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Supporting Online Material for

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Xiu-Jun Yu, Kieran McGourty, Mei Liu, Kate E. Unsworth,* David W. Holden†

†To whom correspondence should be addressed. E-mail: d.holden@imperial.ac.uk

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pH Sensing by Intracellular *Salmonella* Induces Effector Translocation

Xiu-Jun Yu[1], Kieran McGourty[1], Mei Liu[1], Kate E. Unsworth[1] and David W. Holden[1*]

[1]*Section of Microbiology*

Centre for Molecular Microbiology and Infection

Imperial College London

Armstrong Road

London SW7 2AZ

United Kingdom

[*]To whom correspondence should be addressed. (e-mail: d.holden@imperial.ac.uk).

Materials and Methods

Bacterial strains, plasmids and antibodies. DNA allelic exchange (*1*) was used to construct the *S.* Typhimurium *ssaL* mutant. The bacterial strains expressing SseF-2HA, SseJ-2HA or SsaN-3FLAG from the chromosome, the *spiCssaM* mutant expressing SsaL-3Flag (Fig.2) and the *sseA-D* mutant with deletion of translocon protein and chaperone genes were constructed by combination of the λ Red recombinase method (*2, 3*) and phage transduction (*4*). The *spiCssaM* mutant was used to construct the wt-3tag strain by allelic exchange. Plasmids expressing SsaL-2HA, SsaL variants or SseF-2HA under the control of the SPI-2 *sseA* promoter were constructed by standard techniques using the low-copy-number plasmid pWSK29 (*5*) as a vector. The following strains and plasmids were described previously: chromosomal *sseL-2HA* (*6*), chromosomal *steC-2HA* (*7*), *ssaV* mutant (*8*), strains expressing SseJ-2HA from the chromosome and plasmids p*gstspiC*, p*ssaM-2HA* and p$ssaM_{104}$*-2HA* (*9*). Plasmid-expressed effector SseF-2HA was labeled SseF-2HA (P).

The following antibodies were used for immunoprecipitation, immunoblotting and immunofluorescence labeling: rabbit polyclonal anti-SseB and anti-SseC (*9*), rat anti-HA (3F10, Roche), mouse anti-HA (HA.11, Covance), mouse anti-T7 (Novagen), mouse anti-Flag (M2, Sigma), mouse anti-LAMP-1 (H4A3, Developmental Studies Hybridoma Bank under the auspices of the NICHD and maintained by the University of Iowa), rabbit anti-LAMP-1 (931A) (*10*), goat anti-*Salmonella* (CSA-1, Kirkegaard and Perry Laboratories), rabbit anti-GST (Covance), rabbit anti-DnaK (*11*). The rabbit anti-*Salmonella* i-H serum (Murex Biotech Limited, UK) was used for detecting FliC. Rhodamine Red X-conjugated donkey anti-mouse or anti-rabbit antibody, donkey anti-rabbit cyanine 2 (Cy2), and Cy5 or Cy2-conjugated donkey anti-goat antibody were purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Horseradish peroxidase (HRP)-conjugated donkey

anti-rabbit and sheep anti-mouse antibodies were purchased from Amersham Life Sciences.

pH shift and protein secretion assay. Bacterial strains were grown overnight in LB broth with or without antibiotic, and sub-cultured in MgM-MES medium pH 5.0 for 4 h to assemble and activate SPI-2 T3SS. For the pH shift experiments, bacterial cells were collected at room temperature by centrifugation, re-suspended into pre-warmed MgM-MES at the required pH and incubated for 1.5 h. To prevent protein synthesis, bacteria were re-suspended in MgM-MES medium containing 50 μg/ml chloramphenicol or 20 μg/ml tetracycline and incubated for different times. Secreted and bacteria-associated (lysate) proteins were then prepared and loaded for SDS-PAGE according to equivalent OD_{600} of culture as described (*9*).

