Supporting Information

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SI Methods

DNA Manipulation. Standard protocols were used for DNA manipulation and for *E. coli* transformations. Plasmid DNA was isolated from *E. coli* using QiaQuick spin columns (Qiagen), or by alkaline lysis (1). PCR amplification procedures were performed using Taq DNA polymerase (Invitrogen). Electroporation procedure for the transformation of *S. pyogenes* is described elsewhere (2).

Deletion of *srtA* in Strain D471. The plasmid pFW15 was used for gene replacement as described by Podbielski *et al.* (3). A 1-kb DNA fragment upstream of the *srtA* gene was amplified from D471 using primers 5-UKOsrtA and 3-UKOsrtA and inserted between the SalI and XhoI sites of pFW15. Primers 5-DKOsrtA and 3-DKOsrtA were used to amplify a 1-kb region downstream of the srtA gene, and this fragment was inserted into the NcoI and SmaI sites of the resulting plasmid, yielding pAR95. This plasmid was transformed into D471, and erythromycin-resistant colonies were screened for a double crossover event by PCR, yielding AR01.

Construction of pAR107. A 150-bp region upstream to the gene encoding GAPDH was constructed by the ligation of primers pGAPDH-F1, pGAPDH-F2, pGAPDH-R1, pGAPDH-R2, and pGAPDH-R3. This DNA fragment, which harbors promoter activity, was inserted into the EcoRI and BamHI sites of pLZ12Spec (4) yielding pAR102. The sortase reading frame was amplified using primers 5-srtA-BamHI 3-srtA-BglII, and inserted into the BamHI and BglII sites of pAR102, yielding pAR107.

Cloning and Purification of Sortase A. The estimated location of the sortase N-terminal transmembrane domain was predicted using the DAS software (5). The soluble portion of sortase was PCR amplified using primers 5-srtA-aa34-salI and 3-srtA-notI, and a hexahistidine-tagged version of the protein (H6-SrtAΔN) was obtained by the insertion of this fragment between the SalI and NotI sites of a modified pET21a vector (a generous gift from Erec Stebbins), yielding pAR86.

An overnight culture of *E.coli* BL21/pAR86 was diluted 1:100 into 1 L of LB medium containing ampicillin and placed in an environmental shaker. Upon reaching OD_{600} 0.5, the expression of H6-SrtAΔN was induced with α-lactose at a final concentration of 0.2% for 4 h. The cells were harvested and resuspended in 60 ml MCAC buffer (30 mM Tris pH 7.4, 0.5 M NaCl, 10% glycerol, 1 mM DTT), homogenized, and sonicated briefly to shear DNA. Cell debris was removed by centrifugation, and the supernatant was filtered through a 0.22-μm filter (Millipore). The cleared lysate was loaded on a NiNTA column equilibrated with MCAC buffer, followed by washes with MCAC containing 20 mM imidazole and elution with MCAC containing 100 mM imidazole. The eluted fraction was concentrated using an amicon ultrafiltration device, fitted with a 3-kDa molecular weight cutoff membrane, and applied to a G-100 gel filtration column, equilibrated with 30 mM Tris pH 7.5, 150 mM NaCl, 1 mM DTT. The final product is shown in supporting information (*SI*) Fig. S1*A*. Purified H6-SrtAΔN showed cleavage activity of bead-bound ^{125}I-labeled KRQ*LPSTG*ET-ANPFY peptide (data not shown) as described (6).

For immunization of mice to produce antibodies, the NiNTA H6-SrtAΔN elution fraction was separated on 16 10% SDS polyacrylamide gels and dyed with Gel-Code blue (Pierce). The H6-SrtAΔN band was excised from the gel and placed into a 3-kDa molecular weight cutoff dialysis bag containing 15 ml 50 mM Tris-glycine buffer pH 8.3, 0.04% SDS. The bag was then placed in a miniprotean transfer device (Bio-Rad) containing the same buffer. Following elution of the proteins at 50 V for 8 h, the liquid fraction was transferred to a new dialysis bag and dialyzed against 100 volumes of PBS overnight. It was then concentrated to 2 ml using a Centricon (Amicon), resulting in a final protein concentration of 1 mg/ml, as determined by BCA assay.

Production and Affinity Purification of Sortase A Antibodies. Three balb/C female mice (Jackson Laboratories) were each immunized with 300 μg of purified H6-SrtAΔN in complete Freund's adjuvant and boosted twice with 100 μg H6-SrtAΔN in incomplete Freund's adjuvant, at 1-month intervals. A week following the last injection, blood was obtained by heart puncture, and serum was separated by centrifugation.

