Legends to the supplemental figures:

Figure S1. Scheme of the adhesin GspB-F construct.

Full-length GspB contains a signal sequence (SS), a Ser/Thr-rich region 1 (SRR1), the binding region (BR), a second Ser/Thr-rich region 2 (SRR2), and a cell wall anchoring (CWA) motif. GspB-F is a fragment containing the indicated regions. GspB-F also contains C-terminal FLAG and His tags.

Figure S2. Analysis of carbohydrates attached to GspB secreted from *S. gordonii*.

(A) Signal sequence containing FLAG-tagged GspB-F was expressed in *S. gordonii*. The secreted protein was purified and subjected to SDS-PAGE. After trypsin treatment, glycopeptides were extracted from gel slices, and the glycans were cleaved off by *β*-elimination, permethylated, and analyzed by MALDI-mass spectrometry (MS). Detected *O*-glycans are indicated. Hex – Hexose, HexNAc – N-acetyl hexosamine.
(B) *β*-eliminated glycans were permethylated and analyzed by ESI-mass spectrometry. Detected *O*-glycans are indicated.
(C) The HexHexNAc and Hex_2HexNAc peaks in (B) with m/z ratios of 534.29 and 728.39, respectively, were further analyzed by tandem MS (collision-induced dissociation (CID) MS/MS), allowing the determination of the glycan sequence.
(D) Tryptic glycopeptides of the purified protein were subjected to hydrolysis with trifluoroacetic acid. Released monosaccharides were analyzed by anion exchange chromatography. The bottom panel shows quantification of the monosaccharide composition based on standards. The experiments in (B) -(D) together identified GlcNAc, GlcGlcNAc, and Glc2GlcNAc as the major glycans attached to GspB, but other glycans were detected that contained GalNAc, Gal, and Man.

Figure S3. Glycans attached to GspB when expressed together with GtfA/B, Nss, and Gly in *E. coli*.

(A) His-tagged GspB-F was purified from *E. coli* cells expressing GtfA/B, Nss, and Gly, and subjected to SDS-PAGE. *β*-eliminated glycans were analyzed by MALDI-mass spectrometry (MS). Detected *O*-glycans are indicated. Hex – Hexose, HexNAc – N-acetyl hexosamine.
(B) *β*-eliminated glycans were permethylated and analyzed by ESI-mass spectrometry. Detected *O*-glycans are indicated.
(C) The HexHexNAc and Hex 2 HexNAc peaks in (B) with m/z ratios of 534.29 and 728.39, respectively, were further analyzed by tandem MS (collision-induced dissociation (CID) MS/MS) to determine the glycan sequence.
(D) Tryptic glycopeptides of the purified protein were subjected to hydrolysis with trifluoroacetic acid. Released monosaccharides were analyzed by anion exchange chromatography. Quantification of the monosaccharide composition is based on standards and is shown in the bottom panel.
(E) Purified GspB-F was treated with trypsin. A tryptic peptide derived from the first Ser/Thr-rich segment (residues 126-135) was analyzed by tandem mass spectrometry.

The identified peaks contain Ser or Thr residues modified with either N-acetyl hexosamine (squares) alone or additionally with hexoses (spheres). Red and green indicate unambiguous and ambiguous assignments, respectively.

Figure S4. The Asp proteins do not affect *O*-glycosylation.

Gsp-F was synthesized *in vitro* in the presence of ^{35}S-methionine and then incubated with the indicated glycosyltransferases and UDP-sugars in the absence or presence of Asp1, Asp1/3, or Asp1/2/3. C, control.

Figure S5. Sequence alignment of regions of Asp1 and Asp3.

(A) Asp1 proteins from different bacterial species contain two conserved Asp residues (D33 and D34 in *S. gordinii*; colored in red). These residues are located in the cleft between R-folds I and II. The conserved residue shown in blue was mutated, but has no effect on secretion (Figure 4B).
(B) Asp3 has a conserved Asn, often preceded by a Glu residue (N46 and E45 in *S. gordonii*; colored in red), which are located at the tip of a β-strand. Residues in blue were mutated and had a moderate secretion defect (Figure 4D).

Figure S6. Complex formation of the Asps.

(A) The Asp1/3 complex was subjected to gel filtration coupled with multi-angle light scattering (SEC-MALS). The refractive index was monitored and the molecular mass of the complex calculated at different elution times.
(B) Asp1, Asp2, and Asp3 were co-purified and subjected to gel filtration. Shown is the UV absorbance profile. Fractions of the peak were subjected to SDS-PAGE and Coomassie blue staining.
(C) Table summarizing the results of SEC-MALS. In the case of Asp1/Asp2/Asp3, both monomers and dimers were detected.

Figure S7. 2D class averages of negatively stained Asp complexes.

(A) 2D class averages of negatively stained MBP-Asp1/Asp3 particles.
(B) As in (A), but for the MBP-Asp1/MBP-Asp2/Asp3 complex.
(C) As in (A), but for the Asp1/MBP-Asp2/Asp3 complex.
(D) As in (A), but for the Asp1/Asp2/Asp3 dimer.
(E) As in (A), but for the Asp1/Asp2/Asp3 monomer.
The box dimensions are 336 Å in (B), and 215 Å in all other panels.

Figure S8. Flotation of liposomes in a Nycodenz gradient.

Liposomes containing Texas Red 1,2-Dihexadecanoyl-*sn*-Glycero-3-Phosphoethanolamine (DHPE) were subjected to flotation in a Nycodenz gradient. Fractions were collected from the top and analyzed for absorbance at 594 nm.

Figure S9. SecA2 has a pronounced positively charged surface patch facing the membrane.

