SUPPLEMENTARY DATA

Indirect fluorescence microscopy revealed defects on the cell surface. Ten mutant constructs that showed wild-type phenotypes in the cell fractionation experiments (Table 1) were not analyzed. The anti-Tsh antibody used in this experiment mainly recognized the passenger domain of the protein rather than the β-domain, as shown by *E. coli* XL1-Blue expressing only the β-domain, which had no fluorescence signal when probed with the antibody (Fig. S2A, top panel, Tshβ). *E. coli* XL1-Blue expressing wild-type Tsh showed a little fluorescence signal, indicating that a small number of Tsh protein still remained surface-bound (Fig. S2A, top panel, Wt Tsh). *E. coli* XL1-Blue expressing N1100A, a mutant with mutated cleavage site in Tsh that abolished processing between the passenger and β-domain (18), had the highest level of signal (Fig. S2A, top panel, N1100A). When compared to wild-type Tsh, most mutants had similar levels of fluorescence signals (Fig. S2, B-E, top panels). Six mutants showed much lower levels of signals (D1154A, Y1227A, G1233Y, G1253Y, F1360A, and F1377A), indicative of reduced amount of Tsh localized on the cell surface (Fig. S2, B-E, top panels). Eight mutants (E1231A, Q1247A, E1249A, N1364A, D1366, N1370A, R1374A, and Y1375A) exhibited fluorescence strengths of 2.5 and higher, equivalent to having 123% or higher levels of signals than the wild type (Fig. S2, C- E, top panels). This could be interpreted in two ways: 1) the mutants might have defects in either processing or releasing mechanism, causing Tsh to linger on the cell surface; or 2) some of these mutations could have perturbed the OM integrity, perhaps through structural or secretion defects. To probe the OM integrity of the mutants, experiments with anti-β-lactamase antibody were further performed on these eight mutants that showed much higher than wild type fluorescence signal when probed with anti-Tsh antibody (Fig. S2, C-E). Seven of these eight mutants probed with anti-β-lactamase showed fluorescence signals (Fig. S2, D and E,

bottom panels), whereas whole cells expressing the empty cloning vector pWKS30 and wild-type Tsh did not have signals when probed with the same antibody (Fig. S2A, middle panel). The results indicate that the OMs of these seven mutant constructs (Q1247A, E1249A, N1364A, D1366, N1370A, R1374A, and Y1375A) were not intact.

When the results from the fluorescence microscopy and the OM fraction data were compared, it was noted that not all of the mutant constructs that showed accumulation of Tsh in the OM had high fluorescence signals when probed with anti-Tsh antibody, for instance, in Ll250Y, A1359Y, F1360A, G1361Y, and F1377A (Fig. 3, lanes 33, 41-43, and 55; Fig. S2, D and E, top panels). These results indicate that in these constructs, the Tsh protein might have been associated with the OM but failed to localize to the cell surface, thus explaining that most of them had degraded Tsh protein products accumulated in the OM fractions.

