Supplementary Information File

"Engineered oligosaccharyltransferases with greatly relaxed acceptor site specificity"

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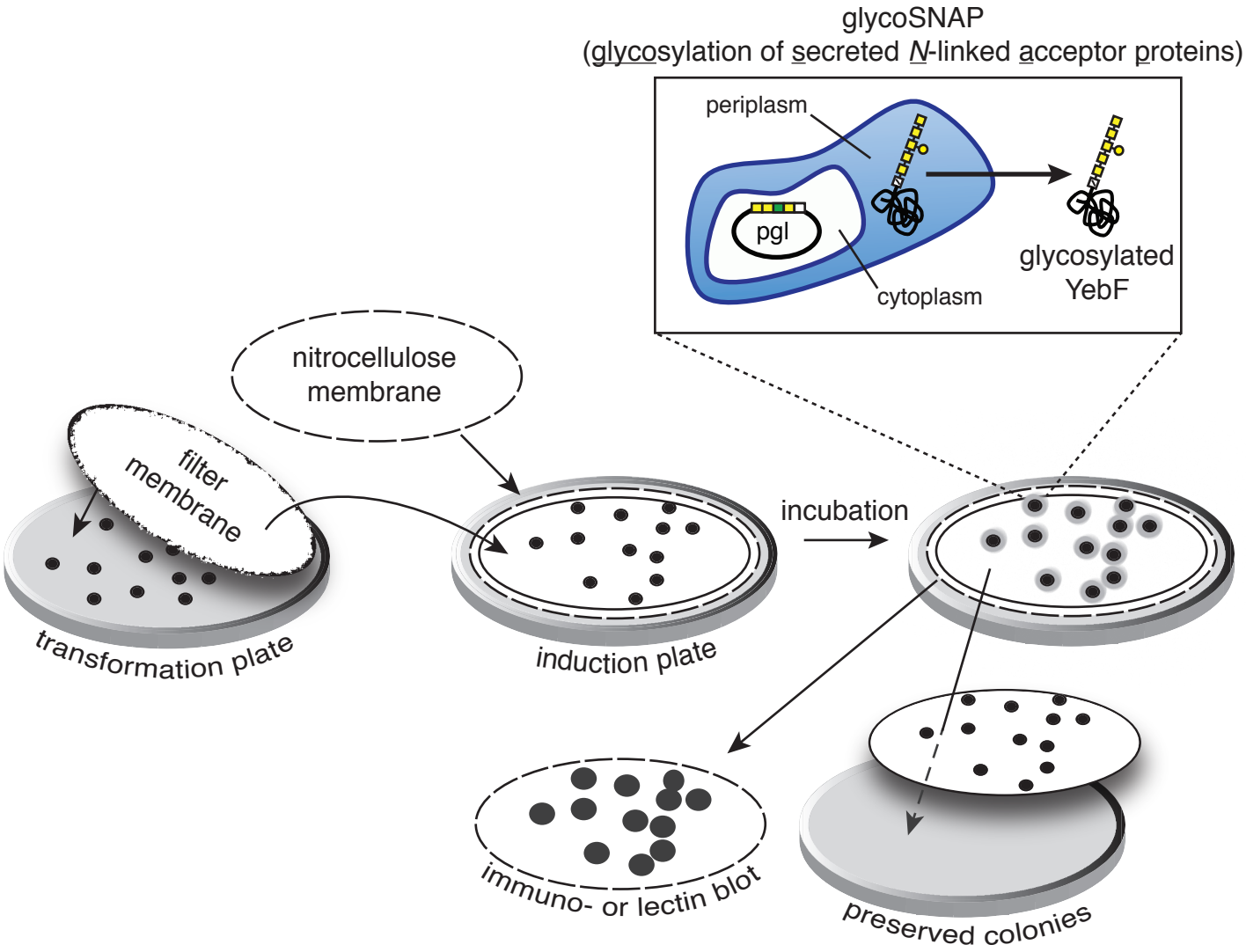
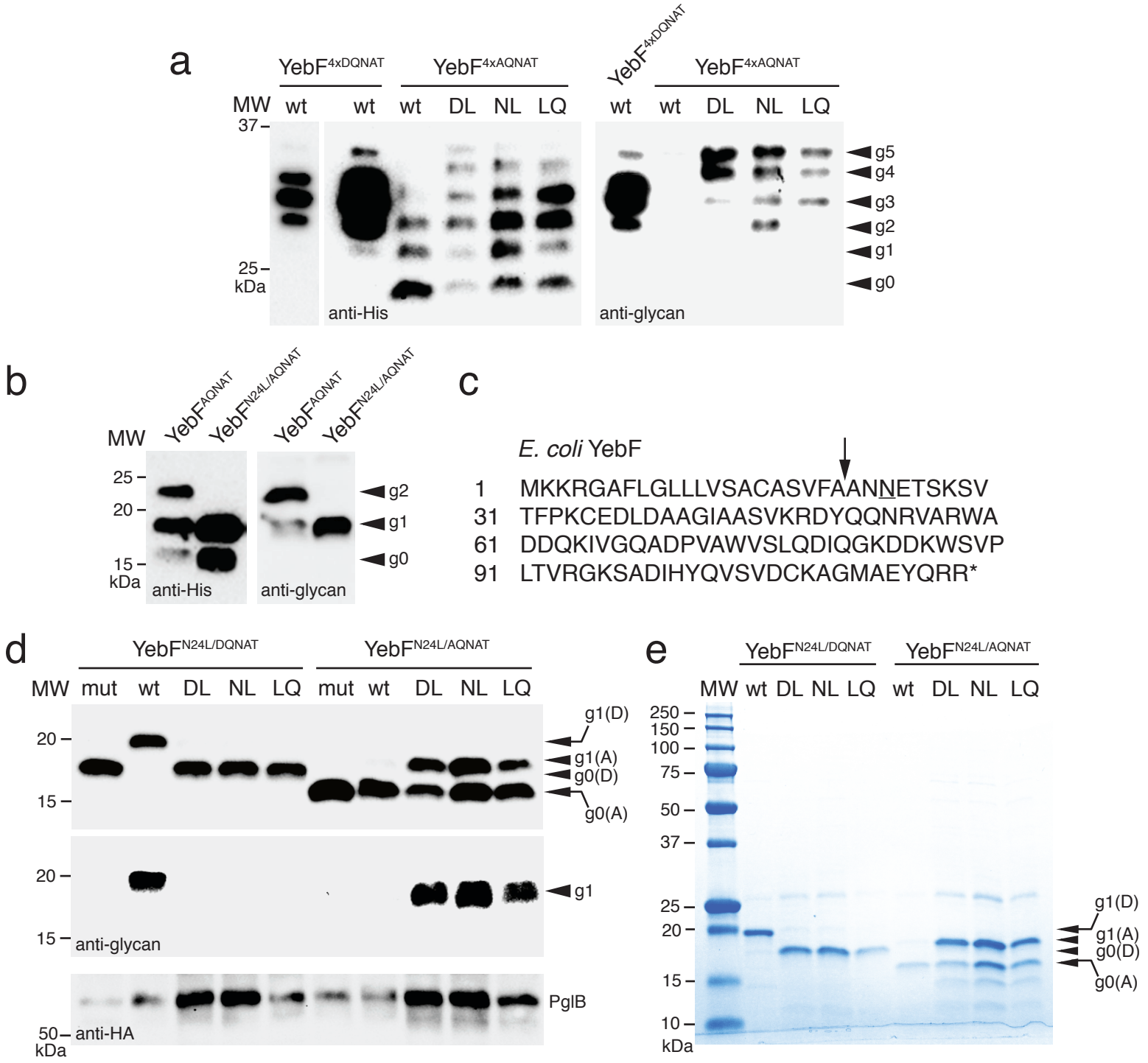
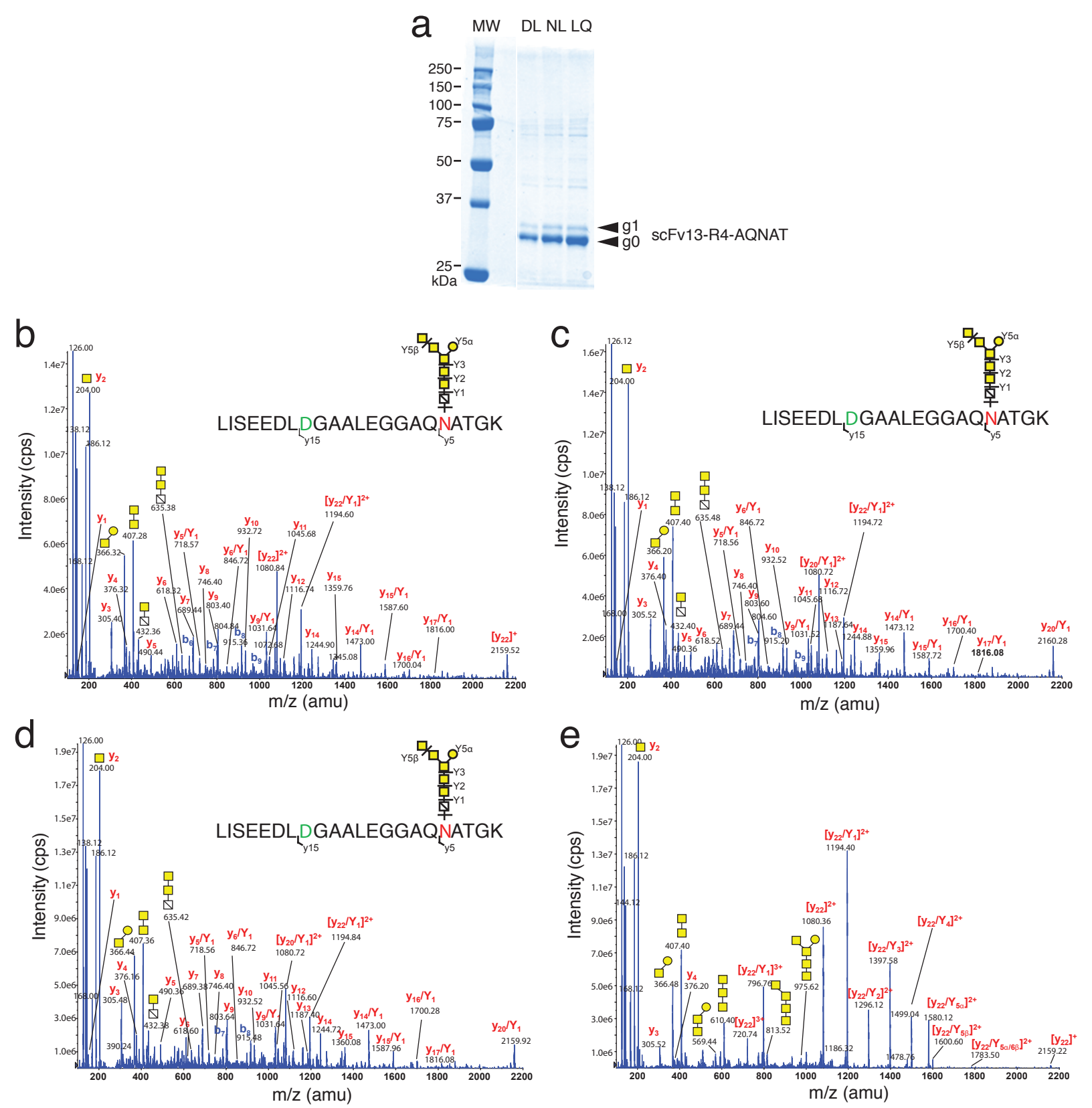
Supplementary Results



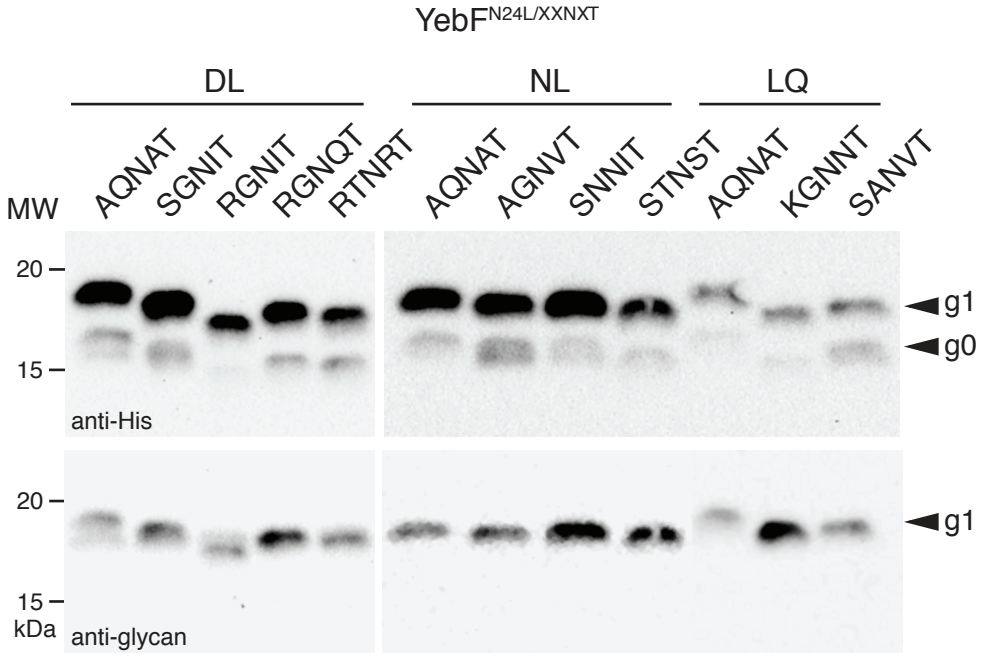
Supplementary Figure 1. The glycoSNAP assay. Schematic of glycoSNAP assay for reporting *N*-glycosylation of secreted acceptor proteins in glycoengineered *E. coli*. Colonies carrying plasmids that encode *N*-glycosylation machinery and *E. coli* YebF modified with an acceptor sequon (e.g., YebF^{4xDQNAT}) were replicated on a filter, and protein expression was induced when the filter was overlaid on a plate layered with a nitrocellulose