(A) Homology model for SecA2 in cartoon presentation, viewed from the membrane. SecA2's domains are shown in different colors. NBD1 and NBD2, nucleotide-binding domains 1 and 2; PPXD, polypeptide crosslinking domain; HWD, helical wing domain; HSD, helical scaffold domain.
(B) Surface of SecA2 facing the membrane. Note that the surface is highly positively charged (blue). The electrostatic surface was calculated with the Adaptive Poisson-Boltzmann Solver, as implemented in Pymol, using a scale from -5.000 to 5.000 (bottom).
(C) As in (B), but for a homology model for *S. gordonii* SecA1. Note that the equivalent surface is much less positively charged than in SecA2.

Figure S1

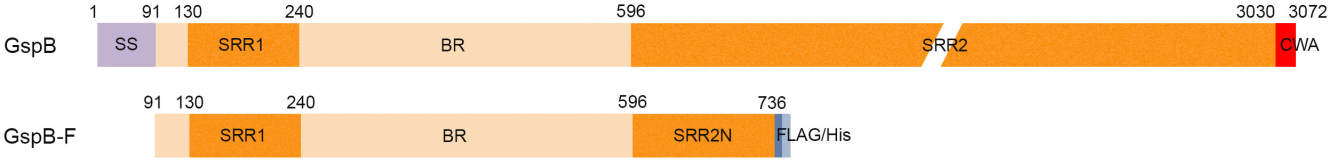
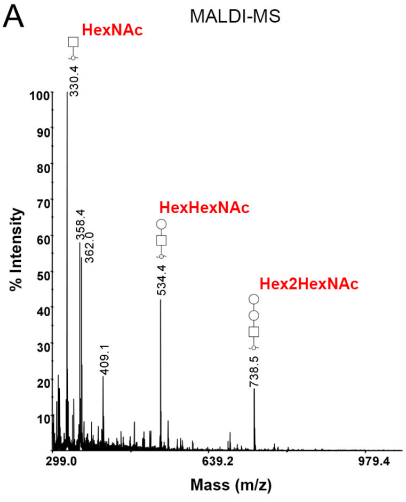
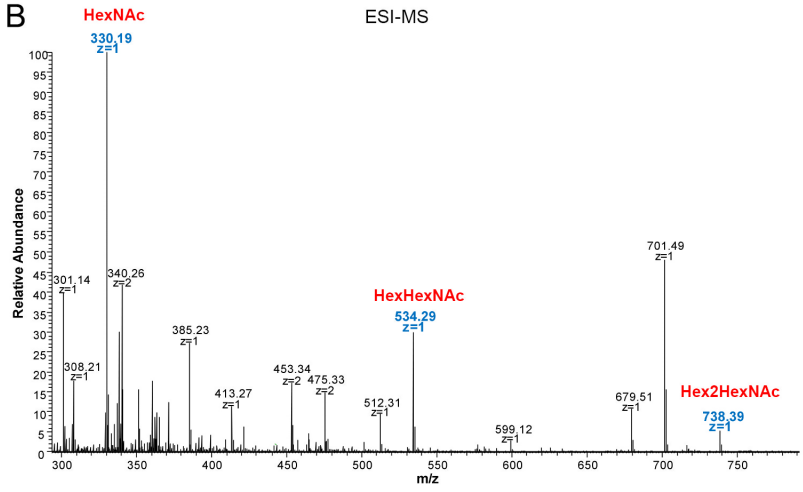
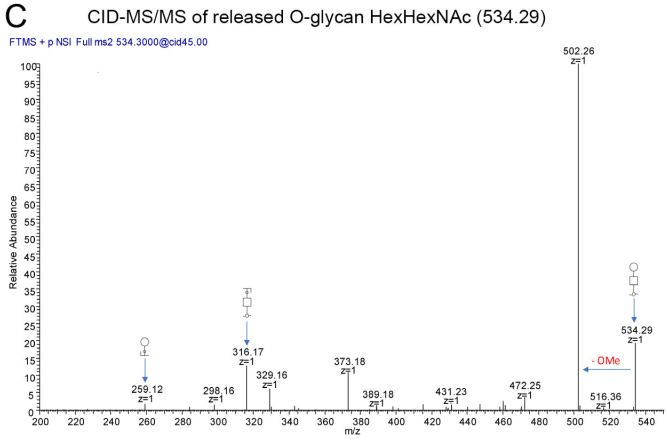
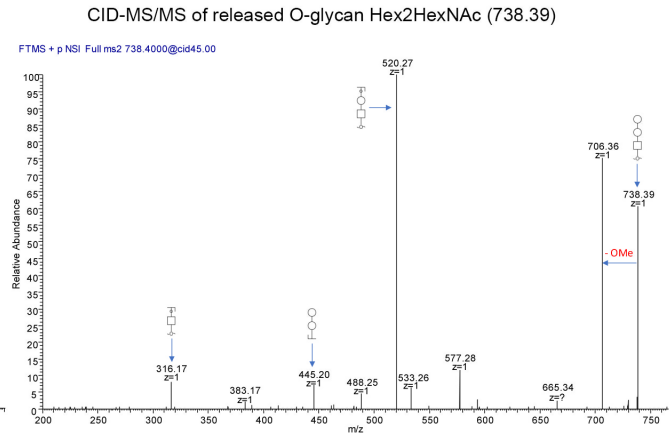