SUPPLEMENTARY TABLE 1. Primers Used in PCR for Site-directed Mutagenesis

Primer	Sequence (5' – 3')
D1154A-fwd	GCA CGA ACT GGG AAG TAT GGC CCT GTT TAC CGG CGT GATG G
D1154A-rvs	CCA TCA CGC CGG TAA ACA GGG CCA TAC TTC CCA GTT CGT GC
L1155Y-fwd	CGA ACT GGG AAG TAT GGA CTA TTT TAC CGG CGT GAT GGC CAC
L1155Y-rvs	GTG GCC ATC ACG CCG GTA AAA TAG TCC ATA CTT CCC AGT TCG
F1156A-fwd	GGA AGT ATG GAC CTG GCT ACC GGC GTG ATG GCC
F1156A-rvs	GGC CAT CAC GCC GGT AGC CAG GTC CAT ACT TCC
T1157A-fwd	GGA AGT ATG GAC CTG TTT GCC GGC GTG ATG GCC AC
T1157A-rvs	GTG GCC ATC ACG CCG GCA AAC AGG TCC ATA CTT CC
G1158Y-fwd	GAA GTA TGG ACC TGT TTA CCT ACG TGA TGG CCA CCT ACA CTG AC
G1158Y-rvs	GTC AGT GTA GGT GGC CAT CAC GTA GGT AAA CAG GTC CAT ACT TC
E1245A-fwd	GAC AGA TAC GAC GTT TGT TGC ACC TCA GGC GGA ACT GGT C
E1245A-rvs	GAC CAG TTC CGC CTG AGG TGC AAC AAA CGT CGT ATC TGT C
P1246Y-fwd	CAG ATA CGA CGT TTG TTG AAT ATC AGG CGG AAC TGG TCT GGG G
P1246Y-rvs	CCC CAG ACC AGT TCC GCC TGA TAT TCA ACA AAC GTC GTA TCT G
Q1247A-fwd	CAG ATA CGA CGT TTG TTG AAC CTG CGG CGG AAC TGG TCT G
Q1247A-rvs	CAG ACC AGT TCC GCC GCA GGT TCA ACA AAC GTC GTA TCT G
E1249A-fwd	GTT TGT TGA ACC TCA GGC GGC ACT GGT CTG GGG AAG AC
E1249A-rvs	GTC TTC CCC AGA CCA GTG CCG CCT GAG GTT CAA CAA AC
L1250Y-fwd	GTT GAA CCT CAG GCG GAA TAT GTC TGG GGA AGA CTG CAG G
L1250Y-rvs	CCT GCA GTC TTC CCC AGA CAT ATT CCG CCT GAG GTT CAA C
V1251Y-fwd	GAA CCT CAG GCG GAA CTG TAC TGG GGA AGA CTG CAG
V1251Y-rvs	CTG CAG TCT TCC CCA GTA CAG TTC CGC CTG AGG TTC
G1253Y-fwd	GCG GAA CTG GTC TGG TAC AGA CTG CAG GGC CAA AC
G1253Y-rvs	GTT TGG CCC TGC AGT CTG TAC CAG ACC AGT TCC GC
S1358A-fwd	GGG GCT GGA AGT TGA ACG CGC TGC ATT TGG TAA ATA CAA CAC AG
S1358A-rvs	CTG TGT TGT ATT TAC CAA ATG CAG CGC GTT CAA CTT CCA GCC CC
A1359Y-fwd	GGG GCT GGA AGT TGA ACG CTC TTA CTT TGG TAA ATA CAA CAC AGA TG
A1359Y-rvs	CAT CTG TGT TGT ATT TAC CAA AGT AAG AGC GTT CAA CTT CCA GCC CC
F1360A-fwd	CTG GAA GTT GAA CGC TCT GCA GCT GGT AAA TAC AAC ACA GAT GAT GC