membrane. YebF that was secreted and glycosylated was specifically detected by Western blot and correlated to glycosylation-competent colonies.



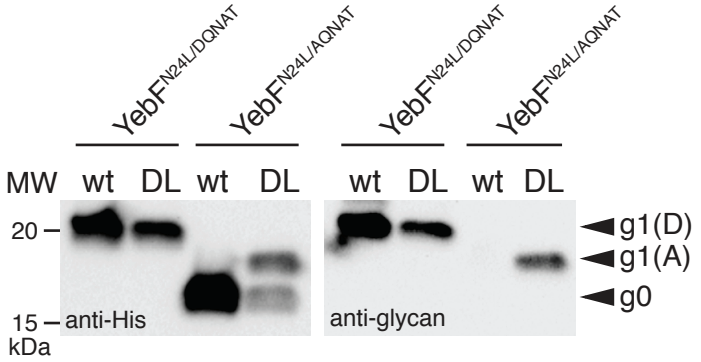
Supplementary Figure 2. Characterization of YebF glycosylation by isolated OST variants. (a) Western blot analysis of YebF^{4xAQNAT} glycosylation by wt *Cj*PglB or DL, NL, and LQ mutants isolated using glycoSNAP. Glycosylation of YebF^{4xDQNAT} by wt *Cj*PglB is shown for comparison. Shorter exposure of YebF^{4xDQNAT} glycosylation clearly shows doubly through quadruply glycosylated YebF (far left panel). The g0 through g5 labels denote aglycosylated and singly through quintuply glycosylated forms of YebF. Results are representative of at least three biological replicates. (b) Western blot analysis to