1
91
130
240
596
3030
3072
GspB
SS
SRR1
BR
SRR2
CWA
91
130
240
596
736
GspB-F
SRR1
BR
SRR2N
FLAG/His

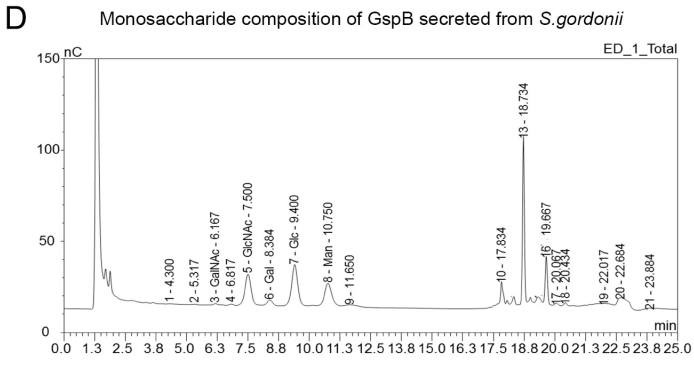
Figure S2





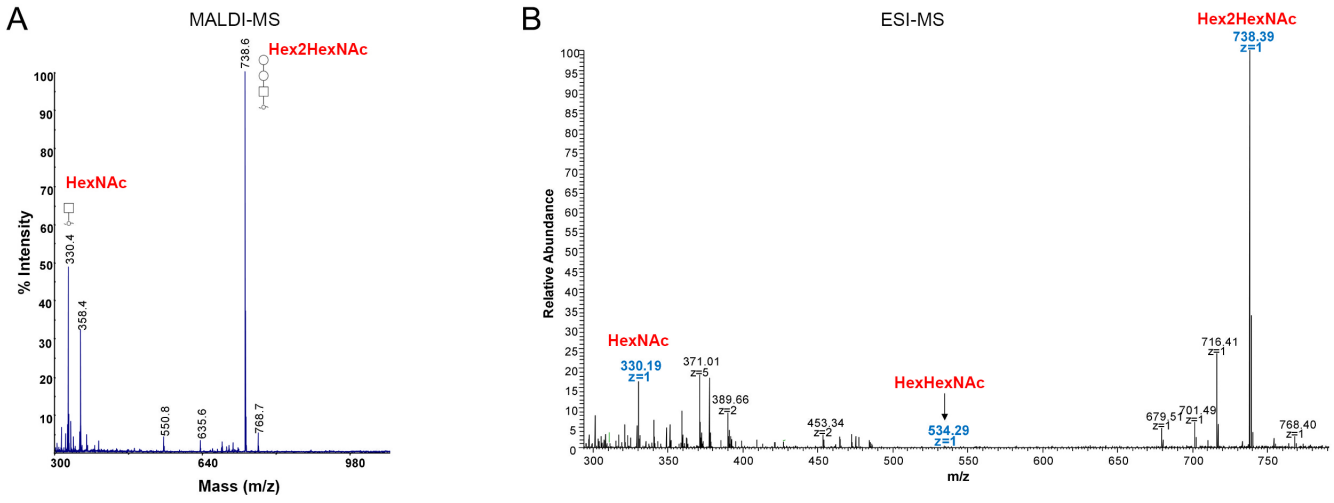


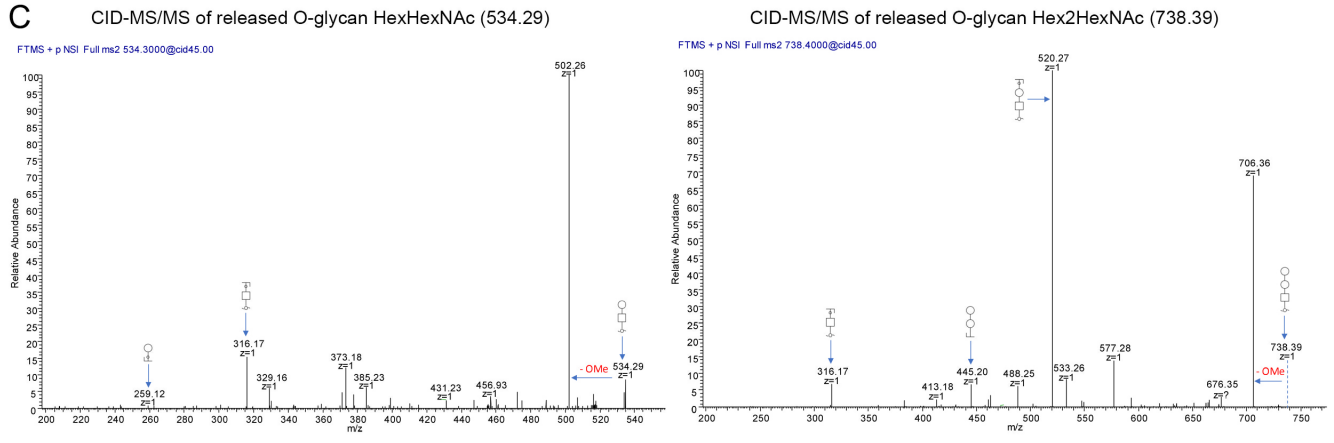


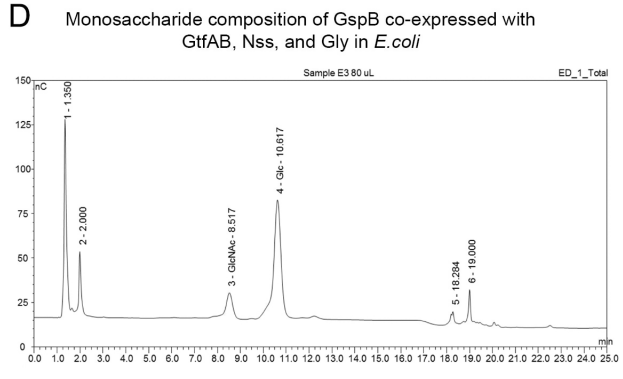


Residue	Micrograms	Nanomoles	% by mole
Fuc	nd	-	-
GalNAc	0.009	0.04	2.29
GlcNAc	0.158	0.71	38.11
Gal	0.024	0.13	6.98
Glc	0.102	0.56	30.13
Man	0.076	0.42	22.49