F1360A-rvs	GCA TCA TCT GTG TTG TAT TTA CCA GCT GCA GAG CGT TCA ACT TCC AG
F1360Y-fwd	GCT GGA AGT TGA ACG CTC TGC ATA TGG TAA ATA CAA CAC AGA TGA TGC G
F1360Y-rvs	C GCA TCA TCT GTG TTG TAT TTA CCA TAT GCA GAG CGT TCA ACT TCC AGC
G1361Y-fwd	GGG GCT GGA AGT TGA ACG CTC TGC ATT TTA TAA ATA CAA CAC AGA TGA TGC G
G1361Y-rvs	CGC ATC ATC TGT GTT GTA TTT ATA AAA TGC AGA GCG TTC AAC TTC CAG CCC C
Y1363A-fwd	GAA CGC TCT GCA TTT GGT AAA GCC AAC ACA GAT GAT GCG ATA AAC GC
Y1363A-rvs	GCG TTT ATC GCA TCA TCT GTG TTG GCT TTA CCA AAT GCA GAG CGT TC
N1364A-fwd	GAA CGC TCT GCA TTT GGT AAA TAC GCC ACA GAT GAT GCG ATA AAC GC
N1364A-rvs	GCG TTT ATC GCA TCA TCT GTG GCG TAT TTA CCA AAT GCA GAG CGT TC
D1366A-fwd	CGC TCT GCA TTT GGT AAA TAC AAC ACA GCT GAT GCG ATA AAC GCT AAT ATT C
D1366A-rvs	GAA TAT TAG CGT TTA TCG CAT CAG CTG TGT TGT ATT TAC CAA ATG CAG AGC G
N1370A-fwd	GGT AAA TAC AAC ACA GAT GAT GCG ATA GCC GCT AAT ATT CGT TAT TCA TTC TG
N1370A-rvs	CAG AAT GAA TAA CGA ATA TTA GCG GCT ATC GCA TCA TCT GTG TTG TAT TTA CC
A1371Y-fwd	CAC AGA TGA TGC GAT AAA CTA TAA TAT TCG TTA TTC ATT CTG ATG CTG TGC AGC
A1371Y-rvs	GCT GCA CAG CAT CAG AAT GAA TAA CGA ATA TTA TAG TTT ATC GCA TCA TCT GTG
R1374A-fwd	GAT GAT GCG ATA AAC GCT AAT ATT GCT TAT TCA TTC TGA TGC TGT GCA GC
R1374A-rvs	GCT GCA CAG CAT CAG AAT GAA TAA GCA ATA TTA GCG TTT ATC GCA TCA TC
Y1375A-fwd	GCG ATA AAC GCT AAT ATT CGT GCT TCA TTC TGA TGC TGT GCA GCA AAG
Y1375A-rvs	CTT TGC TGC ACA GCA TCA GAA TGA AGC ACG AAT ATT AGC GTT TAT CGC
F1377A-fwd	GAT AAA CGC TAA TAT TCG TTA TTC AGC CTG ATG CTG TGC AGC AAA GGG
F1377A-rvs	CCC TTT GCT GCA CAG CAT CAG GCT GAA TAA CGA ATA TTA GCG TTT ATC
S1376A-fwd	GCG ATA AAC GCT AAT ATT CGT TAT GCA TTC TGA TGC TGT GCA GCA AAG GGA C
S1376A-rvs	GTC CCT TTG CTG CAC AGC ATC AGA ATG CAT AAC GAA TAT TAG CGT TTA TCG C
F1291A-fwd	GGC GTT GTT TCC GGT AAA ACC GCC AGT GGT AAG GAC TG
F1291A-rvs	CAG TCC TTA CCA CTG GCG GTT TTA CCG GAA ACA ACG CC

