the SDH-immunized rabbit serum using an SDH-linked diaminodipropylamine M-phase (Pierce) affinity column as described previously (33, 37). The purified IgG was then treated with immobilized papain enzyme (Pierce) at 4°C overnight to cleave them between their Fab′ and Fc regions. The latter was then removed by passing the cleaved products on a protein A column (Pierce) per the manufacturer's instructions. The unbound initial fall-through was used as Fab′-specific anti-SDH IgG.

Electrophoresis on SDS-polyacrylamide gels and Western blotting. Purified SDH, SDH_{HBtail}, and cell wall-associated proteins were separated by SDS-PAGE and Western blotted onto polyvinylidene difluoride (PVDF) membranes. Subsequently, the presence of SDH was detected by reacting PVDF membranes with anti-SDH monoclonal antibody (clone 13D5) (16) or monospecific rabbit anti-SDH IgG followed by corresponding alkaline phosphatase-labeled conjugate antibody (Fab′ specific). SDH-specific protein bands on PVDF membranes were visualized using a chromogenic or a chemiluminescence-based substrate as described previously (16, 36, 37).

GAPDH activity. The ability of SDH and SDH_{HBtail} to convert D-glyceraldehyde-3-phosphate (G-3-P) to 1,3-diphosphoglycerate in the presence of NAD^+ was measured spectrometrically at 340 nm essentially as described before with some modifications (33). Briefly, the reaction was initiated by adding 3 μg of purified SDH or SDH_{HBtail} proteins or 6 μg of each cell wall extract (strains M1-SF370 and M1-SDH_{HBtail}) in a reaction buffer at a final volume of 600 μl containing 50 mM Tris-HCl, pH 7.4, 4 mM NaH_2PO_4, 4 mM NAD, 4 mM G-3-P, and 1 mM dithiothreitol. The conversion of NAD to NADH was monitored every 10 s spectrometrically (Beckman Coulter model DU640). To measure the GAPDH activity of intact bacteria, instead of a kinetic measurement as described above, only an endpoint OD_{340} was measured. M1-WT and $M1_{HBtail}$ strains were grown until late log phase (OD_{600} = 0.8) and washed twice in phosphate-buffered saline (PBS) to finally adjust the OD_{600} of the bacterial suspension to 1.0 (~5×10^8 CFU). One milliliter of washed bacterial suspension was centrifuged, and the 600 μl of complete enzyme reaction was resuspended as described above for 5 min. At the end of incubation, the bacteria were removed by centrifugation and the absorbance of the supernatant was measured at 340 nm as described above.

Real-time quantitative PCR. Total RNA was extracted from M1-SF370 and M1-SDH_{HBtail} strains grown to an OD of 0.8 using a commercially available RNA isolation kit (RNAwiz; Ambion). The total RNA (2 μg) isolated from the wild-type and mutant strains were converted to first-strand cDNA using an SYBR green iScript cDNA synthesis kit (Bio-Rad) in a final volume of 40 μl. Real-time PCR was performed in a reaction mixture (25 μl) containing 2.5 mM $MgCl_2$, 250 μM deoxynucleoside triphosphate mix, 1.25 U of AmpliTaqGold DNA polymerase, 500 pM of each primer, and 1 μg of template. Gene-specific primer pairs used in this study were as follows: *sdh* (sdh-F, 5′-ATTAACGGTTTCGGTCGTATCGGACG-3′; sdh-R, 5′-GGATCACGTTCAGCAGAAACTTTG-3′), *gyrA* (gyrA-F, 5′-TTCGTATGGCTCAGTGGTTTAGTT-3′; gyrA-R, 5′-CTGGTTCTCTTTCGCTTCCATCGT-3′), and *proS* (proS-F, 5′-GGGTGGTTCTTGACAAGTCTATTGCG-3′; proS-R, 5′-TTCTGCCAAGGCATCTTCAGCA-3′). The mRNA copies specific to *sdh* were normalized with those specific to the housekeeping genes *gyrA* and *proS* for the M1-WT and M1-SDH_{HBtail} strains, and data were evaluated as relative mRNA copy number ratios.

Different concentrations of genomic DNA were used to obtain a standard curve for each gene. The reaction mixtures were incubated for 10 min at 95°C to activate DNA polymerase, followed by 40 cycles each of denaturing at 95°C for 30 s, annealing at 58°C for 50 s, and extension at 72°C for 30 s. Fluorescence was measured in every well during each annealing step throughout the course of each reaction. Data were automatically analyzed by software of the real-time PCR instrument (Stratagene Mx-4000).

Immunofluorescence microscopy. The M1-WT and M1-SDH_{HBtail} strains (10^6 CFU/ml of PBS) were attached to polylysine-treated eight-well glass slides and blocked with DAKO blocking buffer. Adherent bacteria were incubated with affinity-purified anti-SDH IgG (Fab′ fragment, diluted 1:10 in DAKO dilution buffer) at 4°C for 8 h. The wells were washed several times with PBS and reacted with fluorescein isothiocyanate-conjugated anti-rabbit IgG antibody (1:50 dilution) and incubated at room temperature for 1 h. The slides were washed several times with PBS, and the bacteria were stained with DAPI (4′,6′-diamidino-2-phenylindole, 1 μg/ml) in SlowFade equilibration buffer (Molecular Probes) for 5 min. The stained bacteria were kept in SlowFade buffer containing glycerol and observed under a Nikon Eclipse C600 fluorescence microscope coupled with a Diagnostic RT color camera and the Spot software (v3.5.9). Bacterial cells stained with only conjugated antibody were treated as a control.

Fluorescence labeling of plasminogen. Human Plg was labeled with AlexaFluor-488–carboxylic acid succinimidyl ester (antibodies Em 495 and Em 519; Molecular Probes) using EDC (1-ethyl-3-[3-dimethylamino-propyl]carbodiimide) as per the manufacturer's instructions. Fluorescence of the labeled plasminogen was measured with a POLARstar Galaxy fluorimeter (BMG Labtechnologies GmbH, Offenburg, Germany). The values of fluorescence units versus different amounts of labeled plasminogen (1 to 5 μg) were plotted to obtain a standard curve.

Plasminogen-binding assay. Plg-binding activities of purified SDH proteins (SDH and SDH_{HBtail}) were determined using a solid-phase ligand-binding assay and a slot blot apparatus (Bio-Rad) essentially as described before with some modifications (12). The binding of Plg to serially diluted SDH proteins was detected by rabbit anti-human Plg antibody (DAKO, Denmark), followed by corresponding alkaline-phosphatase-labeled conjugate. To determine Plg-binding activities of the intact M1-WT and M1-SDH_{HBtail} strains, late-log-phase THY broth cultures (OD_{600}, 0.8) were adjusted to an OD_{600} of 1.0 after two washes in 50 mM Tris, pH 8.0, buffer. The cultures were then centrifuged, and the resulting bacterial pellets were resuspended in TBST (Tris-buffered saline containing 0.5% Tween 20, 0.5% gelatin, and 0.5% bovine serum albumin) blocking buffer in a final volume that was 1/10 of the original volume. GAS strains (100 μl, ~2×10^9 CFU) were then mixed with twofold serially diluted labeled Plg in a deep-well 96-well plate for 2 h under constant mixing at room temperature. At the end of incubation, unbound labeled Plg was removed and the amount of bound Plg was determined with a fluorimeter as described above. Each dilution of Plg was tested in triplicate wells. Based on the standard curve, the exact amount of Plg bound to GAS strains was calculated. Data were statistically evaluated by unpaired *t* test with Welch's correction.