Figure S3







Residue	Micrograms	Nanomoles	% by mole
Fuc	nd	-	-
GalNAc	nd	-	-
GlcNAc	0.181	0.82	16.83
Gal	nd	-	-
Glc	0.729	4.05	83.17
Man	nd	-	-

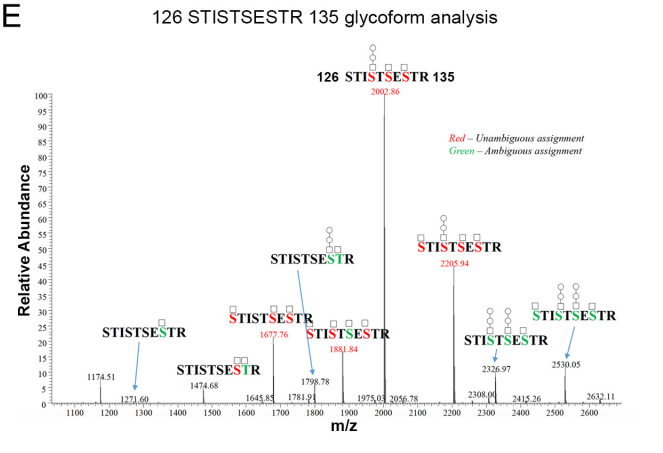


Figure S4

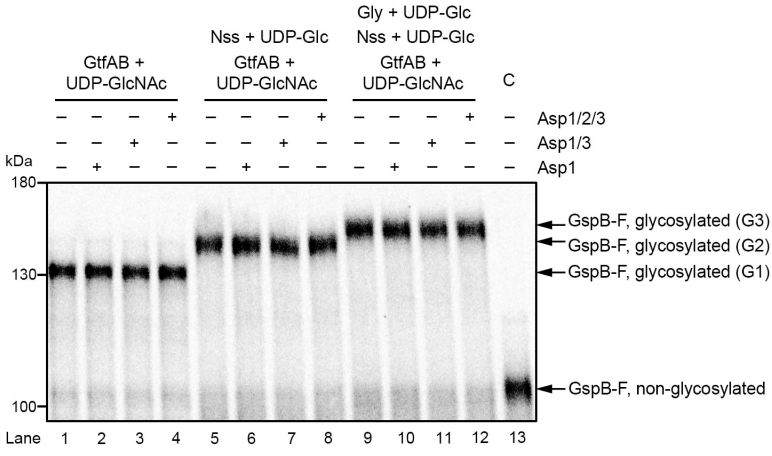

GtfAB + UDP-GlcNAc
Nss + UDP-Glc
GtfAB + UDP-GlcNAc
Gly + UDP-Glc
Nss + UDP-Glc
GtfAB + UDP-GlcNAc
C
Asp1/2/3
Asp1/3
Asp1
kDa
180
130
100
GspB-F, glycosylated (G3)
GspB-F, glycosylated (G2)
GspB-F, glycosylated (G1)
GspB-F, non-glycosylated
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13

Figure S5

A Asp1 (Partial sequences)

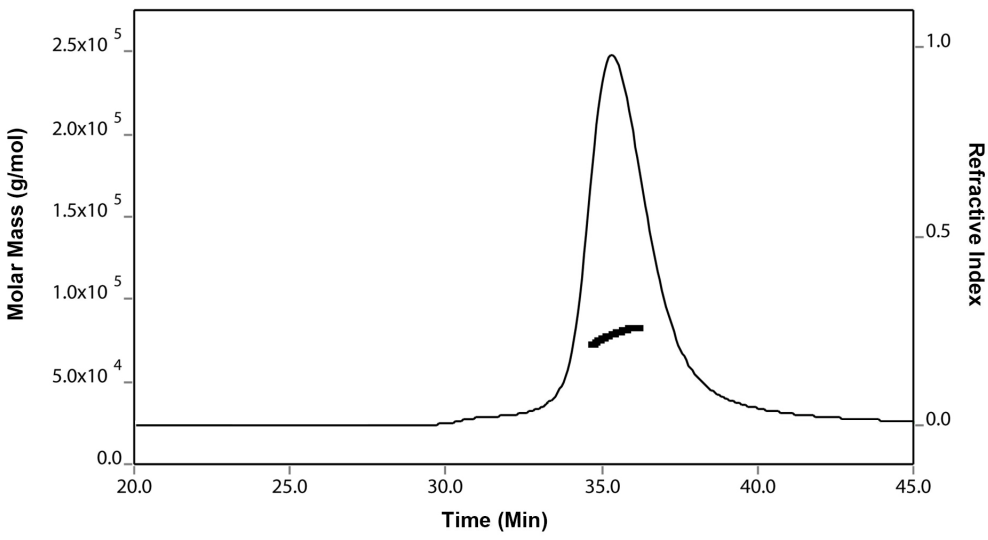
```
Streptococcus gordonii          19 DIIPWYRSMQRLEFDDTIHQIRIFHSENLP 48     254 ILSFFHERNQASN 266
Streptococcus pneumoniae        18 DVTPWYYSSFRFEFDDTFNQIRLFQRQDIP 47     253 ILSLFINRNAQAS 265
Streptococcus sanguinis         19 DIIPWYRSMQRLEFDDTIHQIRIFQSENLP 48     254 ILSFFHERNHTVD 266
Streptococcus parasanguinis     18 DTPWWFRVKNRMTFDDSVNQVKMFLQGQEE 47     256 IYSFFGNRFNLEN 268
Streptococcus salivarius        19 VIDPWYRIRQKIEFDDSLHQVRIFQDEDLA 48     252 ILTWFAERNQDDS 264
Streptococcus mitis             18 DMIPWYHSQFRFEFDDTFNQIRLFQRQGIP 47     253 ILSLFINRNSQAS 265
Streptococcus oralis            18 DLTPWYFSHFKLEFDDTFNQIRLMNRQGIP 47     253 VLSLFIGRNPQEQ 265
Streptococcus agalactiae        18 NNYLWYFKPTNVGFDDTINQMKMFDYAGKE 47     252 ILSVFSERNNAHN 264
Streptococcus vestibularis      19 AIDPWYRIRQKIEFDDSLHQVRIFQDEDLA 48     252 ILTWFAERNQEDK 264