confirm glycosylation of residue N24 in YebF by *Cj*PglB DL variant. The g0 through g2 labels denote aglycosylated, singly, and doubly glycosylated forms of YebF. Results are representative of at least two biological replicates. (c) Amino acid sequence of native *E. coli* YebF. Arrow indicates signal peptide cleavage site for processing to mature protein. The underlined Asn indicates the *N*-glycosylated residue in this study. (d) Glycosylation efficiency of *Cj*PglB variants assessed using YebF^{N24} with single DQNAT or AQNAT acceptor site. Catalytically inactive *Cj*PglB$^{D54N/E316Q}$ (mut) is shown for an aglycosylated YebF control. The g0 and g1 labels denote aglycosylated and singly glycosylated forms of YebF, respectively. Samples were from four-hour inductions and representative of at least three biological replicates. The bottom blot corresponds to membrane fractions prepared from osmotically lysed spheroplasts from the same cultures as the blots above. Note that both aglycosylated and glycosylated YebF$^{N24L/AQNAT}$ (g0(A) and g1(A), respectively) migrate faster than aglycosylated and glycosylated YebF$^{N24L/DQNAT}$ (g0(D) and g1(D), respectively). (e) SDS-PAGE analysis of YebF$^{N24L/DQNAT}$ or YebF$^{N24L/AQNAT}$ purified from culture supernatants of the same cells in (d). The g0 through g1 labels denote aglycosylated and singly glycosylated forms of YebF. Molecular weight (MW) markers are indicated at left of all Western blots and the SDS-PAGE gel.



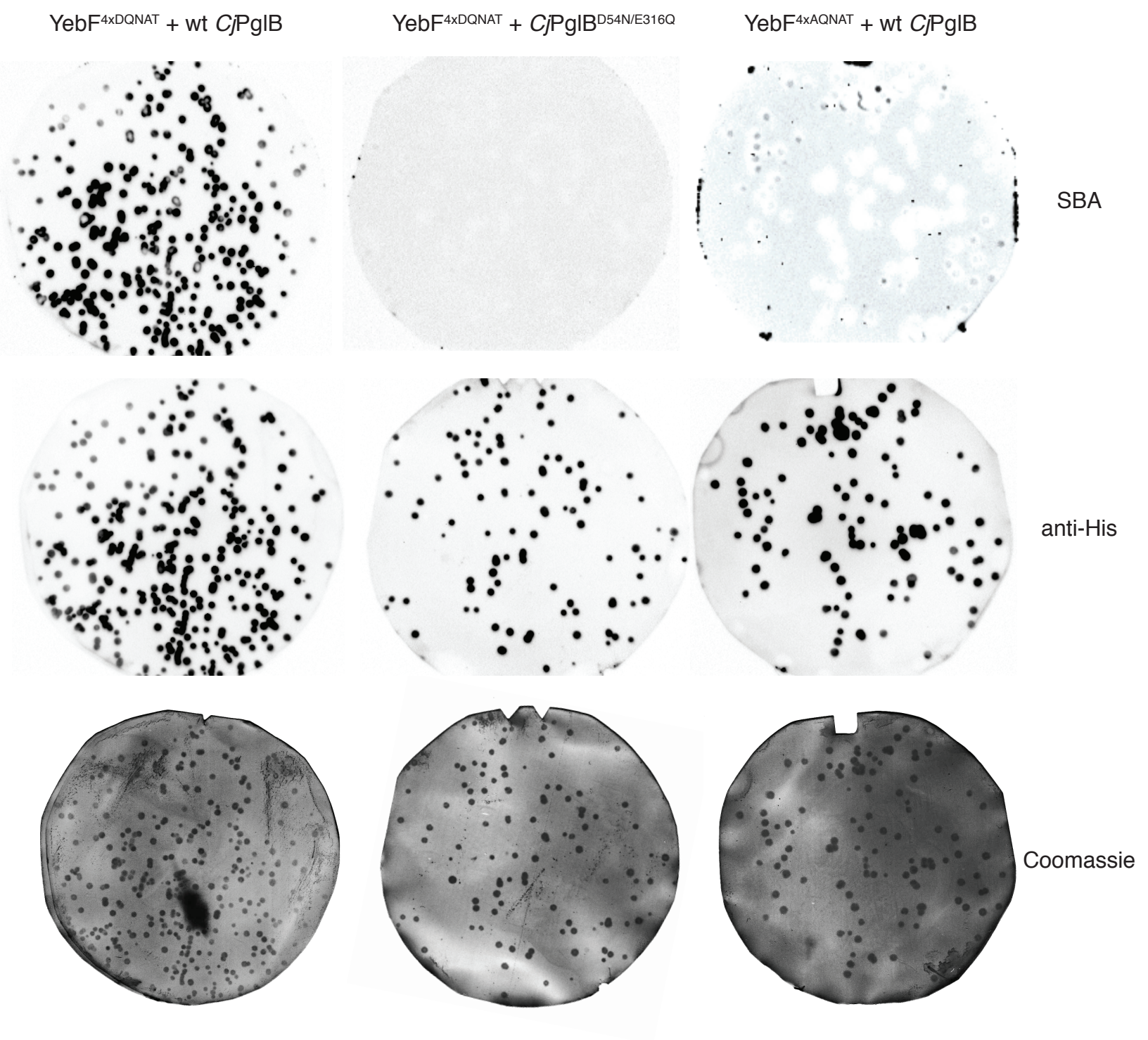