W1296A-fwd	CTT CAG TGG TAA GGA CGC GAG TCT GAC AGC CCG TG
W1296A-rvs	CAC GGG CTG TCA GAC TCG CGT CCT TAC CAC TGA AG
W1262A-fwd	ACT GCA GGG CCA AAC ATT TAA CGC GAA CGA CAG TGG AAT GGA TGT CT
W1262A-rvs	AGA CAT CCA TTC CAC TGT CGT TCG CGT TAA ATG TTT GGC CCT GCA GT
D1130A-fwd	CTG AAC GGT TCC GGC TCT GCT GCT GGC GGT TTC ACT GAC CAC
D1130A-rvs	GTG GTC AGT GAA ACC GCC AGC AGC AGA GCC GGA ACC GTT CAG
S1187A-fwd	GGG GTG GTG GTT TCT ATG CCG CTG GTC TGT TCC GGT CCG G
S1187A-rvs	CCG GAC CGG AAC AGA CCA GCG GCA TAG AAA CCA CCA CCC C
R1334A-fwd	GGC AGA AAA GAC AGT GCT ATG CTT TAC GGT GTG GGG TTA AAT GCC CGG
R1334A-rvs	CCG GGC ATT TAA CCC CAC ACC GTA AAG CAT AGC ACT GTC TTT TCT GCC
N1342A-fwd	GCT TTA CGG TGT GGG GTT AGC TGC CCG GTT TGG CGA CAA TAC GG
N1342A-rvs	CCG TAT TGT CGC CAA ACC GGG CAG CTA ACC CCA CAC CGT AAA GC
H1224A-fwd	GTA AAC AGA ACT TCC GCA GCG CTT CAC TGT ATG CAG GTG CAG
H1224A-rvs	CTG CAC CTG CAT ACA GTG AAG CGC TGC GGA AGT TCT GTT TAC
S1225A-fwd	GGT AAA CAG AAC TTC CGC AGC CAT GCA CTG TAT GCA GGT G
S1225A-rvs	C ACC TGC ATA CAG TGC ATG GCT GCG GAA GTT CTG TTT ACC
Y1227A-fwd	GAA CTT CCG CAG CCA TTC ACT GGC TGC AGG TGC AGA AG
Y1227A-rvs	CT TCT GCA CCT GCA GCC AGT GAA TGG CTG CGG AAG TTC
G1229Y-fwd	CGC AGC CAT TCA CTG TAT GCA TAT GCA GAA GTC GGA TAC CGT TAT C
G1229Y-rvs	G ATA ACG GTA TCC GAC TTC TGC ATA TGC ATA CAG TGA ATG GCT GCG
E1231A-fwd	CCA TTC ACT GTA TGC AGG TGC AGC AGT CGG ATA CCG TTA TCA TC
E1231A-rvs	GA TGA TAA CGG TAT CCG ACT GCT GCA CCT GCA TAC AGT GAA TGG
G1233Y-fwd	CTG TAT GCA GGT GCA GAA GTC TAC TAC CGT TAT CAT CTG ACA GAT ACG
G1233Y-rvs	CGT ATC TGT CAG ATG ATA ACG GTA GTA GAC TTC TGC ACC TGC ATA CAG
Y1234A-fwd	GCA GGT GCA GAA GTC GGA GCC CGT TAT CAT CTG ACA GAT AC
Y1234A-rvs	GT ATC TGT CAG ATG ATA ACG GGC TCC GAC TTC TGC ACC TGC
R1235A-fwd	GGT GCA GAA GTC GGA TAC GCC TAT CAT CTG ACA GAT ACG ACG
R1235A-rvs	CGT CGT ATC TGT CAG ATG ATA GGC GTA TCC GAC TTC TGC ACC
D1197A-fwd	GTT CCG GTC CGG CGC TTA CTT TGC TGT GAT TGC CAA ATA TAT TCA C
D1197A-rvs	G TGA ATA TAT TTG GCA ATC ACA GCA AAG TAA GCG CCG GAC CGG AAC