Fractionation and protein-protein interaction assays. Bacterial cells collected from MgM-MES cultures were sonicated in ice cold PBS containing 1 mM PMSF, and unbroken cells and debris were removed by centrifugation (10 min at 16,000 *g*). The supernatant (whole lysate) was mixed with Triton X-100 to a final concentration at 0.2% and subjected to pull-down with immobilized glutathione beads (Pierce) according to the manufacturer's instructions, or immuno-precipitation. For fractionation assays, the supernatant was subjected to ultracentrifugation for 1 h at 185,500 *g* to separate membrane and cytosolic fractions. The membrane pellet was washed twice with PBS-PMSF, and resuspended into a volume of PBS-PMSF equal to that of the cytosolic fraction. To immunoprecipitate epitope-tagged proteins from the membrane and cytosolic fractions, Triton X-100 was added to a final concentration at 0.2%. Before incubating with antibody, the whole lysate and different fractions were pre-cleaned with protein G-immobilized beads (Pierce) for 1 h at 4°C. The antibody was incubated with pre-cleaned lysate or fraction for 2 h, then with protein G-

immobilized beads for 1.5 h. The beads were washed four times with PBS-PMSF-0.2% Triton X-100, resuspended into 2 × sample buffer, and analysed by SDS-PAGE.

pH manipulation of HeLa cell cytoplasm. To manipulate cytoplasmic pH, HeLa cells were permeabilised with 25 μg/ml digitonin in KHM (110 mM KOAc, 20 mM Hepes pH 7.2, 2 mM MgOAc) for 5 mins on ice (*12*), then incubated in ISB (5 mM NaCl, 140 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5 mM D-glucose, 50 mM MES buffered to different pH). To verify that cytoplasmic pH changed in response to external pH following digitonin treatment, HeLa cells were preloaded with the pH-sensitive fluorescent probe 2', 7'-bis(2-carboxyethyl)-5,6-carboxyfluorescein acetoxymethyl ester (BCECF-AM, Invitrogen) to a final concentration of 2 μM. After 20 min incubation at 37^0 C, cells were washed twice and treated with digitonin, before resuspension in ISB and incubation for 15 mins at 37^0 C. A total of 5×10^4 cells in each sample were then analysed on a FACS Calibur cytometer (Becton Dickinson) for fluorescence intensities in channels FL-1 and FL-3 (*13*). A calibration curve was also produced using 10 μM nigericin as described (*13*). Data were analysed with FlowJo 8.6.3 software. The emission ratio (FL-1/FL-3) was calculated for each pHe value.

pH measurements of *Salmonella*-containing vacuoles. Measurements of pH_{SCV} were made as described (*14*), with the following modifications. HeLa cells were grown on 35 mm glass bottomed dishes (Matek) in DMEM containing 10% fetal calf serum (FCS) at a concentration of 1.5-6 x 10^5. The medium was replaced after 12 h with DMEM/FCS containing dextran-coupled fluourescein (488 nm) (300 μg/ml) and dextran-coupled Alexa Fluor 647 (647 nm) (200 μg/ml) and incubated for a further 12 h. The cells were then

infected with dsRED-expressing wild-type *S.* Typhimurium strain 12023 in the same medium using standard protocols (12). 3.5 h post-invasion some samples were exposed to 25 μg/ml digitonin (for 5 mins on ice followed by 2 washes in the absence of digitonin) in KHM buffer. After 2.5 h incubation in ISB at pH 6.0, cells were imaged by confocal microscopy. Three-dimensional images of SCVs were selected and quantification of the mean fluorescent ratios 488 nm/647 nm was determined using Volocity software (Perkin Elmer). A standard curve for each experiment was generated to determine the pH value of the SCVs as described (*14*). Groupings of SCVs within 0.5 pH units were generated and the percentages of SCVs in each binned group were calculated. Microscopy was performed at the Facility for Imaging by Light Microscopy (FILM) at Imperial College London.