Phagocytosis/bactericidal assays. The ability of M1-WT and M1-SDH_{HBtail} to resist phagocytosis and survive in heparinized human blood was determined by a bactericidal test as described previously (26). An overnight culture of each bacterial strain was diluted 1:50 in THY broth and grown without agitation to an OD_{600} of 0.15 at 37°C in a CO_2 incubator. The bacterial suspension was diluted in THY broth (1:10,000) to obtain ~4×10^3 CFU/ml for the initial inoculum of the assay. The exact number of bacteria added to the blood was determined by plating the bacterial culture and counting CFU on sheep blood agar plates. Fifty microliters of the suspension containing approximately 200 CFU was added to 1 ml of freshly heparinized blood in sterile glass tubes (100 by 10 mm) and was incubated with end-over-end rotation at 37°C for 3 h. The number of CFU in the inoculum used and those obtained after growth in human blood were quantified by the pour plate method using sheep blood agar plates. Blood for these experiments was drawn from healthy volunteers in 10-ml Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) containing 143 U of freeze-dried heparin. Opsonophagocytosis assays were performed essentially in the same way as the bactericidal assays except that the former assays were carried out in the presence of increasing amounts of purified anti-SDH antibody (SDH-specific IgG). Prior to these experiments, the necessary approval of the Institutional Review Board of UMDNJ, New Jersey, for human subject use was obtained.

Estimation of the hyaluronic acid of the bacterial capsule. The hyaluronic acid capsule produced by wild-type, mutant, and complemented mutant strains was measured essentially as described previously (40). Briefly, a standard curve was generated by the addition of 2 ml of a chromogenic reagent {20 mg of 1-ethyl-2-[3-(1-ethylnaphtho-[1,2-*d*]thiazolin-2-ylidene)-2-methylpropenyl]naptho-[1,2-*d*] thiazolium bromide (Kodak) and 60 μl of glacial acetic acid in 100 ml of 50% formamide} into different concentrations of hyaluronic acid (0 to 30 μg/ml) in a 50-μl volume (ICN/MP Biomedicals LLC) and measuring absorbance at 640 nm. Bacterial cells from a 10-ml late-log-phase culture were washed with water and resuspended in 0.5 ml water. Capsule was released by shaking with 1 ml of chloroform and centrifuged. The aqueous phase (50 μl) was then processed as described above, and the hyaluronic acid content was determined by comparing the absorbance values with the standard curve as described above.

Streptococcal adherence assays. Streptococcal adherence to Detroit pharyngeal cells was carried out in 24-well tissue culture plates essentially as previously described (36, 38). Briefly, overnight cultures of M1-WT and M1-SDH_{HBtail} were washed and adjusted to an OD_{600} of 1.0 in minimal essential medium without fetal bovine serum. Cells were infected with these strains (multiplicity of infection, 50 bacteria to 1 cell, ~4×10^7 to 5×10^7 CFU/well) and incubated in a

humidified CO_2 incubator at 37°C for 3 h. Nonadherent bacteria were removed by repeated washing, Detroit cells with adherent bacteria were lysed, and the resulting cell lysates containing cell-associated bacteria were counted as CFU on sheep blood agar plates as described previously (24).

RESULTS

Fusion of a hydrophobic tail to SDH. Gram-positive bacterial surface proteins contain a signal sequence, an LPXTGX hexapeptide motif, and a hydrophobic tail for anchoring in the membrane lipid bilayer and subsequently for surface export (30). We, therefore, hypothesized that the addition of only a hydrophobic tail at the C-terminal end of SDH would allow SDH to anchor to the membrane without being exported to the cell surface, as the resulting protein would not have either a sorting signal for cell wall anchoring or a signal sequence for secretion. To create such a construct, we designed a putative hydrophobic tail based on the amino acid sequence of the hydrophobic tail region of the *epf* gene (spy0737) product in the M1-SF370 genome database (13). The translated amino acid sequence of the *epf* gene (2,045 aa, 221,959 Da) contains an LPXTGX motif and a hydrophobic tail with 18 amino acids (^{2020}IVLVGLG^{2026}VMS^{2029}LLLG^{2033}MVLY^{2037}) with a predictable hydrophobicity score of 3.5 according to the DAS method (http://www.sbc.su.se/~miklos/DAS/) (8). This score is well above the recommended optimum score (2.2) required for a transmembrane protein (8, 13). We edited this sequence and shortened the size of the de novo hydrophobic tail by removing Gly^{2026}, Ser^{2029}, and the last 5 amino acid residues (^{2033}GMVLY^{2037}). The resulting sequence (IVLVGLVM LLL) gave a DAS score of 3.1. The removal of the last 5 residues and only the Gly^{2026} residue gave DAS scores of 2.8 and 3.2, respectively. However, the deletion of the serine residue (Ser^{2029}) of the sequence IVLVGLVMS^{2029}LLL and its addition at the end of a resulting sequence, i.e., IVLVGLVM LLLS, increased the DAS value from 3.2 to 3.5. Thus, the amino acid sequence IVLVGLVMLLLS with a DAS score of 3.5 was considered the putative hydrophobic transmembrane region for SDH to be inserted at its C-terminal end (Fig. 1A and B).

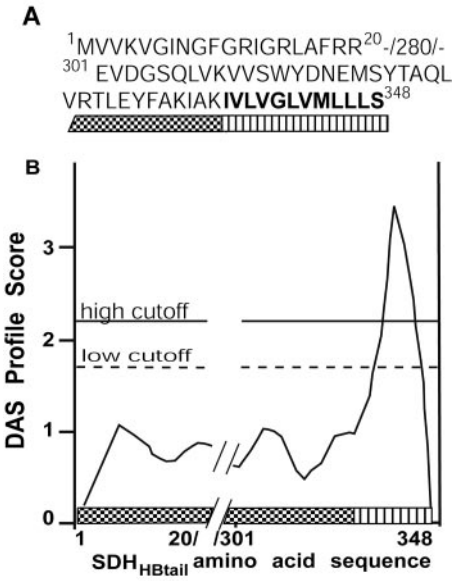

FIG. 1. Strategy to insert a hydrophobic tail at the C-terminal end of SDH. (A) Amino acid sequences of the SDH protein (only residues 1 to 20 and 301 to 336 are noted for brevity) and an inserted hydrophobic tail (^{337}IVLVGLVMLLLS^{348}; in bold) at the C-terminal end (*sdh*/*plr* in the M1 genome is annotated as SPy0274 [13]). The sequence of the hydrophobic tail for SDH was derived from that of the hydrophobic tail portion (^{2020}IVLVGLGVMSLLLGMVLY^{2037}) of the product of the *epf* gene (SPy0737) of the M1-SF370 genome (13). (B) DAS hydrophobicity index of the putative hydrophobic tail sequence (IVLVGLVMLLLS) to be inserted at the C-terminal end of the SDH molecule.