SUPPLEMENTARY FIGURES

Fig. S1.

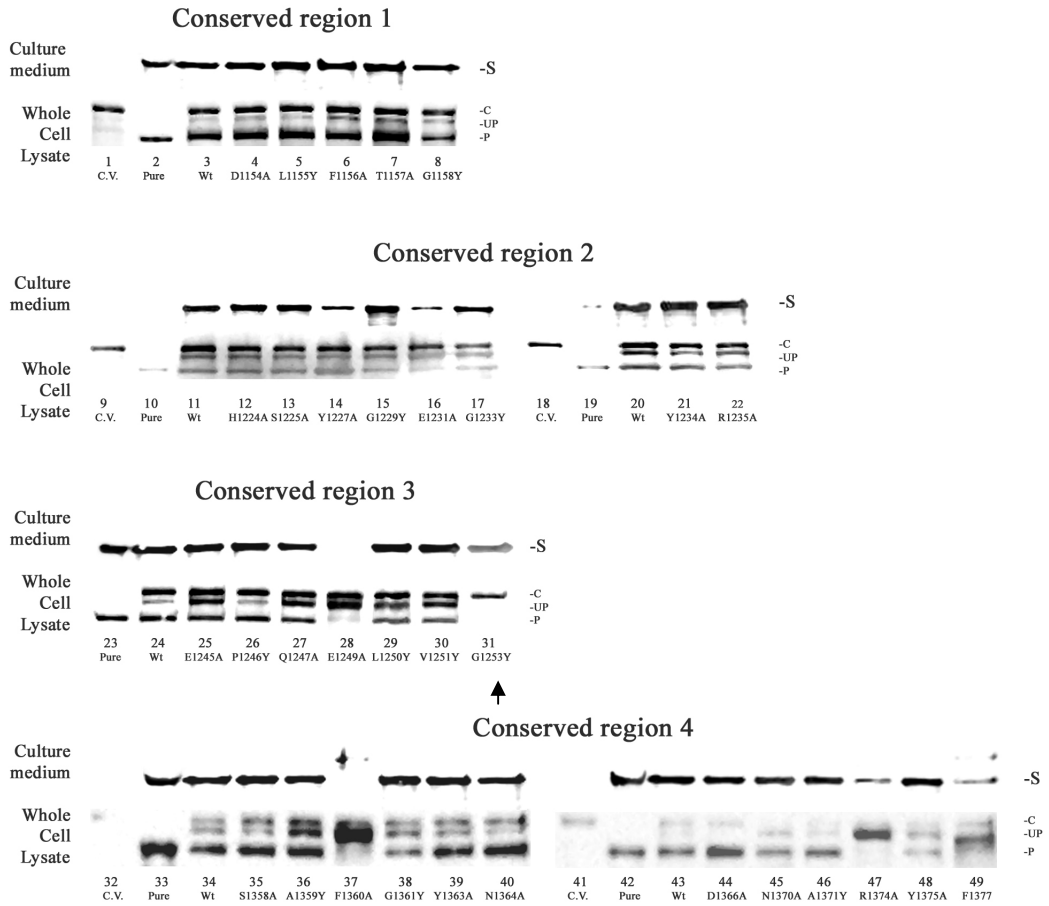
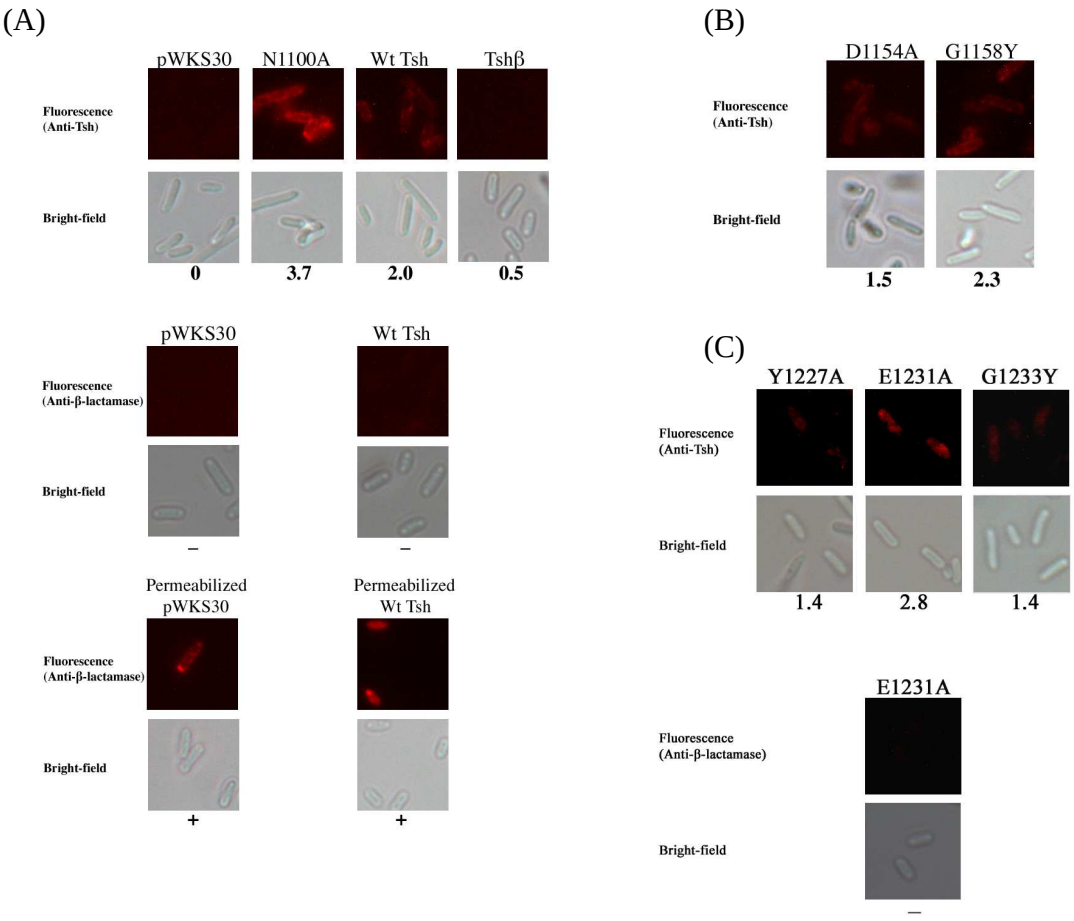
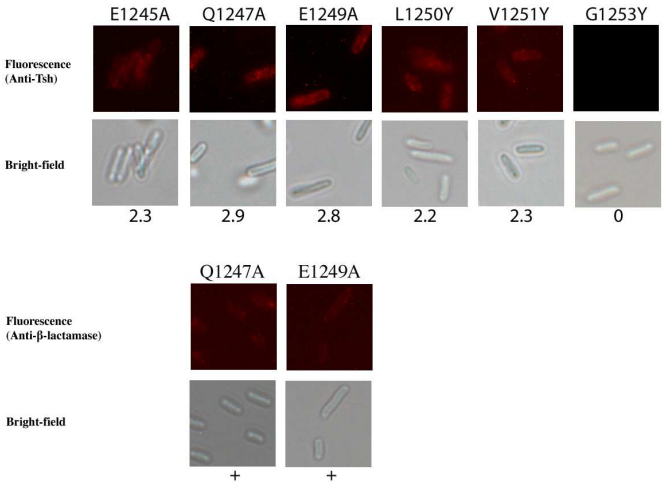