Supplementary Figure 3. Mass spectrometry analysis of nonconsensus glycosylation. (a) Ni-NTA-purified scFv13-R4^{AQNAT} samples used in MS analysis, stained with Coomassie Brilliant Blue G-250. Glycosylated bands, indicated by g1 arrow, were excised and submitted for MS analysis. MS/MS spectrum of the triply-charged precursor ion [*m/z* 1189.01 for *Cj*PglB DL variant (b); *m/z* 1189.03 for *Cj*PglB NL variant (c), and *m/z* 1189.08 for *Cj*PglB LQ variant (d)], identifying the glycopeptide and a 1405.56 Da glycan with bacillosamine as the innermost saccharide attached to the N273 site (shown in red) in scFv13-R4^{AQNAT}. A series of y-ions covering from y1 to y15 was observed with the complete knockout of glycan molecule, leading to the confident identification of tryptic peptide 256-LISEEDLDGAALEGGAQNATGK-277, in which N263 residue was found to be deamidated to Asp (shown in green), consistent with commonly observed deamidation of Asn residues that are followed by Gly. A second series of y-ions with the added mass of 228.11 Da at N273 site was also found covering from y9/Y1 to y17/Y1, providing direct evidence for bacillosamine as the innermost saccharide (Y1) attached to N273 site. This result is also consistent with the previous observation that a relatively tight bond exists for Y1-peptide compared to the fragile internal glycan bonds. (e) Representative MS/MS spectrum (result from *Cj*PglB DL variant is shown) for the quadruply-charged precursor (*m/z* 892.05) with low collision energy (CE = 29 eV) applied. A complete Y-type series ions (from Y1 to Y6β) attached to the core peptide reveals the expected *C. jejuni* heptasaccharide glycan.