Streptococcus urinalis          18 KTSVWYRRAAGMDFDDTINHVKMFQYIDYQ 47     253 LVSVFSLRNKKED 265
Streptococcus porcinus          18 DTKEWTHINQYLQFDDSVNQIKVFHDNQEN 47     252 IFSFYQSRQRLDS 264
Staphylococcus aureus           18 TTVPYYQLQNKTEFDDMISLMGMHLENDLD 47     251 SYSFFKNRNETVS 263
Staphylococcus epidermidis      18 TSRPFYLKKQYTDFDDMISLMTMHSSNNVD 47     251 CFSIFTERNKVVT 263
Staphylococcus capitis          18 TSVPFYQKRQYTDFDDMISLMTMHSKNNVD 47     251 CFSVFTERNKKVT 263
Staphylococcus hyicus           18 QAQPFFRKRTITEFDDIISLMSMHYQNDNH 47     251 CYSVFKQRNTNLD 263
Staphylococcus saprophyticus    18 NTLPFYYKPDKTDFDDMISLMSMHKKNGYE 47     250 CFSLFSNRNREIT 262
Staphylococcus pasteuri         18 LNQPFYIKNAMIEFDDSISLMSMHEKNDED 47     250 YYSIFSKRNRTID 262
Lactobacillus salivarius        18 AFPEWYFTTNKMEFDDTVNQLMMFRQSEEK 47     249 IMSIFSNRLDIDQ 261
Lactobacillus oris              15 ----WAYTVPMVEFDDAVSHLQILRDDQQP 40     244 VFSAS--RWHPYR 254
Lactobacillus reuteri           15 ----WAHTIPKLEFDDAVSHIKVFQTNQKP 40     243 IFSLS--RWHRYH 253
Leuconostoc gelidum             13 ---------KATTFDDTVNQIRMFEAVHEN 33     240 VLSFFKNRYEYKK 252
Aerococcus urinae               17 ----GEPSYMDLYFDDTVNQLRLFQRAEEA 42     249 VYSLYSQRSFDSQ 261
Oenococcus oeni                 13 --------FRPTDFDDTVHQIKIFKKGNLP 34     239 VLSYFSNRYDFQD 251
Facklamia sourekii              18 RLDGSSLRRGFLEFDDTVNQLRMFQEAGEA 47     250 ALSLFQLRNPSYQ 262
                                                *** .  : :                 :    *
```