Fig. S1. Total Tsh protein expression analyzed by Western blotting. Top of each panel: Tsh secreted into the culture medium and detected with anti-Tsh antibody. These data are the same ones shown in the top panel of Fig. 2. Bottom of each panel: Tsh in the whole cell lysate detected with anti-Tsh antibody. C.V.: cloning vector, pWKS30. Pure: purified secreted Tsh protein, Tsh_s (17), 106 kD. Wt: wild-type Tsh. S: secreted Tsh protein, 106 kD. C: contaminant protein from the cloning vector or bacterial strain, 150 kD. UP: unprocessed form of Tsh, 140 kD. P: processed form of Tsh, 106 kD. The arrow (↑) points to G1253Y, the mutant having secreted Tsh in the culture medium but missing Tsh in the whole cell lysate.

Fig. S2.


(A)
pWKS30
N1100A
Wt Tsh
Tshβ
Fluorescence (Anti-Tsh)
Bright-field
0
3.7
2.0
0.5
pWKS30
Wt Tsh
Fluorescence (Anti-β-lactamase)
Bright-field
–
–
Permeabilized pWKS30
Permeabilized Wt Tsh
Fluorescence (Anti-β-lactamase)
Bright-field
+
+
(B)
D1154A
G1158Y
Fluorescence (Anti-Tsh)
Bright-field
1.5
2.3
(C)
Y1227A
E1231A
G1233Y
Fluorescence (Anti-Tsh)
Bright-field
1.4
2.8
1.4
E1231A
Fluorescence (Anti-β-lactamase)
Bright-field
–


(D)
E1245A
Q1247A
E1249A
L1250Y
V1251Y
G1253Y
Fluorescence (Anti-Tsh)
Bright-field
2.3
2.9
2.8
2.2
2.3
0
Q1247A
E1249A
Fluorescence (Anti-β-lactamase)
Bright-field
+
+

(E)