Bacterial infection of HeLa cells, immunofluorescence, immunoblotting and replication assays. HeLa cells were infected, fixed, permeabilized with saponin (except for one sample shown in fig. S6B where 0.1% Triton X-100 was used), immunolabeled as described (*9, 15*), and analysed using a confocal laser scanning microscope (LSM 510, Zeiss). Translocation of SseF-2HA expressed from plasmid was not detected at 3.5 h post-invasion, but was clearly visible at 4 h (data not shown). Therefore, for digitonin treatment, cells were infected for 3.5 h, then extracellular medium was changed to KHM containing 25 μg/ml digitonin and incubated for 5 mins on ice, followed by washing in KHM to remove excess digitonin. Infected cells were then incubated in ISB at pH 6.0 or 7.2 for 2.5 h, followed by fixation in 3% PFA. In one digitonin-treated sample, pHe was changed from 6.0 to 7.2 1 h before fixation. Immunoblot analysis was performed as described previously (*9*). Band intensities were quantified using Image J software. Intracellular bacterial replication assays were performed as previously described (*16*).

Supplementary Figure legends

Supplementary Figure 1. Hypersecretion of the SPI-2 T3SS effector SseF by *ssaL, spiC* and *ssaM* deletion mutants of *S*. Typhimurium at pH 5.0. In one strain, SseF-2HA is expressed in place of endogenous protein from the chromosome, and in the other it is expressed from a plasmid (SseF-2HA (P)). Strains were grown in minimal medium pH 5.0, and secreted and bacterial-associated (lysate) proteins were examined by immunoblotting to detect the HA epitope and DnaK.

Supplementary Figure 2. Phenotypes of the wt-3tag strain. (A) HeLa cells were infected with the wild-type (wt) or wt-3tag strain for 8 h, then fixed and labeled for LAMP-1 (red) and bacteria (green). Scale bar = 5 μM. **(B)** Intracellular replication of wt, *ssaV* mutant and wt-3tag strains in HeLa cells and RAW264.7 macrophages. The fold increase represents the increase in cfu obtained after lysing host cells at 2 h and 16 h.

Supplementary Figure 3 Alignment of N-terminal regions of SsaL and YopN. The amino acids deleted in the three variants assayed in Fig. 2B and C are indicated by solid lines above the sequence. *, identical residues; :, conserved residues; ., semi-conserved residues. The chaperone-binding domain of YopN extends from residues 32-76, and wraps around the SycN-YscB heterodimer in a horseshoe conformation (*17*).

Supplementary Figure 4 Effect of chloramphenicol and pH on effector and SseB secretion. **(A)** Wild-type *Salmonella* expressing 2HA epitope-tagged SPI-2 T3SS effectors SseL or SteC, or the translocon protein SseB, all from the chromosome, were examined as in Fig 3A. **(B)** Wild-type bacteria expressing SseF-2HA from a plasmid were grown in minimal medium pH 5.0 for 4 h then re-suspended in the same medium with (+) or without (-) chloramphenicol (Cm) at pH 7.2 or pH 5.0. Samples were harvested after 5, 15, 30 or 45 min and secreted and bacterial-associated (lysate) fractions were subjected to immunoblotting using an antibody to detect the HA epitope. **(C)** Immunoblot of SseF-2HA secreted after growth in minimal medium pH 5.0 for 4 h, followed by a 90 min incubation in the same medium buffered to the indicated pH. An anti-*Salmonella* i-H serum was used as a control to detect secreted FliC.

Supplementary Figure 5 Intracellular pH manipulation. **(A)** Exposure of HeLa cells to different extracellular pH values in the presence of digitonin changes cytoplasmic pH. HeLa cells were loaded with BCECF-AM for 20 min, then incubated in ISB at different pH values following exposure to 25 μg/ml digitonin or 10 μM nigericin. After 15 min, cells were analysed by laser scanning cytometry with excitation at 488 nm and emission at 530 nm (green; FL-1) and 640 nm (red; FL-3). The FL-1: FL-3 ratio was calculated; in the presence of digitonin or nigericin, intracellular BCECF becomes increasingly protonated by lower extracellular pH (pHe), causing an increase in red fluorescence (*13*). **(B)** Measurement of pH_{SCV}. HeLa cells were incubated with dextran-coupled fluourescein (488 nm) and Alexa Fluor (647 nm) for 12 h, then infected with dsRED-expressing wild-type *S.* Typhimurium. 3.5 h post-invasion some samples were permeabilised with digitonin or treated with 20 μM nigericin (to provide a positive control) and exposed to ISB at pH 6.0. After 2.5 h

incubation, cells were imaged by confocal microscopy. Three-dimensional images of SCVs were selected and quantification of the mean fluorescent ratios 488 nm/647 nm was determined. pH values were generated by reference to a standard curve. Groupings of SCVs within 0.5 pH units were generated and the percentages of SCVs in each binned group were calculated.