B Asp3 (Partial sequences)

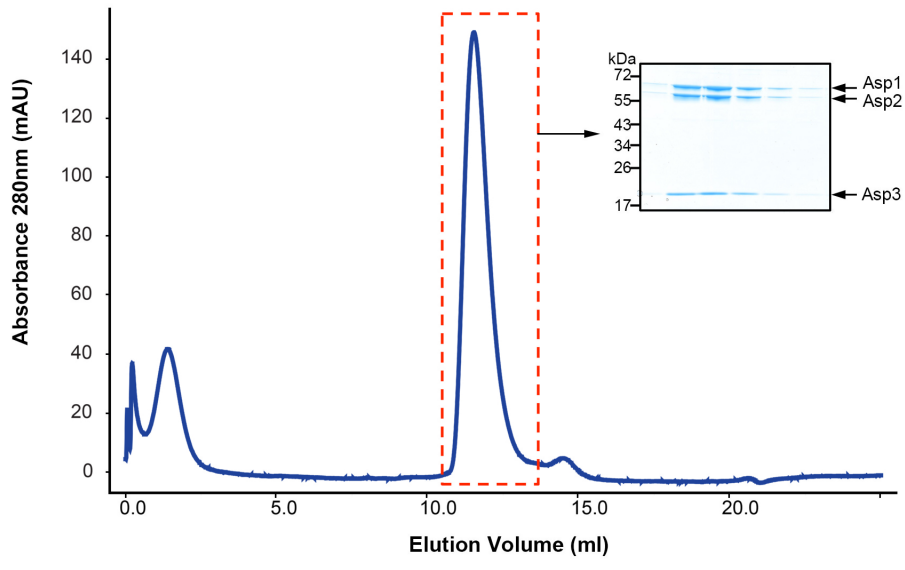
```
Streptococcus gordonii          44 FENKLIASGQTIHEW 58   97 FLKLIFFDRYNREVSNHVERSDKMTFTYPEEAYSYKVQLL 136
Streptococcus pneumoniae        43 FENEMISSGTMVHEW 57   96 YFRLIFKDRYDEKVSQLIKKDLTFTFTYPEEAYNYSIQLL 135
Streptococcus mitis             43 FENSMVNSGTMIHEW 57   96 YFRLIFMDRYDKQVDQVIEKDFDFTFTYPEEAYHYKVQLL 135
Streptococcus sanguinis         44 FENKLMASGQTIHEW 58   97 FLKIIFFDRYDKEISNQVERSESMIFTYPKEAYSYKVQLL 136
Streptococcus infantis          43 FENSMVNSGTMIHEW 57   96 YFRLIFMDRYNKQVDQVIEKDFDFTFTYPEEAYHYKVQLL 135
Streptococcus parasanguinis     38 FKNPLMPSGQILKTW 52   91 MVEIVFLDRFGVPVKRQVTSDGSLAFVYPENAYAYEVRLL 130
Streptococcus salivarius        34 FYNPLVPSGTEIQSW 48   87 FLKVSFLDRYDNEIKQLIEKGTHMTFVYPHEAYTYRISLL 126
Streptococcus vestibularis      34 FYNPLVPSGTEIQSW 48   87 FLKVSFLDRYDNEIKQLIEKSTQMTFVYPHEAYTYRISLL 126
Streptococcus oralis            34 LKNPLLASGETLKTW 48   87 YLKLTFLNRYEEIIEEKIERQFSFSFTYPETAYTYRLSLI 126
Streptococcus thoraltensis      30 FNNSLMPSGQVINRW 44   83 ILQITFYDRFNRSLNSVTLKNDNHSFVYPEGAYAYDISLV 122
Streptococcus halotolerans      30 FKNHLMPSGQVINCW 44   83 MLQITFYDRFDRSLETITLKNGKTSFVYPQGAYAYDISLI 122
Staphylococcus aureus           34 FNNPLMPSGTVIHDW 48   87 YIKITFYRKNDTEHSNLIIQNSDAEFEFPEEAYAYKIELI 126
Staphylococcus schleiferi       34 FQNELMPSGIVIHQW 48   87 YFKIIFKHRDNTVCDVQIILGHEAEVRMPQQAFNYELQMI 126
Staphylococcus epidermidis      34 FENPLMPSGTIIHSW 48   87 YFKMKFYRKNKEILSHQILKNKKENIVYPREAYSYELELI 126
Staphylococcus capitis          34 FENPLMPSGTVIHNW 48   87 YFKIKFYRNNKEQFEYKIIKNKEEEIVYPLEAHSYQLELV 126
Lactobacillus oligofermentans   34 FKNERVSPGLAVHRW 48   87 GLQIQFFDEAGLVIDECLVRTSQVDFVVPAGMVSYEIDLI 126
Lactobacillus uvarum            34 FENKLMSPGKTVNTW 48   87 YLRLDFYDRSDLVIKQIFIKEMEEAFEYPDGAYYYTISLL 126
Lactobacillus murinus           34 FENELMSPGKALVTW 48   87 YLKVSFYSRFGEVVQTTFIKDLQGEFEYPKDAYEYRLELI 126
Leuconostoc pseudomesenteroides 34 FKNDRFPSGTKIHTW 48   87 YVQIIFFNRFHDIVDRVIAKNEEQKFLYPEAAYYYEISLL 126
Leuconostoc gelidum             34 FQNDLFSAGSFVHTW 48   87 YLQFKFYDRYEDMIDSVTIKNGVGKFIYPNNAYAYSMALI 126
Enterococcus cecorum            34 FKNVQLASGKQIVAW 48   87 YLKIIFYNRLDEEIGTVIQREDVQEFVYPQGAFRYDILVI 126
Aerococcus urinae               33 FKNTLMPSGERITSW 47   86 FFKISFFDYYGGPIEEYYLRTPSDYFTVPDTYNYYEIALH 125
Oenococcus oeni                 36 FENSLMPSSVIIHSW 50   89 YTQISFYDIAKTRIKTINIKESRGTFIYPKNSFSYRIDLI 128
Facklamia sourekii              39 FENELLPSGKRIHTW 53   92 FVELSFYDRYDNLIQAVYVKDGQEDFQVPLGTYYYHMSLF 131
                                   : *  .  .  : *        .. *                  .  *    * : :
```

Figure S6

A



B



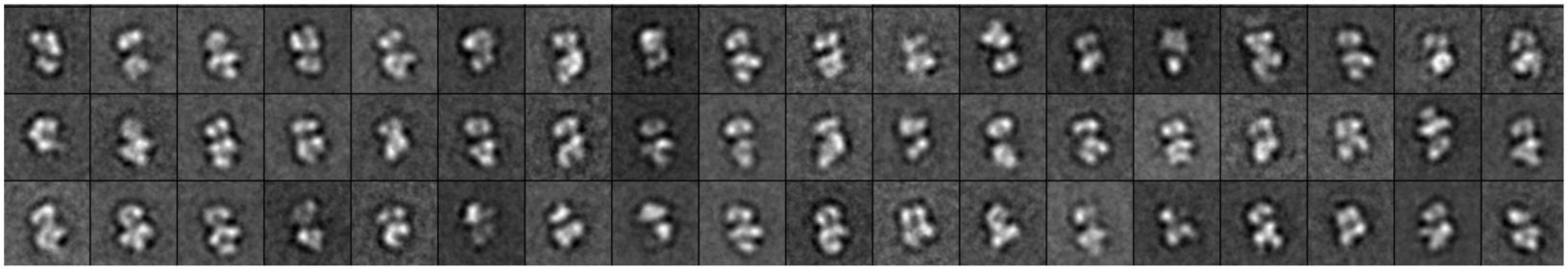
C

Protein complex	Molecualr weight (kDa)		
	Measured	Calculated (1:1:1)	Calculated (2:2:2)
Asp1/Asp2/Asp3	241.50	141.60	283.20
Asp1/MBP-Asp2/Asp3	181.80	184.13	
Asp1/GST-Asp2/Asp3	335.00		335.48