Phenotypic characteristics of M1-SDH$_{HBtail}$. PCR, DNA sequencing, and Southern hybridization assays with the mutant strain M1-SDH$_{HBtail}$ confirmed that the two-crossover allelic-exchange event between the M1-SF370 genome and pFW5-*sdh*$_{HBtail}$ occurred only at the desired locus without any polar effect (Fig. 2A).

Growth characteristics. M1-WT and M1-SDH$_{HBtail}$ strains grew similarly in THY broth throughout the lag and log phases of the growth period and until the stationary phase (Fig. 2B). Thus, the hydrophobic tail at the C-terminal end of SDH had no effect on bacterial growth.

SDH-specific mRNA and protein expression. To determine the effect of the insertion of a hydrophobic tail at the C-terminal end of SDH on protein expression, first SDH-specific mRNA expression was measured in wild-type and mutant strains grown to the late log phase (OD_{600} of 0.8) by real-time quantitative PCR. The expression of SDH-specific mRNAs with respect to housekeeping gene (*gyrA* and *proS*)-specific mRNAs in both the wild-type and mutant strains was found to be essentially the same without a statistically significant difference (*gyrA*, $P = 0.137$; *proS*, $P = 0.213$) (Fig. 2C). Western immunoblot analysis of whole-cell extract using anti-SDH antibody also indicated the presence of SDH and the SDH$_{HBtail}$ protein in their respective strains (Fig. 2D). We concluded that the introduction of hydrophobic residues at the C terminus of SDH does not affect metabolic functions or the growth of the resultant M1-SDH$_{HBtail}$ strain. The SDH$_{HBtail}$ protein, however, migrated faster than SDH during gel electrophoresis. The faster migration of the SDH$_{HBtail}$ protein could be attributed to the insertion of additional hydrophobic residues at the C terminus.

GAPDH activity of purified SDH and SDH$_{HBtail}$. To determine the impact of the insertion of a hydrophobic tail in SDH on its enzymatic activity, the wild-type SDH and mutant SDH$_{HBtail}$ proteins were purified as described under Materials and Methods from whole-cell extracts and their GAPDH activities were determined using the same amount of protein (Fig. 3A). Both SDH and SDH$_{HBtail}$ demonstrated similar kinetics for the conversion of G-3-P to 1,3-diphosphoglycerate in the presence of NAD, indicating that the hydrophobic tail fused to the C-terminal part of SDH does not affect or interfere with the catalytic GAPDH activity of the SDH$_{HBtail}$ protein (Fig. 3A).

Surface localization of SDH and SDH$_{HBtail}$. To demonstrate the effect of insertion mutagenesis in the *sdh* gene on the surface export of SDH, initially GAPDH activities per unit amount of total protein of the whole-cell extract of the wild-type and mutant strains were determined. In both cases, the

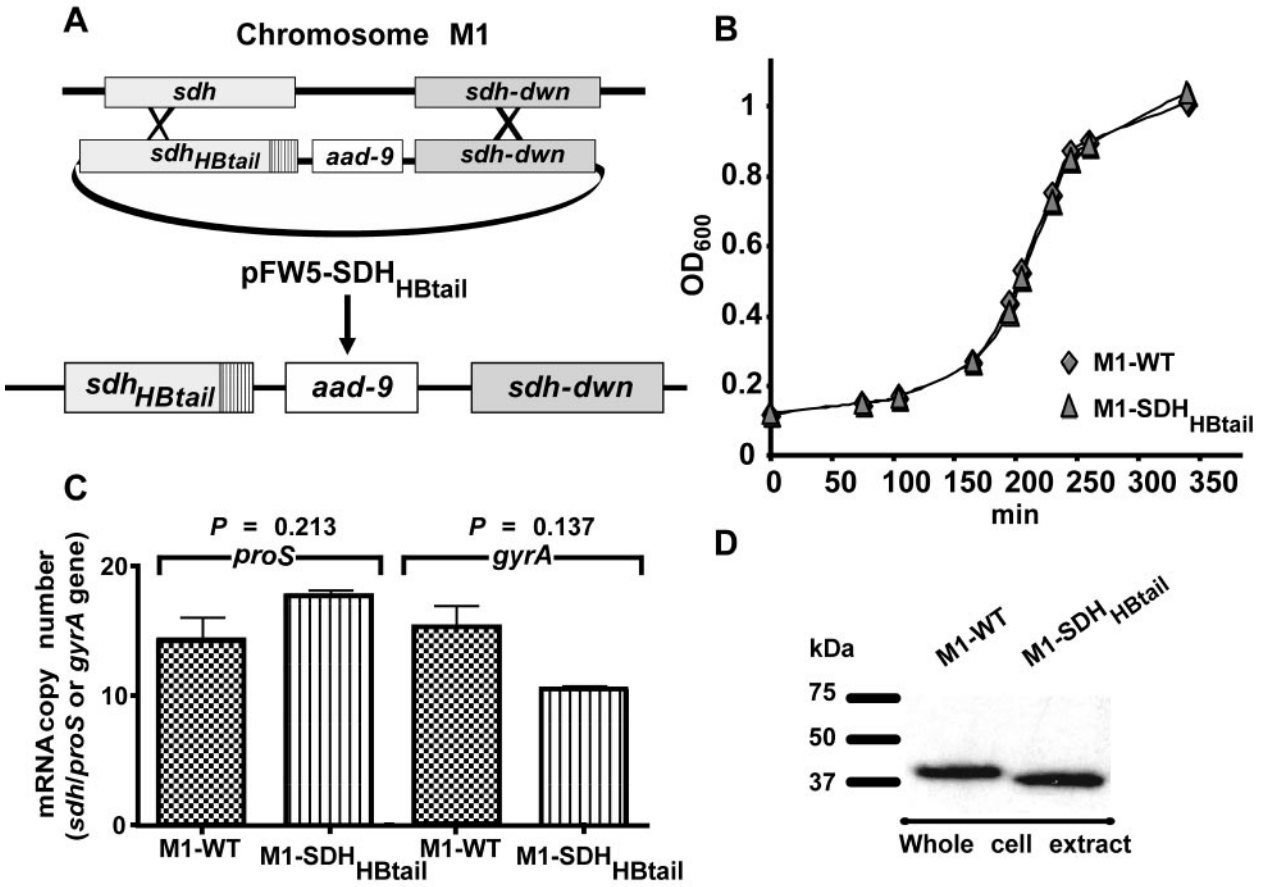

FIG. 2. Growth characteristics of wild-type (M1-WT) and mutant (M1-SDH$_{HBtail}$) GAS strains. (A) The mutant strain (M1-SDH$_{HBtail}$) was derived from the wild-type strain (M1-SF370) using the pFW5-SDH$_{HBtail}$ suicide shuttle vector and by achieving double crossover during allelic-exchange events. (B) Comparison of the growth curves of the wild-type (M1-WT) (diamonds) and mutant (M1-SDH$_{HBtail}$) (triangles) strains. (C) Comparison of the levels of *sdh*-specific mRNA expression with housekeeping gene (*gyrA* and *proS*)-specific mRNA expression in the late log phase of growth of the wild-type (M1-WT) and mutant (M1-SDH$_{HBtail}$) strains by real-time quantitative PCR. *P* values were determined statistically using Student's *t* test. (D) Western immunoblotting of whole bacterial lysates (mutanolysin extract without raffinose buffer) of equal numbers of wild-type (M1-WT) and mutant (M1-SDH$_{HBtail}$) strains using anti-SDH monoclonal antibody (13D5). Numbers on the left indicate positions of the molecular mass markers (kDa).