For the affinity purification of sortase-specific antibodies, a NiNTA column was loaded with purified H6-SrtAΔN and equilibrated with TSA (10 mM Tris pH 8.0, 0.14 M NaCl, 0.2% NaN_3). Serum from one of the mice was applied to the column, and nonspecific antibodies were removed by washes with TSA and 50 mM Tris pH 8.8, 0.5 M NaCl. Sortase-specific antibodies were eluted with 50 mM diethanolamine pH 11.5, 0.15 M NaCl and the pH was adjusted to 7.5.

Fractionation of *S. pyogenes* Cells and Western Blot Analysis. Overnight *S. pyogenes* cultures were diluted 1:100 into 5 ml TH + Y supplemented with spectinomycin when appropriate. Cells were grown to OD_{600} 0.5, at which time 1 ml was harvested by centrifugation. The cell pellet was washed with 1 ml PBS/30% raffinose, resuspended in 200 μl of the same buffer containing 300 U/ml PlyC (7), and incubated for 15 min at room temperature. The resulting spheroplasts were harvested by centrifugation, washed once with PBS/30% raffinose, resuspended in 60 μl Laemmli buffer, and sonicated briefly ("spheroplasts" fraction). The culture supernatant ("supernatant" fraction) and the PlyC digestion supernatant ("wall" fraction) were supplemented with 5% trichloroacetic acid (TCA) and incubated on ice for 1 h. Protein precipitates were collected by a 10-min centrifugation at 20800 g in a cooled Eppendorf 5417R centrifuge, incubated with 1 ml acetone for 1 h with agitation, centrifuged, and resuspended in 60 μl Laemmli buffer.

Samples were boiled for 10 min and separated by electrophoresis on a 10% SDS polyacrylamide gel (8). Blotting to polyvinylidene difluoride membranes was according to Matsudaira (9). PBS supplemented with 5% nonfat dry milk, and 0.1% Tween 20 was used to block the membranes and for antibodies and HRP-conjugates dilution. Blots were developed using SuperSignal West Pico chemiluminescent substrate (PIERCE).

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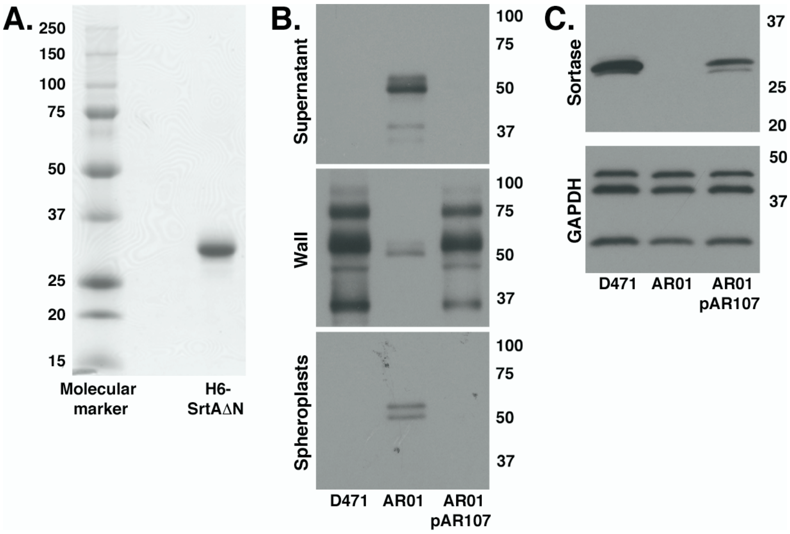


Fig. S1. Production and validation of the anti-sortase antibodies specificity. (*A*) H6-SrtAΔN was purified by NiNTA column and G-100 gel filtration chromatography, loaded on 10% SDS/PAGE, and stained by Coomassie blue. (*B*) Wild-type D471, sortase mutant AR01, and reconstituted AR01 + pAR107, were grown in TH + Y to OD_{600} 0.5, and fractionated into supernatant, wall, and spheroplasts. The distribution of M protein in the different fractions was assessed by Western blot. (*C*) Cells grown in the same manner were harvested, washed, and lysed. The presence of sortase and cytoplasmic GAPDH (loading control) was assessed by Western blot.