Figure S7

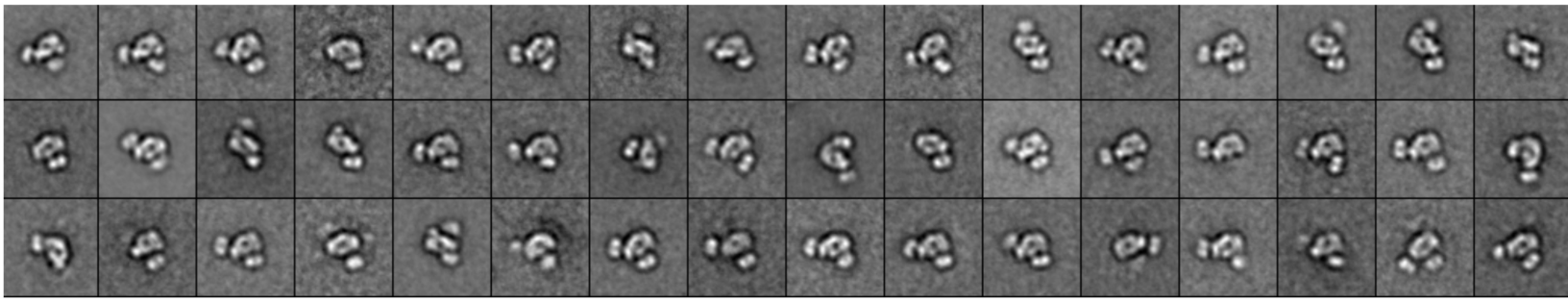
A

MBP-Asp1/Asp3



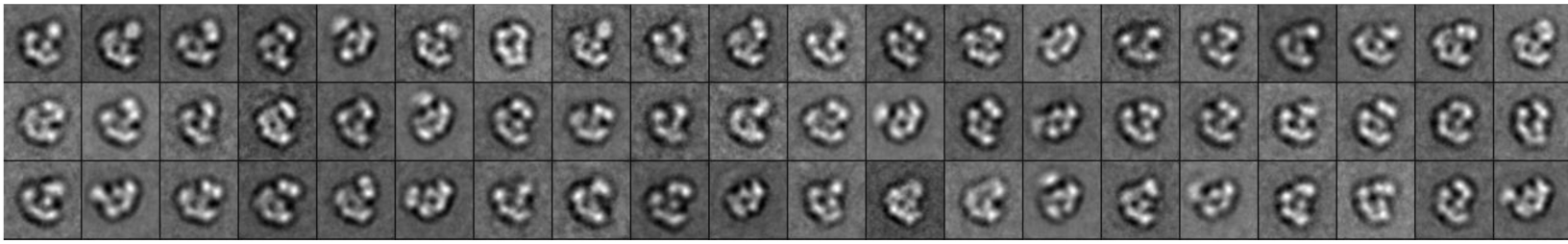
B

MBP-Asp1/MBP-Asp2/Asp3



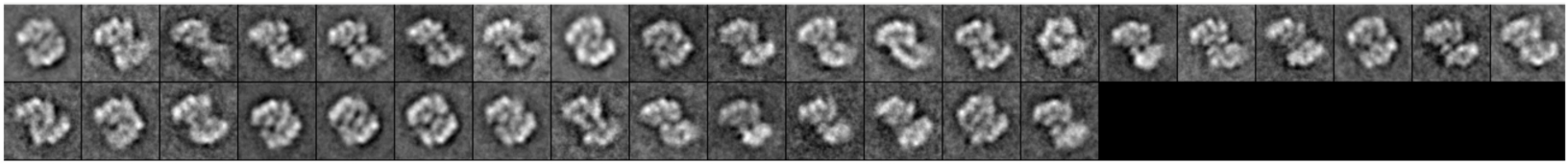
C

Asp1/MBP-Asp2/Asp3



D

Asp1/Asp2/Asp3 (Dimer)



E

Asp1/Asp2/Asp3 (Monomer)

