Supplementary data 4

Standard protein purification and analysis protocols

Purification of GST-hybrid proteins

GST-YopO (pML10), GST-YopO_{20-77} (pML11) and GST-YopO_{D267A} (pML12) were produced in *E. coli* Top10. GST-YopO (pML10) and SycO (pML15) were co-expressed in *E. coli* BL21. Bacteria were cultivated in 100 ml LB medium at 37 °C up to an OD$_{600}$ of 0.6. Expression of the fusion proteins was induced by addition of 0.125 mM IPTG. Cultures were then grown for 3 hours at room temperature (RT). Bacterial pellets were resuspended in Phosphate Buffered Saline (PBS) containing Triton X100 0.1 % (v/v), proteases inhibitors (Complete mini, Roche) and lysed by sonication. Unbroken cells and debris were removed by centrifugation (30 min, 6000 g). The supernatant was incubated with Glutathione Sepharose 4B (Amersham) for 2 hours at 4 °C. The matrix was pelleted by centrifugation 500 g for 2 min. The supernatant was discarded and the matrix was washed 4 times with ice-cold PBS. GST-YopO and GST-YopO$_{D267A}$ were eluted by addition of PBS with 10 mM reduced glutathione to the matrix and incubated for 30 min at 4 °C. GST-YopO$_{20-77}$ and GST-YopO bound to SycO were eluted using Prescission Protease from Amersham (3 h at 4 °C). Matrix was sedimented at 500 g for 2 min, and discarded. The supernatant containing the proteins was checked by SDS PAGE.
Purification of SycO

E. coli Top10 containing pML14 were grown in 25 l LB medium at 37 °C. Expression of sycO was induced at an OD<sub>600</sub> of 0.6 by the addition of 0.2 % (w/v) arabinose and the culture was grown for three additional hours. 30 grams of bacteria were lysed in a French press. Unbroken cells and debris were pelleted by centrifugation during 30 min at 6000 g. SycO was precipitated with 30 % (w/v) ammonium sulfate, dialyzed and further purified by successive chromatographies on DEAE sepharose (Amersham), Q-Sepharose (Amersham) and Sephacryl S-300 (Amersham). All purifications and dialyses were done in Hepes 50 mM, NaCl 50 mM, DTT 1 mM buffer containing proteases inhibitors (Complete mini, Roche). 1.5 mg of SycO was used for antibody production.

Purification of YopO<sub>his</sub>-(SycO)<sub>2</sub>

YopO<sub>his</sub> and SycO were co-produced in E. coli BL21 from the pET22-derived plasmid pML9. Bacteria were grown overnight at 37 °C, diluted to an OD<sub>600</sub> of 0.1 in LB with ampicillin 100 µg ml<sup>-1</sup> and incubated at 37 °C until they reached an OD<sub>600</sub> of 0.6. Then the expression of the proteins was induced by adding 0.125 mM IPTG and the cultures were incubated at room temperature (RT) with shaking for 3 hours. Cells were harvested by centrifugation, resuspended in PBS containing Triton X100 0.1 % (v/v), proteases inhibitors (Complete mini, Roche) and lysed using a French press. The lysate was spun for 30 min at 6000 g to eliminate unbroken cells and debris. YopO<sub>his</sub> was purified on Ni<sup>2+</sup>- sepharose (Amersham). After several washing steps with 50 mM imidazole, YopO<sub>his</sub> and SycO were eluted with 300 mM imidazole.
Size exclusion chromatography

Purified proteins were run on Superdex™ 200 PC 3.2/30 gel filtration column (Amersham) in PBS containing 1 mM DTT.

GST-Pulldown assay

Yersinia bacteria expressing GST fusions were grown in conditions inducing expression of the yop regulon (see here before). Bacterial pellets were re-suspended in Phosphate Buffered Saline (PBS) containing Triton X100 0.1 % (v/v), proteases inhibitors (Complete mini, Roche) and lysed by sonication. The lysate was centrifuged 30 min at 6000 g to remove unbroken cells and debris. The supernatant was incubated with Glutathione Sepharose 4B (Amersham) for 2 hours at 4 °C. The matrix was separated by centrifugation at 500 g for 2 min. The supernatant was discarded and, after four washing steps with ice-cold PBS, proteins were eluted by the addition of SDS-PAGE loading buffer. Aliquots of each step were analyzed by SDS-PAGE.

Electrophoresis and immunoblotting

Electrophoresis was performed in 12% (w/v) polyacrylamide gels with SDS. The proteins were stained with Coomassie brilliant blue (Pierce) or transferred onto hybond C extra membrane (Amersham). The membranes were incubated with polyclonal rabbit anti-YopO, anti-YopP, anti-SycO, or commercial anti-CyaA (Santa Cruz Biotechnology, Inc.), anti-flag M2 (Sigma), and anti-his (Amersham) antibodies. The proteins were detected using secondary antibodies conjugated to horseradish peroxidase (1:2000; Dako) before development with supersignal chemiluminescent substrate (Perkin Elmer).