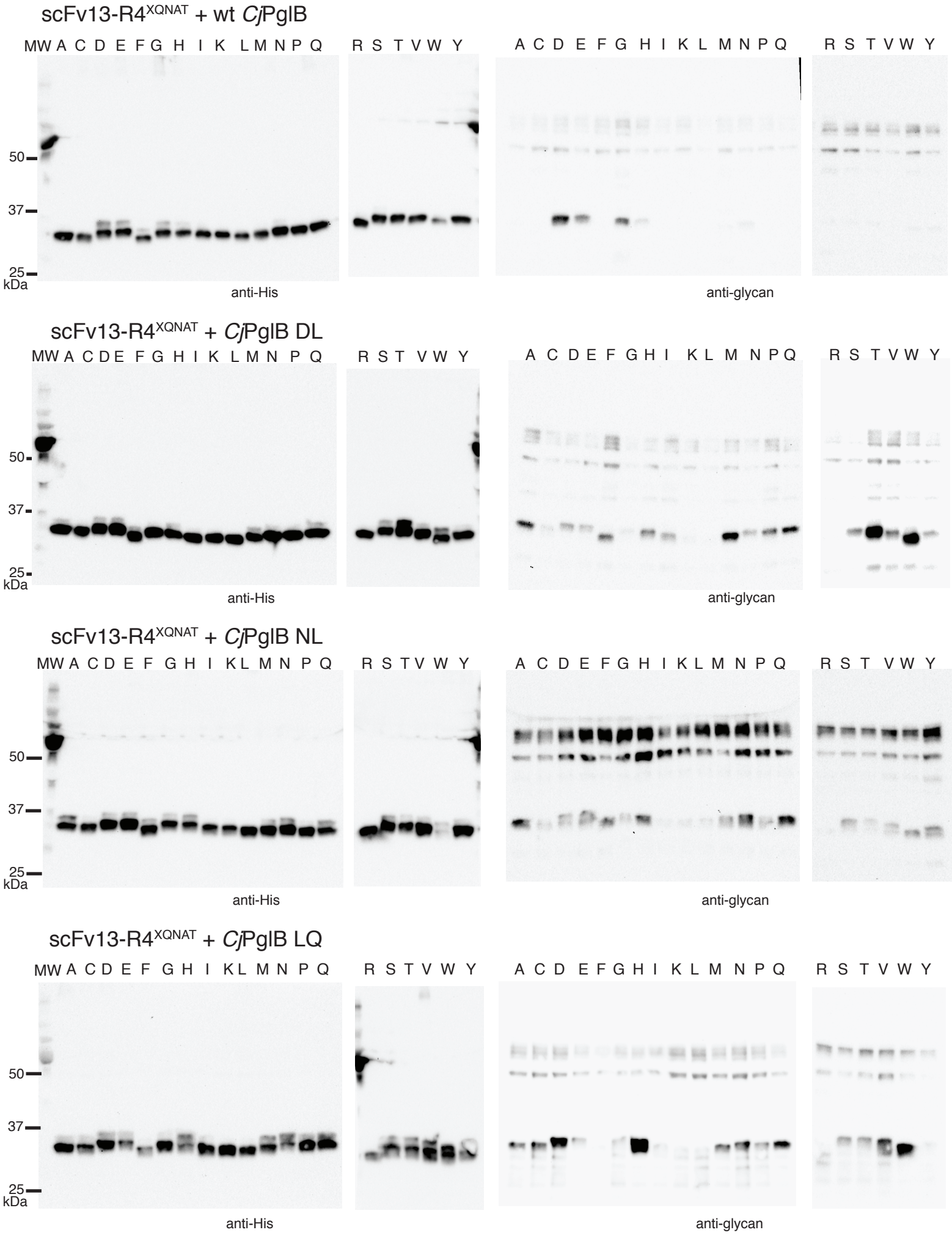
Supplementary Figure 4. Glycosylation of glycoSNAP-isolated YebF$^{N24L/XXNXT}$ variants. Western blot analysis of the most efficiently glycosylated YebF$^{N24L/XXNXT}$ targets for each *Cj*PglB variant (DL, NL, or LQ) compared to glycosylation of YebF$^{N24L/AQNAT}$. Molecular weight (MW) markers are indicated at left. The g0 and g1 labels denote aglycosylated and singly glycosylated forms of YebF, respectively. Results are representative of at least three biological replicates.