ratios of the slopes of the kinetic activities were found to be similar, as shown in Fig. 3A (and data not shown). However, the GAPDH activity of the cell wall extract of the mutant M1-SDH$_{HBtail}$ strain was 5.5-fold or 76% less than in the cell wall extracts of the wild-type M1-SF370 strain, indicating that the insertion of the hydrophobic tail at the C-terminal end of SDH results in its retention in the cytoplasm, while significantly affecting its export to the surface and, hence, its association with the cell wall (Fig. 3B). GAPDH activity of the intact mutant M1-SDH$_{HBtail}$ strain was also found to be significantly less (approximately threefold or 60%) than that of the wild-type M1-WT strain, confirming the above results (Fig. 3C).

The presence of SDH on the surfaces of the wild-type and mutant strains was observed by immunofluorescence microscopy using the Fab′ fragment of affinity-purified anti-SDH-antibody. An SDH-specific punctate green fluorescence pattern was seen on the surface of the wild-type strain (Fig. 3D). The M1-SDH$_{HBtail}$ mutant strain showed DAPI staining similar to that of the wild-type strain; however, it did not show any SDH-specific green fluorescence on its surface except where occasional lysed bacteria were observed (Fig. 3D). These data thus support our hypothesis and indicate that it is possible to retain an anchorless protein, SDH, in the cytoplasm and prevent its export to the cell surface by inserting a hydrophobic tail at its C terminus.

To confirm these findings, we determined the presence of SDH in the culture supernatants and other subcellular fractions, such as the cell wall, cytoplasm, and membrane fractions obtained from the M1-WT and M1-SDH$_{HBtail}$ bacterial suspensions, each possessing the same optical density. The presence of SDH was detected by Western immunoblot analysis using SDH-specific monoclonal antibody (13D5). The results showed the minimal amount of secreted SDH in the supernatant of both the wild-type and mutant strains (Fig. 3E). The immunoblot analysis of serially diluted samples derived from equal amounts of cell wall-associated proteins from these two strains showed that the cell wall fraction of the mutant strain contained approximately fourfold less SDH than the cell wall fraction of the wild-type strain (Fig. 3E). On the other hand, the cytoplasmic fraction of the mutant strain contained more

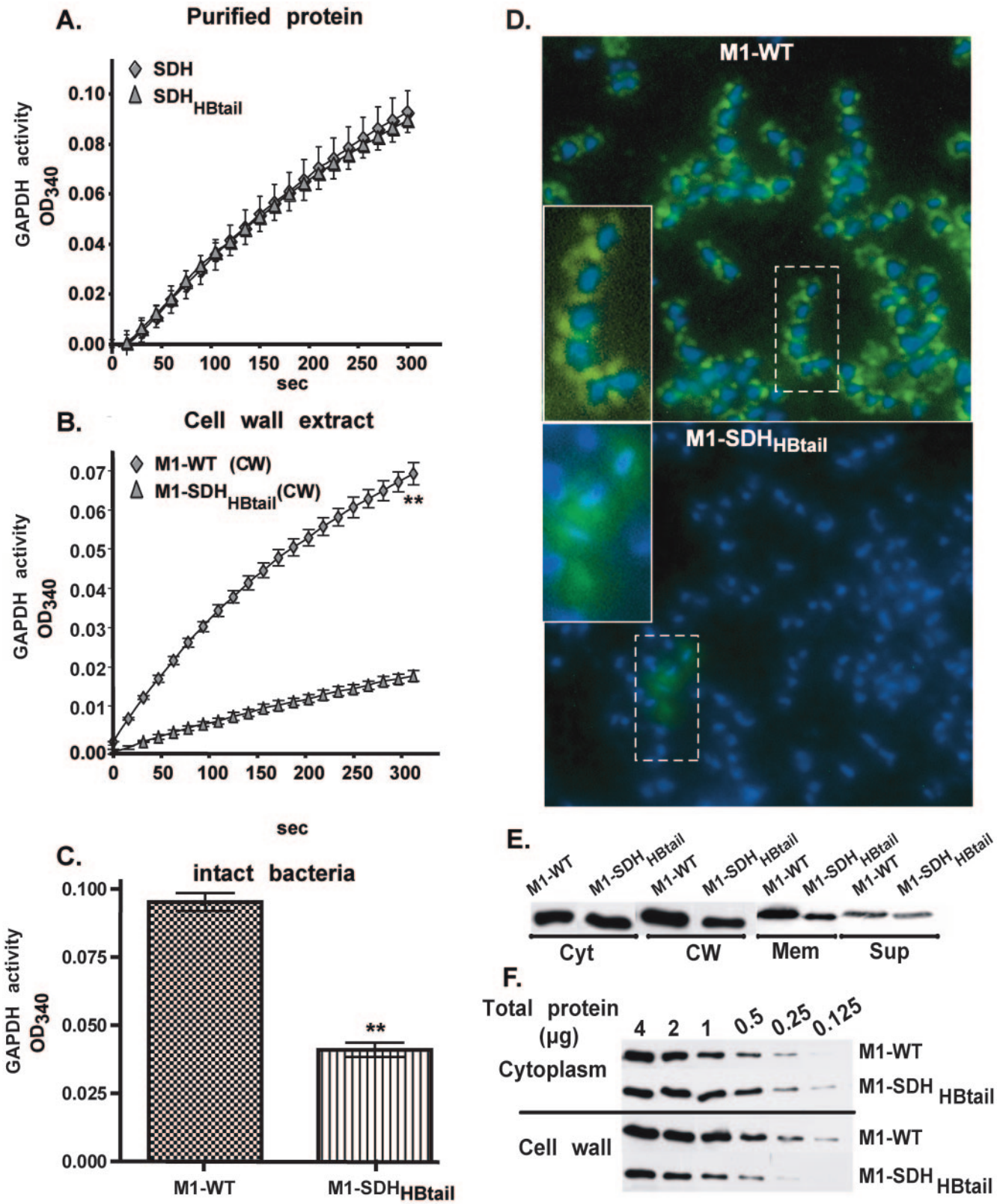

FIG. 3. Surface and cell wall-associated GAPDH activities of the M1-WT and M1-SDH$_{HBtail}$ strains. The GAPDH activity of purified SDH, cell wall extract, or intact bacteria was measured by monitoring the conversion of G-3-P to 1,3-diphosphoglycerate spectrometrically (OD_{340}) at different time intervals or as an endpoint reading after 5 min of incubation. (A) Comparison of GAPDH enzymatic activities of purified wild-type SDH (diamonds) and SDH$_{HBtail}$ (triangles) (3 μg of each). (B) Comparison of GAPDH enzyme activities of the cell wall extracts (CW) of the wild-type (M1-WT) (diamonds) and the mutant (M1-SDH$_{HBtail}$) (triangles) strain (6 μg of each cell wall extract). (C) Comparison of GAPDH

SDH than that in the cytoplasmic fraction of the wild-type strain. These results corroborated those obtained by enzyme kinetic assay (Fig. 3B) and confirmed that the insertion of a hydrophobic tail at the C-terminal end of SDH results in the retention of SDH primarily in the bacterial cytoplasm and to some extent also within the bacterial cell walls. These results also showed that, contrary to our expectation, the SDH_{HBtail} protein was not retained or trapped in the membrane, suggesting that the hydrophobic tail may interfere with the secretion of SDH.