Supplementary Figure 6 Analysis of secretion and translocation of SsaL-2HA. (A) Bacterial strain *ssaL* p*ssaL-2HA* was grown in minimal medium pH 5.0 for 4 h. The medium was then changed to pH 5.0 or 7.2 for 1.5 h. Bacterial associated (lysate) and secreted fractions were analysed by immunoblot using anti-HA and SseB antibodies. **(B)** HeLa cells were infected for 6 h with the above strains and with a strain expressing SseF-2HA from a plasmid, then fixed and immunolabeled for HA-tagged proteins (red) and bacteria (green), after permeabilization with either saponin (to detect translocated protein) or triton x-100 (to detect intrabacterial protein) (*9*). Scale bar = 2 μM.

Supplementary Figure 7 Deletion of translocon proteins and their chaperones does not affect enhanced secretion of SseF-2HA upon pH upshift. The wild-type strain and a deletion mutant encompassing the *sseA-sseD* region of SPI-2 expressing SseF-2HA from a plasmid were grown in minimal medium pH 5.0 for 4 h. The medium was then changed to pH 5.0 or 7.2 for 1.5 h. Bacterial associated (lysate) and secreted fractions were analysed by immunoblot using anti-HA antibody.

Supplementary Figure 8 Model for control of effector translocation by the SPI-2 T3SS. (i) Following uptake into host cells, acidification of the vacuole lumen induces assembly of

the secretion apparatus. (ii) Membrane-associated SsaL/SsaM/SpiC regulatory complex (in purple, black and blue, respectively) prevents premature secretion of effectors (in brown). Translocon proteins (in green), connected to the T3SS apparatus, form a pore in the vacuolar membrane. (iii) The pore enables a component(s) of the T3SS to sense the elevated pH of the host cell cytosol, and a signal is transduced to the SsaL/SsaM/SpiC complex, which dissociates. (iv) Relief of effector secretion suppression enables their translocation.

Supplemental References

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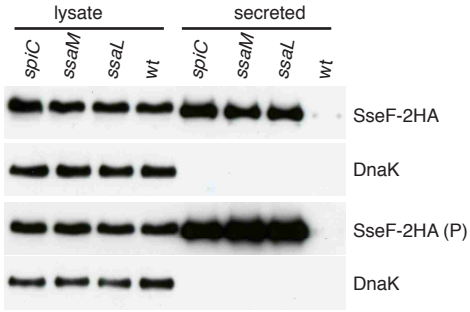
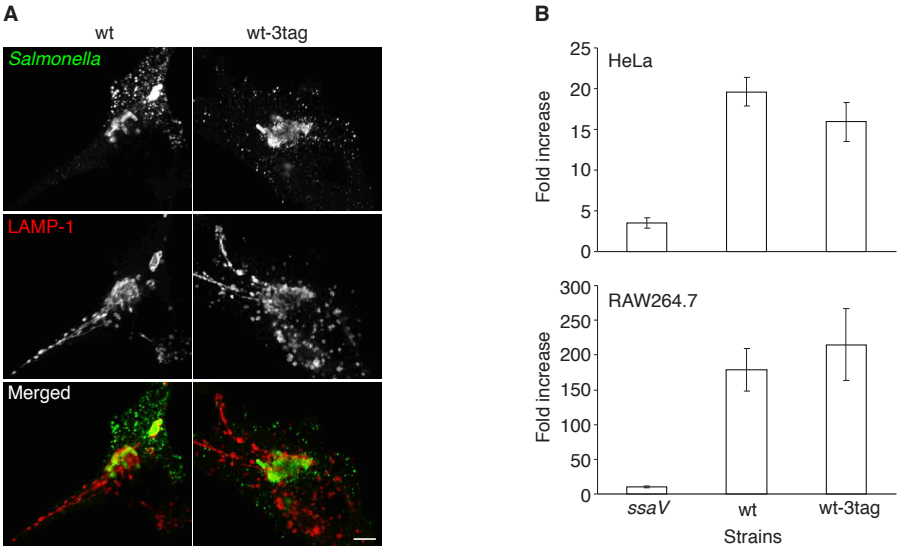