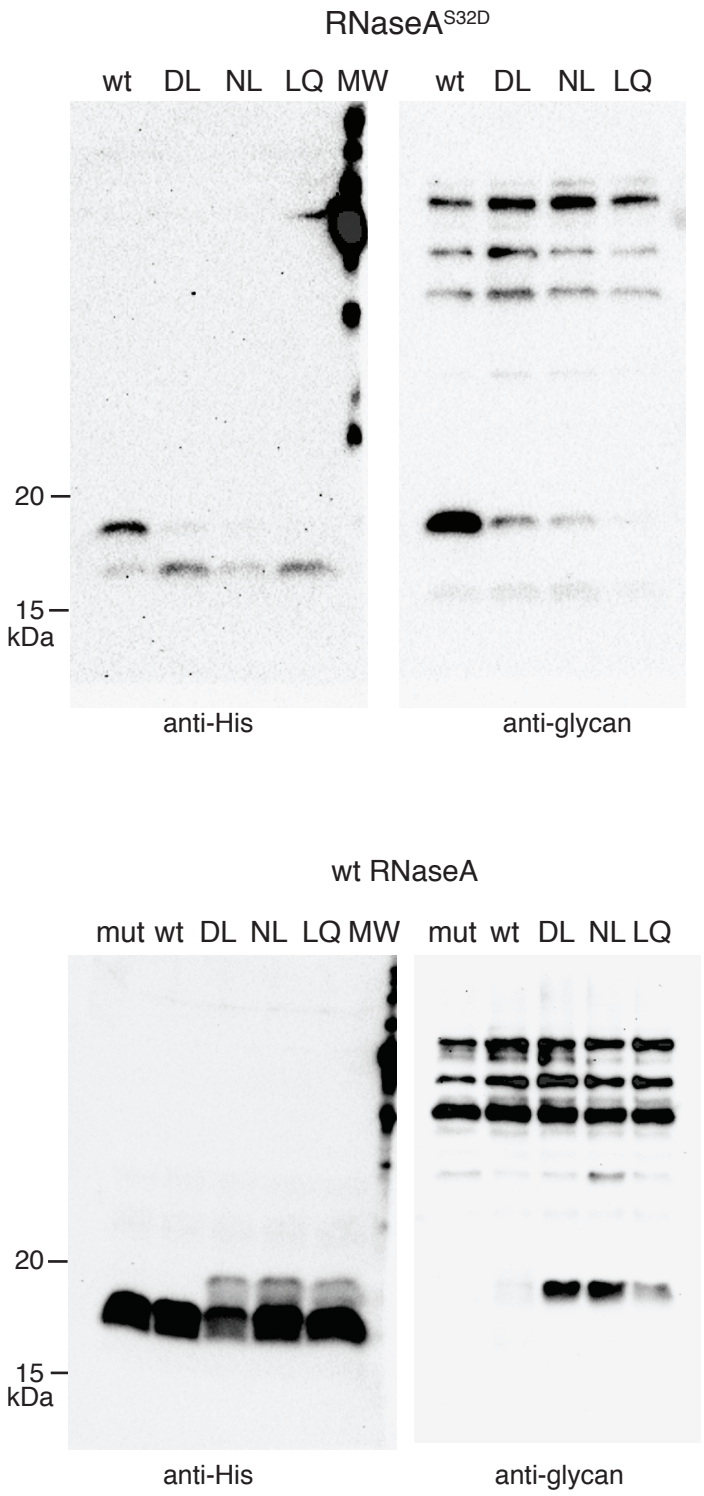
Supplementary Figure 5. Substrate specificity is transferable between bacterial OSTs. Western blot analysis of YebF$^{N24L/DQNAT}$ and YebF$^{N24L/AQNAT}$ glycosylation by wt *Cj*PglB and DL variant. Molecular weight (MW) markers are indicated at left. The g0 and g1 labels denote aglycosylated and singly glycosylated YebF, respectively. Note that glycosylated YebF$^{N24L/AQNAT}$ (g1(A)) migrates faster than glycosylated YebF$^{N24L/DQNAT}$ (g1(D)). Results are representative of two biological replicates.



Supplementary Figure 6. Uncropped images of Figure 1 blots and Coomassie-stained membranes. Each circle was approximately 90 mm in diameter (cut to fit a standard 100 mm petri dish).



Supplementary Figure 7. Uncropped images of Figure 3 immunoblots.



Supplementary Figure 8. Uncropped images of Figure 4 immunoblots.