Role of SDH in plasminogen binding. Both SDH/Plr (27, 33) and SEN (12, 37, 38) directly bind Plg; however, their relative contributions to the overall Plg-binding activity of GAS are not known. To determine the contribution of SDH to the Plg-binding activity of GAS, serially diluted purified SDH and SDH_{HBtail} proteins (starting concentration, 2 μg each) were subjected to Plg binding and then detection of Plg binding by anti-Plg antibody in a slot blot-based direct ligand-binding Western blot assay. Plg-binding activities of serially diluted SDH and SDH_{HBtail} revealed that both proteins bind to Plg equally (Fig. 4A), indicating that the insertion of hydrophobic tail at the C-terminal end of SDH does not interfere with the Plg-binding activity of the SDH molecule. To determine Plg-binding activities of the wild-type and mutant strains, each strain (10^9 CFU) was mixed with various concentrations of AlexaFluor-488-labeled Plg, and the amount of bound Plg was estimated based on the standard curve (labeled Plg versus fluorescence units) incorporated in each assay. At the highest concentration (300 nM) of exogenously added labeled Plg, the mutant strain acquired almost 60% less Plg than the wild-type (M1-WT) strain (~2.4 pmol in the mutant strain versus ~6.0 pmol in the wild type; $P < 0.001$), indicating that surface-expressed SDH plays a significant role in plasminogen-binding activity (Fig. 4B). Immunofluorescence microscopy showed the reduced Plg-binding activity of the mutant strain (Fig. 4C) and also confirmed the results obtained with the solid-phase ligand-binding assay (Fig. 4B). The residual plasminogen-binding activity of the mutant strain (Fig. 4C) is likely due to the presence of SEN, which is the major Plg-binding protein on the surface of GAS (12, 37, 38). The unique crescent-shaped Plg-binding pattern at the bacterial poles displayed by M1-SDH_{HBtail} (Fig. 4C, right panel inset) indicates that in the absence of the surface display of SDH, the distribution of other plasminogen-binding proteins, such as SEN (37), is likely affected.

Contribution of SDH to the antiphagocytic activity of GAS. Antiphagocytic properties of the wild-type and mutant strains were determined by measuring their abilities to multiply in fresh human blood during a 3-h incubation period as described in Materials and Methods. The numbers of live bacteria present before and after incubation were determined by counting CFU on blood agar plates. The results expressed in the form of multiplication factors (MF) (the number of CFU at 3 h over the number of CFU at time zero) revealed that, whereas the wild-type (M1-WT) strain survived in the blood (MF = 59.63 ± 6) as expected, the mutant strain (M1-SDH_{HBtail}) were readily killed (MF = 0.58 ± 0.36), indicating that the antiphagocytic activity of the mutant strain was completely inhibited (99% inhibition; $P < 0.0001$) as was found for the $M1_{\Delta emm1}$ strain, which lacks expression of a major virulence factor, the Emm1 protein (Fig. 5A). SDH, therefore, either directly or indirectly is involved in the antiphagocytic process of GAS. Since the M protein and the bacterial capsular polysaccharide are the major surface components responsible for the antiphagocytic activity of GAS, we questioned whether the loss of antiphagocytic activity in the mutant strain was due to the loss of the expression of the M1 protein and/or the polysaccharide capsule. To determine this, Western immunoblot analysis of the cell wall as well as cytoplasm-associated proteins of the wild-type and mutant strains was carried out using the class I M protein-specific 10B6 mouse monoclonal antibody (raised against the M6 protein [25]). The results demonstrated the absence of the expression of M1 protein in both fractions of the M1-SDH_{HBtail} mutant strain (Fig. 5B). In a parallel study of capsular polysaccharide estimation, we observed no significant change ($P > 0.05$) in the capsule polysaccharide associated with the mutant M1-SDH_{HBtail} strain (22.6 ± 0.1 μg/ml) from that in the wild-type strain (20.3 ± 0.6 μg/ml). Together, these results demonstrate that the insertion of the hydrophobic tail at the C-terminal end of SDH affects M1 protein expression but not capsule formation in GAS.

Alternatively, it is also conceivable that SDH also contributes to the antiphagocytic activity of GAS since in the absence of a surface display of SDH, the bacteria are readily phagocytosed and, as in the presence of anti-M protein antibody (14),

activities of intact M1-WT and M1-SDH_{HBtail} GAS strains (200 μl of a bacterial suspension with an OD_{600} of 1.0, i.e.,10^8 CFU) as an endpoint reading of the supernatant obtained after 5 min of incubation in 600 μl of the reaction buffer followed by pelleting of the bacteria. Each reading point in panels A, B, and C represents an average from three independent experiments ± standard errors. (D) Indirect immunofluorescence microscopy of the wild-type (M1-WT) and mutant (M1-SDH_{HBtail}) strains using affinity-purified monospecific rabbit anti-SDH IgG (Fab′ fragment) as the primary antibody followed by fluorescein isothiocyanate-labeled anti-rabbit Fab′-specific IgG (green fluorescence) as the secondary antibody. In parallel control experiments, both bacterial strains did not stain with the secondary conjugate antibody (not shown). The inset in each panel (dotted-line rectangle) is magnified (solid-line rectangle) to highlight bacterial-surface-associated SDH-specific punctate fluorescence. Note the absence of green fluorescence in the M1-SDH_{HBtail} mutant strain. The blue fluorescence represents the DAPI-stained bacterial DNA. The background fluorescence in M1-SDH_{HBtail} (inset) is due to lysed bacteria as revealed by the absence of DAPI stain. (E) Western blot analysis of the presence of SDH in culture supernatant (Sup) and subcellular fractions (CW, cell wall; Cyt, cytoplasm; and Mem, membrane) of the wild-type (M1-WT) and mutant (M1-SDH_{HBtail}) strains using SDH-specific monoclonal antibody (13D5) (16). Both strains were grown to an OD of 0.8 in THY broth, and the supernatants were separated by centrifugation. The resulting bacterial pellets obtained from each strain were subjected to subcellular fractionation. Twenty-five percent-TCA-precipitated proteins obtained from 125 μl of the supernatant from each strain were tested for the presence of SDH. (F) Semiquantitative analysis of the presence of SDH in the cytoplasm and cell wall fractions of equal numbers of M1-WT and M1-SDH_{HBtail} cells. Serial twofold dilutions of 4 μg of total proteins of the cytoplasmic and cell wall fractions obtained from both strains were resolved on PVDF membranes by SDS-PAGE and electroblotting. The presence of SDH was determined by immunoblotting using anti-SDH monoclonal antibody (13D5) and visualized with a chemiluminescence substrate.