lysate
secreted
spiC
ssaM
ssaL
wt
spiC
ssaM
ssaL
wt
SseF-2HA
DnaK
SseF-2HA (P)
DnaK

Suppl. Fig. 1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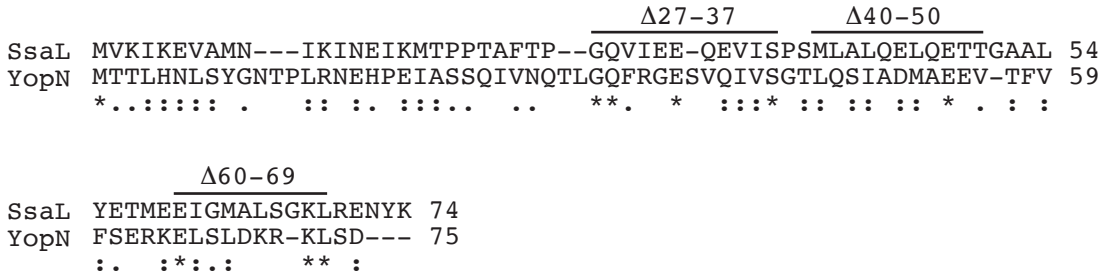
wt
wt-3tag
Salmonella
LAMP-1
Merged
B
HeLa
Fold increase
25
20
15
10
5
0
RAW264.7
Fold increase
300
250
200
150
100
50
0
ssaV
wt
wt-3tag
Strains

Suppl. Fig. 2


Δ27-37
Δ40-50
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YopN MTTLHNLSYGNTPLRNEHPEIASSQIVNQTLGQFRGESVQIVSGTLQSIADMAEEV-TFV 59
Δ60-69
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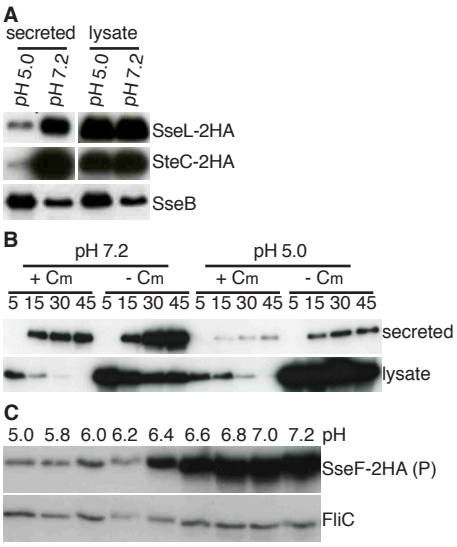
YopN FSERKELSLDKR-KLSD--- 75