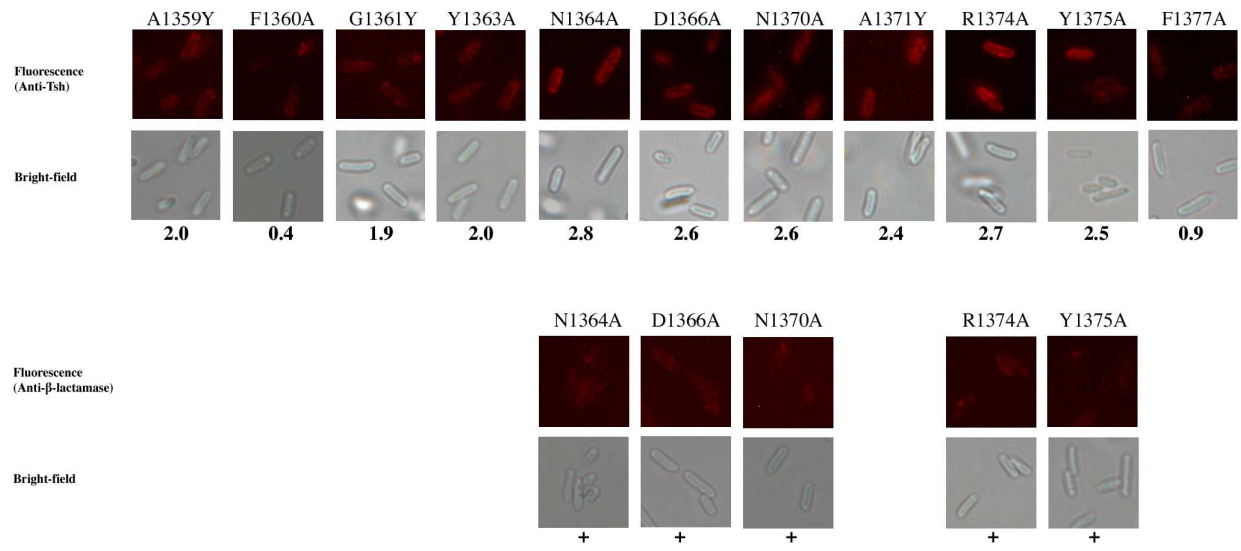


Fig. S2. Cell surface defects of single-mutant constructs visualized by fluorescence microscopy. Shown are representative of analyzed cells. (A) Controls. Top panel: Constructs incubated with anti-Tsh antibody to show the level of Tsh passenger domain localized to the cell surface. *E. coli* XL1-Blue cells expressing the cloning vector pWKS30 and those expressing the Tsh β-domain (Tshβ) served as the negative controls. *E. coli* XL1-Blue expressing N1100A, a mutant with abolished cleavage between Tsh passenger domain and β-domain (18), served as the positive control. Numbers indicate the strength of fluorescence signals assessed by ImageJ. Middle panel: Constructs incubated with an antibody against β-lactamase (a periplasmic protein) to probe the integrity of the OM. The negative (-) signs denote the absence of signals. Bottom panel: Constructs permeabilized with EDTA, lysozyme, and detergent, and incubated with anti-β-lactamase antibody to show the presence of Tsh in the cells. The positive (+) signs denote the presence of signals. Note that not all of the cells were permeabilized by this method. Constructs with mutations in the first, second, third, and fourth conserved regions are shown in (B)-(E),

respectively.

Fig. S3.