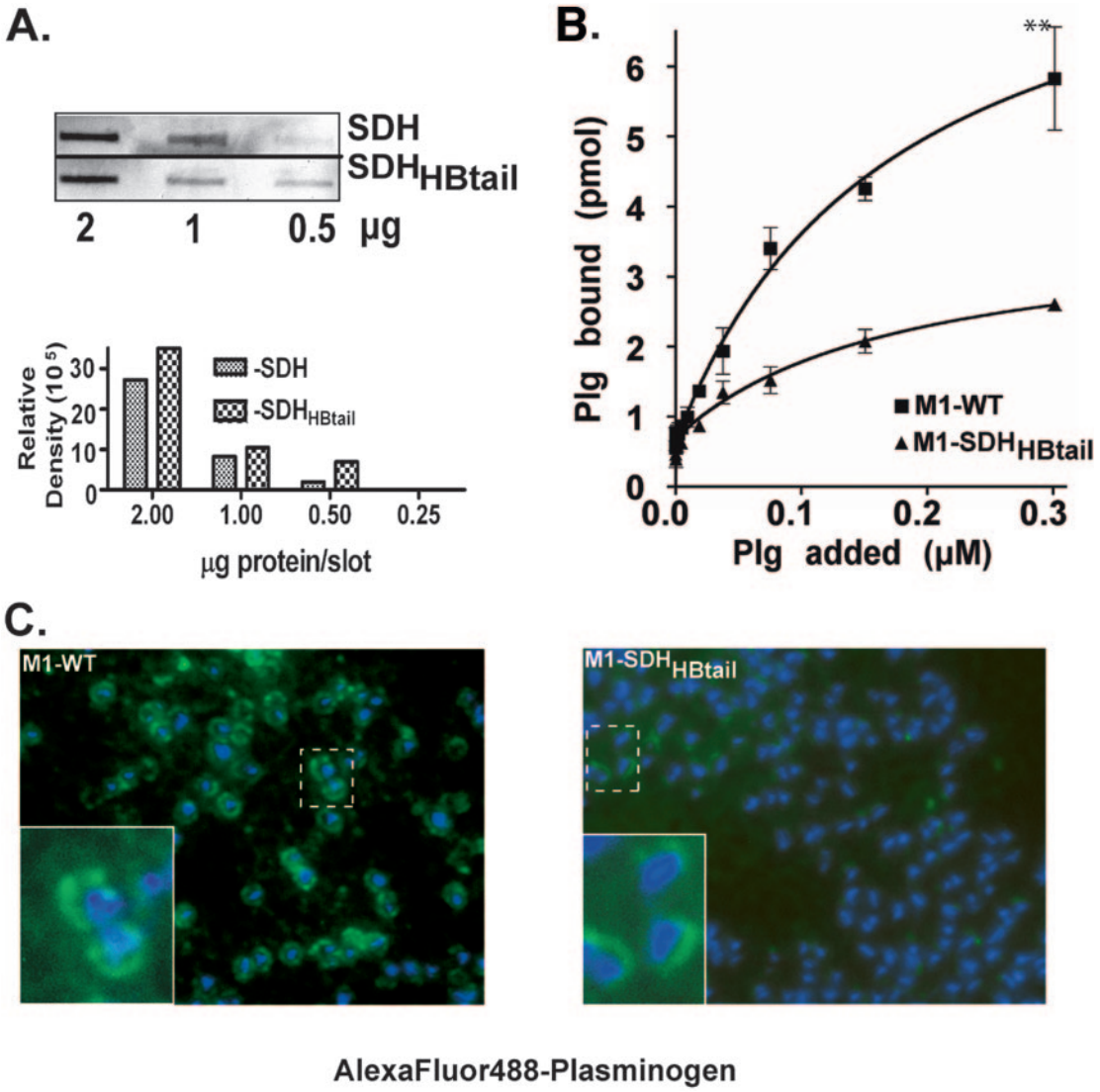

FIG. 4. Plasminogen-binding activities of the wild-type (M1-WT) and mutant (M1-SDH_{HBtail}) GAS strains. (A) Plg-binding activities of serially diluted purified SDH and SDH_{HBtail} (starting with 2 μg) proteins under native conditions using the slot blot device-based protein-ligand-binding method. Specific binding of Plg to purified SDH or SDH_{HBtail} was determined with anti-human Plg antibody, visualized with a chemiluminescence substrate, and analyzed densitometrically. Bovine serum albumin was used as an internal control and showed no plasminogen binding (not shown). (B) Plg-binding activities of M1-WT and M1-SDH_{HBtail} strains as measured by 96-well microtiter plate-based ligand-binding assays using intact bacteria (10^9 CFU) and various concentrations of AlexaFluor-488-labeled human Plg. The amount of bound Plg to bacterial strains was quantitated on the basis of a standard curve (fluorescence units versus the amount of labeled Plg). **, $P < 0.001$. (C) Fluorescence microscopy to reveal the direct Plg-binding ability of the wild-type (M1-WT) and the mutant (M1-SDH_{HBtail}) strains using AlexaFluor-488-labeled human Plg (green fluorescence). The blue fluorescence represents the DAPI-stained bacterial DNA. The inset in each panel (dotted small rectangle) is magnified (solid-line rectangle) to highlight the Plg-binding pattern. Note the punctate Plg-binding pattern in the M1-WT strain versus the crescent-shaped and reduced Plg-binding pattern in the absence of the surface display of SDH in the M1-SDH_{HBtail} strain.

the bacteria may readily be phagocytosed in the presence of anti-SDH antibody. To test this hypothesis, the wild-type GAS strain was subjected to bactericidal assay as described above in the absence and presence of increasing amounts of affinity-purified anti-SDH IgG (50 μg to 200 μg/per reaction mixture). The results showed a dose-dependent decrease in bacterial survival (numbers of CFU) and a corresponding increase in bacterial killing/phagocytosis of the wild-type GAS strain (Fig. 5D). With a 200-μg concentration of anti-SDH IgG, up to a 70% decrease in CFU was observed compared to the total number of bacteria grown under similar experimental conditions in the absence of any exogenously added anti-SDH antibody. These results indicate that either SDH is antiphagocytic in nature or the anti-SDH antibodies are opsonic in nature.