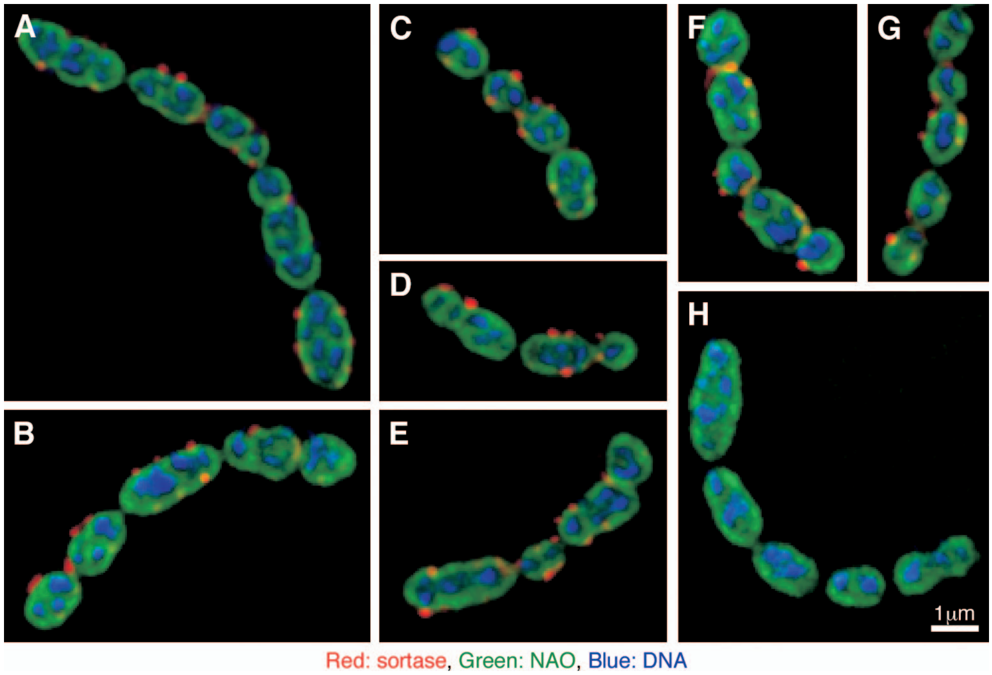


Fig. S2. Sortase localizes to the membrane of *S. pyogenes*. (*A*–*G*) Log-phase D471 cells were fixed, permeabilized, and stained for sortase (Alexa Fluor 647, red), membrane (NAO, green), and DNA (DAPI, blue). (*H*) Control cells not treated with primary sortase antibodies.

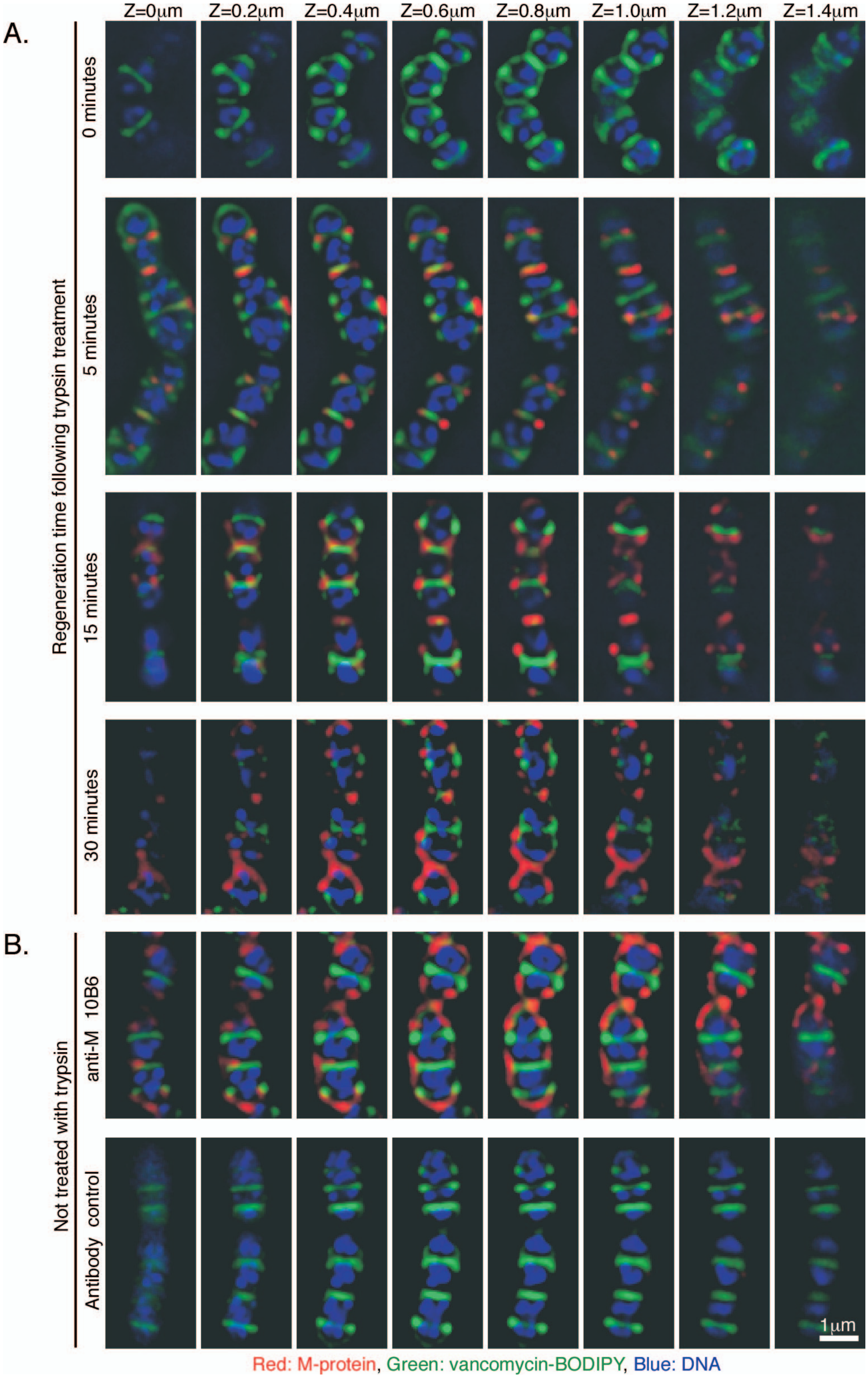
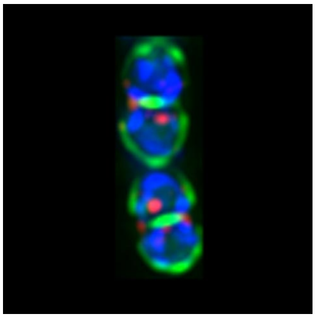
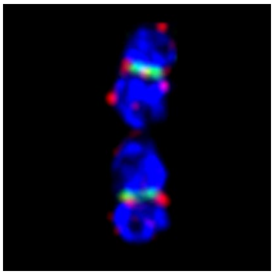