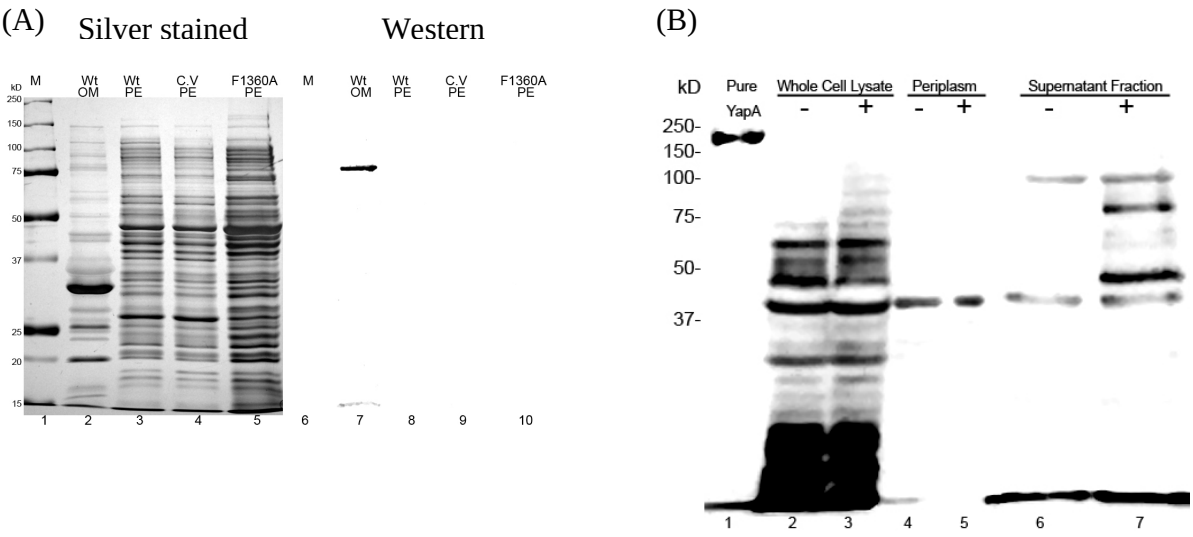


Fig. S3. Periplasmic fractions are free of OM or supernatant/culture-medium protein contamination. (A) Control showing no contamination from OM proteins. Periplasmic fractions (PE) were resolved on a 10% SDS-polyacrylamide gel and silver stained (left panel). Same samples were blotted, and probed with anti-BamA for detection of an outer membrane protein BamA (right panel). An OM fraction reacting with the antibody served as the positive control (wt OM). Lanes 1 and 6: markers (M). Lanes 2 and 7: OM of Wild-type Tsh (Wt OM). Lanes 3 and 8: periplasmic fraction of wild-type Tsh (Wt PE). Lanes 4 and 9: periplasmic fraction of the cloning vector, pWKS30 (C.V. PE). Lanes 5 and 10: periplasmic fraction of the F1360A construct (F1360A PE). (B) Control showing no contamination from supernatant/culture-medium proteins. Exogenous AT protein YapA (43) was mixed into the culture medium of *E. coli* expressing wild-type Tsh before fractionation (+, lanes 3, 5, and 7). Detection of YapA present in each fraction was carried out by Western blotting with anti-YapA antibody. Lane 1: Pure YapA in the unprocessed form, serving as the positive control for Western blotting. Lanes 2, 4, and 6: fractions from *E. coli* expressing wild-type Tsh without the addition of YapA (-).

Fig. S4.

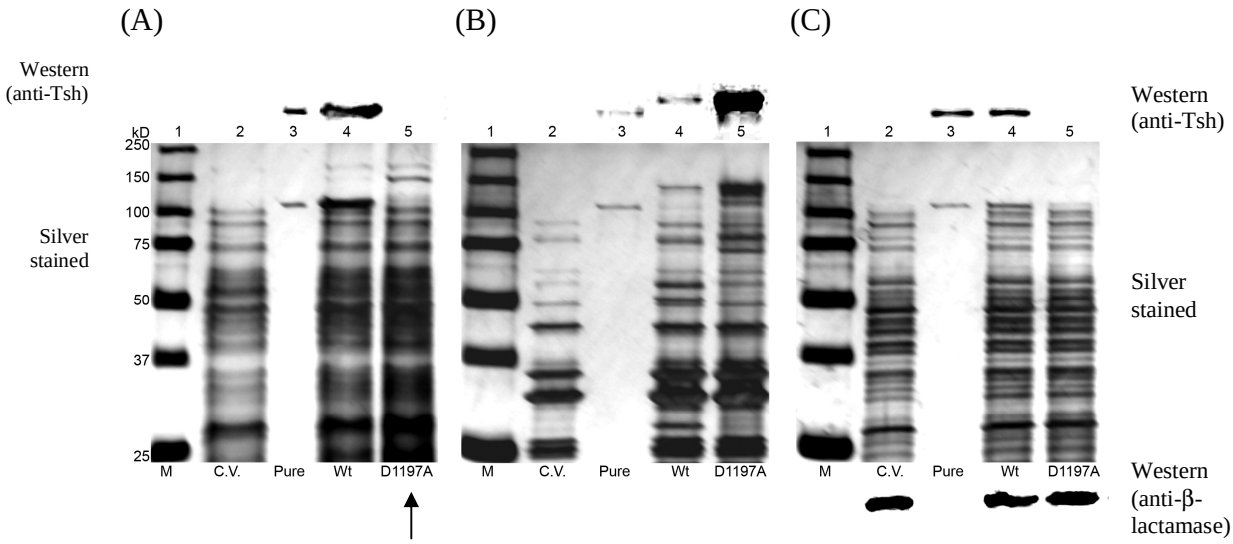


Fig. S4. Cell fractionation of D1197A. The homologous position of this residue in EspP is responsible for assisting complete processing of its passenger domain (3). M: markers. C.V.: cloning vector, pWKS30. Pure: purified secreted Tsh protein, Tsh_s (17), 106 kD. Wt: wild-type Tsh. (A) Culture medium fractions. (B) OM fractions. Top of each panel: results of Western blotting detected with anti-Tsh antibody. Bottom of each panel: silver stained 10% SDS-polyacrylamide gel. The arrow (↑) points to the culture medium fraction of D1197A, into which no Tsh was secreted. (C) Periplasmic fractions. Top of the panel: results of Western blotting detected with anti-Tsh antibody. Middle of the panel: silver stained 10% SDS-polyacrylamide gels. Bottom of the panel: periplasmic β-lactamase from each sample served as the loading control in the Western analysis and was detected with anti-β-lactamase antibody.