Contribution of SDH in GAS adherence. Since purified SDH recognizes CD87/uPAR as its receptor on the surfaces of pharyngeal cells and mediates GAS adherence to pharyngeal cells (24), we hypothesized that the prevention of the cell surface export of SDH might adversely affect GAS adherence to pharyngeal cells. To test this hypothesis, M1-WT and M1-SDH_{HBtail} strains were examined for their ability to adhere to pharyngeal

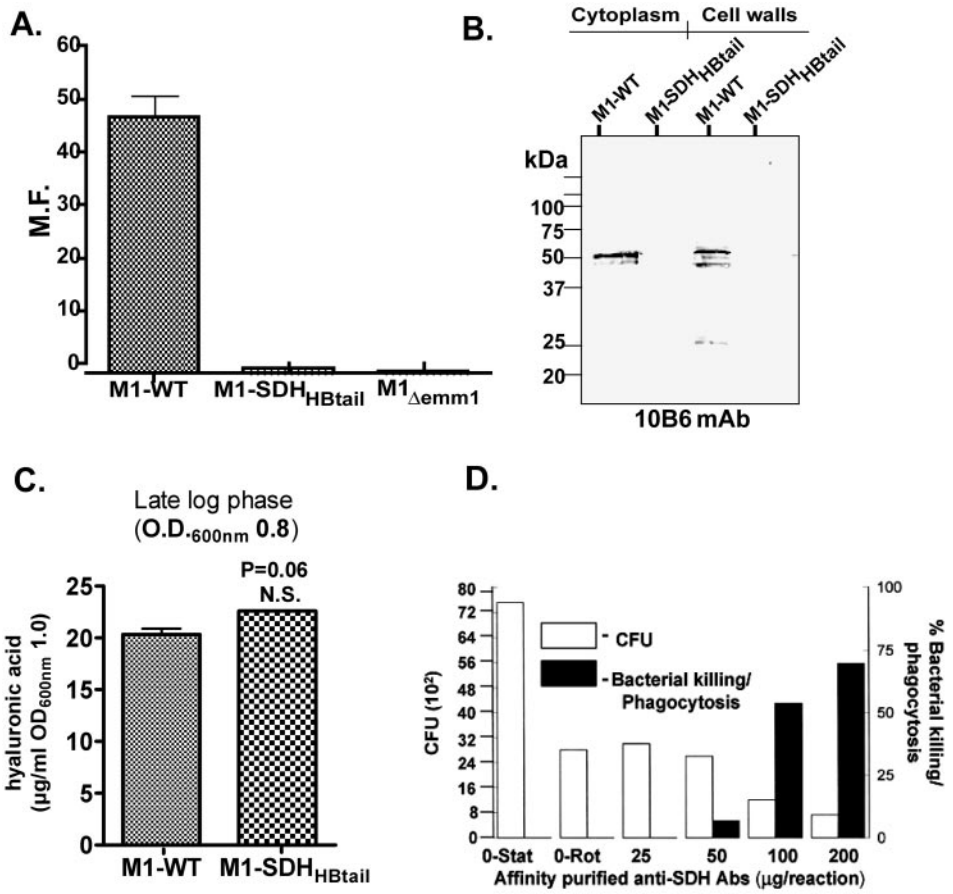

FIG. 5. Phagocytosis/bactericidal assays. (A) Growth of the wild-type (M1-WT) and mutant (M1-SDH_{HBtail}) strains in human blood is expressed as a multiplication factor. The MF is the number of CFU obtained at the end of the incubation period over the number of CFU obtained at time zero. The $M1_{\Delta emm1}$ strain was used as a negative control for antiphagocytic activity. Bars and error bars represent the means ± standard errors obtained from two independent experiments, each with triplicate samples. ***, $P < 0.0001$. (B) Western blot analysis of cell wall- and cytoplasm-associated proteins of the wild-type (M1-WT) and mutant (M1-SDH_{HBtail}) strains using M1 protein-reacting 10B6 monoclonal antibody (mAb). Proteins were visualized with a chromogenic substrate. Each lane received a total of 5 μg of total cell wall-associated or cytoplasmic proteins. (C) Estimation of the hyaluronic capsule associated with the wild-type and mutant (M1-SDH_{HBtail}) strains grown until late log phase (OD_{600} of 0.8). N.S., not significant ($P > 0.05$). (D) Opsonophagocytosis or inhibition of the antiphagocytic activity of the wild-type GAS strain in the presence of various concentrations of affinity-purified anti-SDH antibodies. The bactericidal assay was performed as described for panel A. The left *y* axis denotes the numbers of bacteria that survived at different concentrations of anti-SDH antibodies in the assay mixture at the end of the incubation period. The right *y* axis denotes percentages of bacteria being killed/phagocytosed at different concentrations of anti-SDH antibodies. Stat, stationary; Rot, rotation.

cells. The GAS adherence assay revealed that the mutant strain, M1-SDH_{HBtail}, adhered to pharyngeal cells threefold less (~70% less) than the wild-type strain (Fig. 6). Since the M1-SDH_{HBtail} mutant strain does not express the M1 protein (Fig. 5B), we also included another mutant strain lacking the expression of the M1 protein ($M1_{Demm1}$) in the bacterial adherence assay as an internal control. The results showed that the $M1_{Demm1}$ mutant strain adhered in a way similar to that of the wild-type strain ($P = 0.268$), indicating that SDH and not the M protein plays an important role in GAS adherence to pharyngeal cells (Fig. 5).

DISCUSSION

SDH belongs to a novel class of anchorless cell wall-associated surface proteins (32). Several proteins, including SDH, that belong to this class of surface proteins are not only multifunctional but also essential for bacterial survival. Hence, the resulting nonfeasibility of the "gene knockout" strategy has been a major limiting factor in our progress in understanding their roles in disease pathogenesis. In the present investigation, we have addressed this limiting factor and devised a mutagenesis strategy that allows an insertion of a hydrophobic tail at the C-terminal end of SDH and prevents the export of SDH to the cell surface. This strategy is important for two main reasons. First, it does not affect the innate GAPDH activity of GAS. Second, this strategy also allows retaining this modified SDH in the cytoplasm and does not affect growth characteristics of bacteria.

SDH and other anchorless surface glycolytic enzymes from GAS and other microorganisms are exported to the cell surface in the absence of a signal sequence and hydrophobic domain. The precise mechanism of their export to the cell

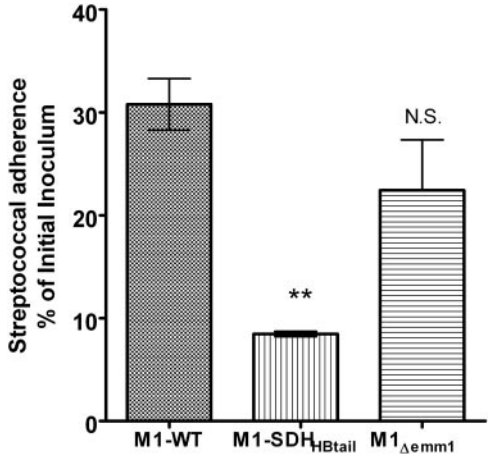

FIG. 6. Bacterial adherence assays. Confluent human pharyngeal cells (Detroit 562) grown in 24-well tissue culture plates were incubated with the wild-type (M1-WT) and mutant (M1-SDH_{HBtail}) strains (multiplicity of infection, 1:50, cells to bacteria) for 3 h. The mutant strain lacking the expression of the M1 protein ($M1_{\Delta emm1}$) was used as an internal control in this assay. At the end of incubation, cell-associated bacteria were counted as numbers of CFU on sheep blood agar plates. The adherence index for each preparation was calculated as a percentage of the initial inoculum (4×10^7 to 5×10^7/well, i.e., 100 μl of an OD 1.0 bacterial suspension). Each error bar represents an average of results from three independent experiments, each from triplicate wells ± standard errors. **, $P < 0.001$. N.S., not significant.