Fig. S3. M protein is initially anchored in septum-associated foci, colocalized with lipid II export regions. (*A*) D471 cells were grown to OD_{600} 0.5 in media containing 0.05% trypsin and either fixed immediately (time 0) or washed and incubated in media without trypsin for 5, 15, or 30 min before fixation. These cells were not treated with methanol and PlyC. The cells were stained for M protein (red), using the 10B6 monoclonal and Alexa Fluor 647 conjugate, and DNA (blue). Vancomycin-BODIPY (green) was used to detect lipid II export regions. Images are presented as sequential Z-stack captures. (*B*) D471 cells, not treated with trypsin, were stained in the same manner. Control cells were not treated with primary M protein antibodies.



Movie S1. 3D reconstruction of D471 cells labeled for sortase (red), cytoplasmic GAPDH (blue), and cell wall material (green).

Movie S1 (MPG)



Movie S2. 3D reconstruction of JRS75 cells labeled for sortase (red), cytoplasmic GAPDH (blue), and cell wall material (green).

Movie S2 (MPG)

Table S1. Primers used in this work

5-srtA-aa34-salI	gaggtcgacaacaaacctatccgaaatacattaattgctc
3-srtA-notI	gtagcggccgcctaggtagatacttggttataagaatgattaaaag
5-UKOsrtA	cccgtcgacgaagaagaagatgttttgattaccttgtc
3-UKOsrtA	ccccctcgagtttttactccttattatgattaattatctttattttttg
5-DKOsrtA	cccccatggatatatcttaaatataatatttaaaaagtaggaccatagagaaaatttatc
3-DKOsrtA	ccccccgggtacagcaggaaccactaataaaacc
pGAPDH-F1	aattcttatttgcatttttctgaaatagttatataatagttctgttgaaaggttgttacagatgactgtaagt
pGAPDH-F2	taatcttttcacaataggtagggagcattccctctaaataatattcttttgattttcataaggaggaaatcactag
pGAPDH-R1	tcaacagaactattatataactatttcagaaaaaatgcaaataag
pGAPDH-R2	ctccctacctattgtgaaaagattaacttacagtcatctgtaacaacctt
pGAPDH-R3	gatcctagtgatttcctccttatgaaaatcaaaagaatattatttagagggaatg
5-srtA-BamHI	gtgggatccatggtaaaaaaacaaaagcgtcg
3-srtA-BglII	gtaagatctctaggtagatacttggttataagaatgattaaaag

Restriction sites are marked by an underline.