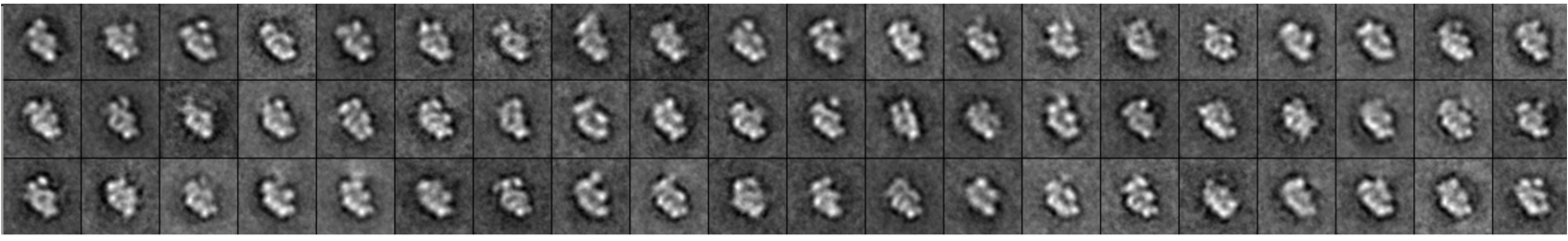


Figure S8

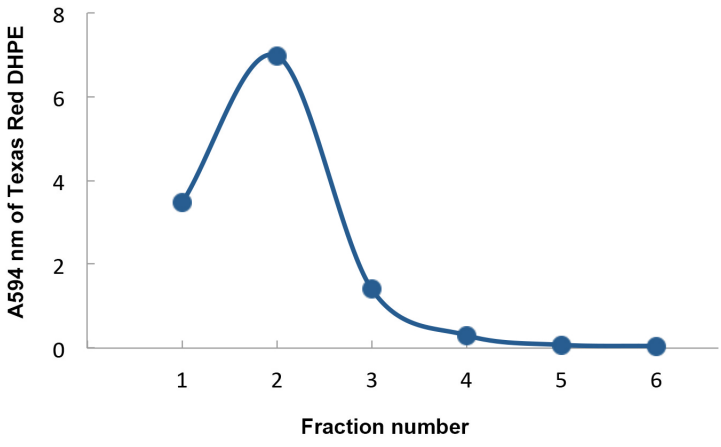


Figure S9

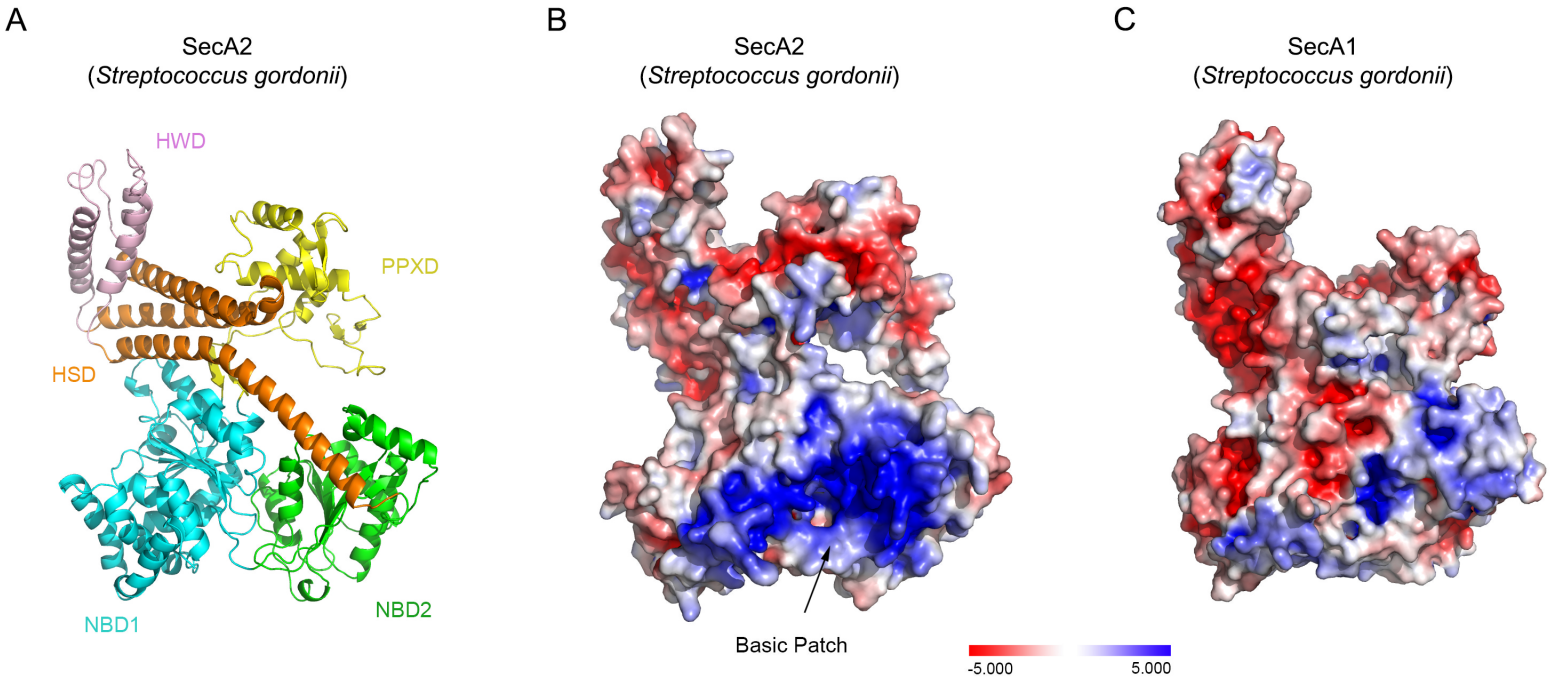

A
SecA2
(Streptococcus gordonii)
HWD
PPXD
HSD
NBD1
NBD2
B
SecA2
(Streptococcus gordonii)
Basic Patch
-5.000
5.000
C
SecA1
(Streptococcus gordonii)

Table S1. Data collection and refinement statistics

	S. gordonii Asp1	*S. gordonii* Asp1/3 complex
Data collection		
Resolution limit (Å)	2.77	3.11
Space group	P 1	C 2 2 2_1
Cell dimensions		
a, b, c (Å)	41.50, 99.90, 179.07	152.60, 257.29, 217.72
α, β, γ (°)	100.7, 90.1, 95.8	90.0, 90.0, 90.0
Total reflections	211247	1164890
Unique reflections	66446	77079
R_{merge}	0.166 (0.493)	0.145 (>1)
CC1/2	0.984 (0.684)	0.999 (0.784)
*CC**	0.996 (0.901)	1 (0.937)
Mean *I/σI*	13.63 (2.72)	25.19 (2.35)
Completeness (%)	92.70 (91.54)	99.48 (95.98)
Multiplicity	3.2 (1.9)	15.1 (15.6)
Wavelength (Å)	0.9789	0.9789
Refinement		
Resolution range (Å)	93.05 - 2.77	49.90 - 3.11
R_{work}/ R_{free} (%)	20.83 / 28.16	20.69 / 24.83
No. non-hydrogen atoms	17260	21840
Protein	17257	21756
Solvent	3	84
Average B-factor (Å^2)	50.57	29.22
Protein	50.57	29.29
Solvent	33.14	11.51
R.m.s deviations		
Bond lengths (Å)	0.009	0.010
Bond angles (°)	1.09	1.29
Ramachandran analysis (%)		
Favored	90.8	92.9
Allowed	8.4	6.7
Outliers	0.8	0.4

Statistics for the highest-resolution shell are shown in parentheses. $R_{merge}=\Sigma_{hkl}\Sigma_i|I_i(hkl)-I(hkl)|/\Sigma_{hkl}\Sigma_i|I_i(hkl)|$, where *I(hkl)* is the integrated intensity of the reflection. $R=\Sigma|F_{obs}-F_{calc}|/\Sigma F_{obs}$, where F_{obs} and F_{calc} are observed and calculated structure factors, respectively. R_{free} was calculated with 5% of the reflections. *CC1/2* is defined as the correlation coefficient between intensities from random half datasets. *CC** describes the CC of the full dataset against the true intensities.