surface and subsequent secretion into the medium is not known. It is, therefore, interpreted that the surface association of this enzyme is possibly due to the reassociation of leaked cytoplasmic proteins as a result of the uncontrolled natural lysis of a few bacteria. We monitored the secretion of SDH in culture supernatant and its retention within bacteria by immunofluorescence, enzyme kinetics, and immunoblot methods. Whereas the immunofluorescence provided a visual proof of the location of wild-type and mutant SDH, the remaining two methods provided quantitative analysis of their association with the GAS cell wall, cytoplasm, and membranes. All methods indicated primarily that the mutated SDH is not secreted and is not displayed on the GAS surface. Further, in the wild-type strain, surface-displayed SDH is not derived from lysed bacteria. Based on the design of the experiment, we had expected a large amount of SDH to be trapped in the membrane. However, our results showed that the large amount of SDH was restricted to the cytoplasm, to some extent in the membrane, and also in the cell wall. The detection of some amount of SDH_{HBtail} in the cell wall fraction of the intact mutant bacteria, albeit three to five times less than that in the wild type, suggest that while the hydrophobic tail primarily interferes with the secretion of SDH and helps to retain the SDH_{HBtail} molecule within the cytoplasm, some molecules may escape during cell division and be retained and trapped within the cell wall while they transgress the cell envelope. These SDH molecules are likely accessible to the glyceraldehydes-3-phosphate substrate during enzyme kinetic assay of the intact bacteria but not accessible to anti-SDH antibody during the immunofluorescence assay. Thus, the presence of SDH_{HBtail} buried within the GAS cell walls was detected in immunoblotting assays, and only the surface-located SDH was detected by the immunofluorescence method. Based on the latter method, more than 95% of the mutant bacterial population (M1-SDH_{HBtail}) showed the absence of SDH on the surface.

SDH and many of the other anchorless proteins are multifunctional. Because of their essential nature, previous studies of mutational analyses of this and other anchorless proteins were restricted to functional regions, such as Plg-binding domains, that do not participate in essential catalytic activities (3, 12, 45). Further, the functional analyses of such domains that overlap catalytic domains were based on in vitro ligand-binding assays, i.e., measuring the Plg-binding activity of internal binding sites of SEN of GAS or a similar protein of *S. pneumoniae*, using truncated recombinant proteins or short relevant synthetic peptides bound to a solid phase (3, 12). In SDH/Plr, the second Plg-binding site is proposed to be located in the N-terminal region (45). The nature of this site is unknown. It is, however, likely that the putative second plasminogen-binding site contributes to the catalytic domain, since several N-terminally truncated recombinant SDH proteins representing deletions of the entire region in the first N-terminal half were catalytically inactive (24; V. Pancholi, unpublished data). It may be emphasized that although the C-terminal lysine residue plays an important role in Plg binding (45), the insertion of an additional hydrophobic tail did not interfere with the Plg-binding activity of the SDH molecule (Fig. 4). The mutagenesis approach described in the present study thus successfully allowed us to study the Plg-binding activity of GAS mediated by the SDH molecule as a whole. By using a similar mutagenesis approach, we propose that secretion of SEN and other anchorless surface molecules of GAS can also be prevented and that they can be localized or restrained to the cytoplasm without affecting their innate enzyme activities and other functions. The successful creation of such mutants may then help understand the contribution of anchorless surface proteins in GAS pathogenesis.

In the present study, the unexpected finding that the isogenic M1-SDH_{HBtail} mutant strain lost the antiphagocytic activity of the wild-type strain raises an important question, namely, whether SDH is indeed antiphagocytic in nature. To understand the contribution of SDH to GAS antiphagocytic activity, we determined the antiphagocytic activity of the wild-type strain in the presence of anti-SDH polyclonal antibody. The increased phagocytosis of the wild-type GAS strain in the presence of anti-SDH antibody is likely due to its opsonic activity, as was also found for the antibody against another well-studied anchorless protein, SEN (16, 37). On the other hand, the absence of antiphagocytic activity in M1-SDH_{HBtail} indeed seems due to the absence of the M1 protein as the expression of another antiphagocytic molecule, the polysaccharide capsule (14), was not affected in the mutant strain. Despite the facts that the *sdh* gene (SPy274) is monocistronic in the M1 genome as well as in other described GAS genomes (1, 2, 13, 29, 43) and hence that the insertion of a hydrophobic tail and the spectinomycin resistance gene *aad-9* does not create any polar effect in the M1-SDH_{HBtail} mutant, the expression of the *emm1* gene (SPy 2018) product (M1 protein) is down regulated. Eukaryotic GAPDH has been shown to bind DNA and RNA, and this binding has been shown to play a role in the transcription regulation of eukaryotic genes (for reviews, see references 41 and 42). It is likely that SDH and its C-terminal

portion in some way may have an important role in M1 expression, possibly at the transcriptional level.

We recently reported that SDH recognizes uPAR/CD87 as its receptor on the surfaces of pharyngeal cell and mediates GAS adherence (24). In that publication, we showed that the mutant GAS strain, displaying SDH with the C-terminal lysine residue (Lys^{336}) deleted or with a C-terminal leucine residue instead of the lysine residue ($Lys^{336}Leu$), adhered poorly to pharyngeal cells (24). It was, however, not possible to study those mutants to understand the contribution of other domains of the SDH molecule in the adherence process, as other domains contribute to the essential GAPDH activity of SDH (24). In the present study, this shortcoming was overcome by preventing the display of the whole SDH protein on the GAS surface. Our results showing the adherence ability of the $M1_{\Delta emm1}$ mutant strain to pharyngeal cells in a way similar to that of the wild-type strain concur with the previously published reports, which indicated that the expression of the M protein has little effect on GAS adherence to human tonsillar/pharyngeal cells and keratinocytes (6, 10). In conjunction with this finding, the poor adherence ability of the $M1\text{-}SDH_{HBtail}$ strain, which does not display SDH, as well as of the M1 protein on its surface underscores the important contribution of SDH in GAS adherence to pharyngeal cells.

In summary, the mutagenesis approach used in the present study revealed new functions of SDH and its role in GAS pathogenesis, such as GAS adherence and antiphagocytic activity. It is not clear at present whether SDH is itself antiphagocytic or whether, in addition, it plays a role in the transcriptional or posttranscriptional control of GAS virulence factors. As the latter is true for the eukaryotic GAPDH (41, 42), it is an important question for future research. Evidence provided in the present study concludes that SDH is an important surface-expressed virulence factor and plays an important role in GAS pathogenesis.

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