Suppl. Fig. 3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pH 7.2
pH 5.0
pH 7.2
SseL-2HA
SteC-2HA
SseB
B
pH 7.2
pH 5.0
+ Cm
- Cm
+ Cm
- Cm
5 15 30 45 5 15 30 45 5 15 30 45 5 15 30 45
secreted
lysate
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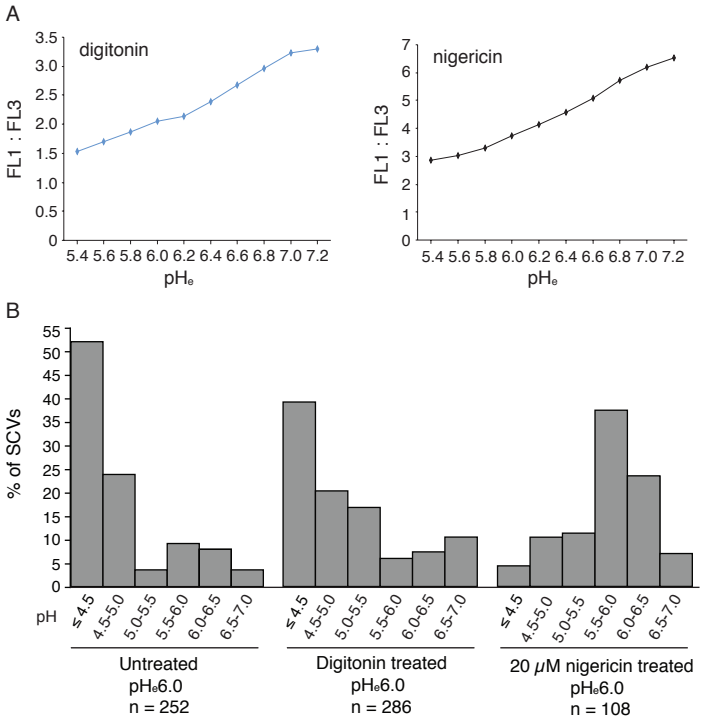
FliC

Suppl. Fig. 4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% of SCVs
pH
≤ 4.5
4.5-5.0
5.0-5.5
5.5-6.0
6.0-6.5
6.5-7.0
Untreated
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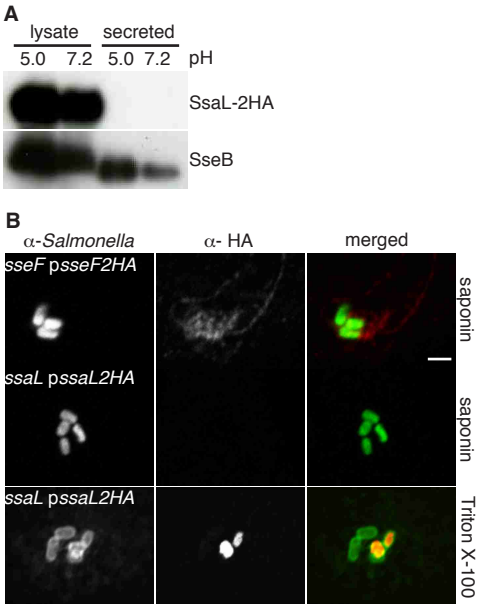
pHe6.0
n = 108

Suppl. Fig. 5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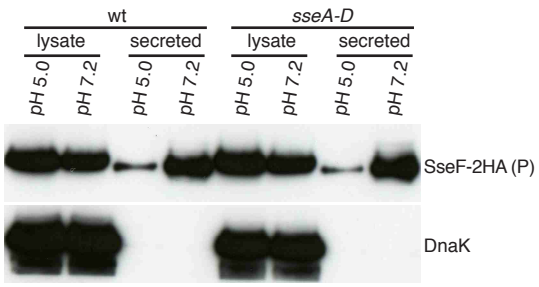
Suppl. Fig. 6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pH 7.2
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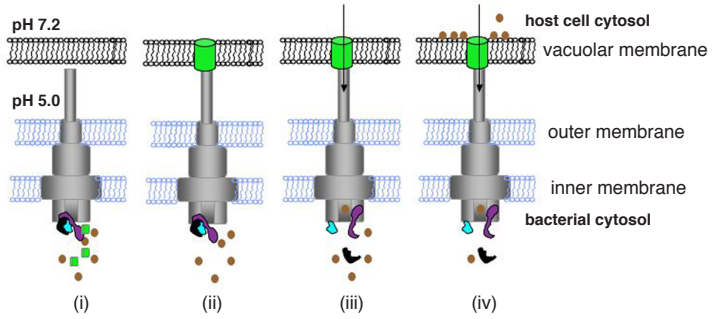
pH 5.0
pH 7.2
SseF-2HA (P)
DnaK

Suppl. Fig. 7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pH 5.0
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outer membrane
inner membrane
bacterial cytosol
(i)
(ii)
(iii)
(iv)

Suppl. Fig. 8