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Limited tolerance towards folded elements during secretion of the autotransporter Hbp

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Supplementary Fig. S1

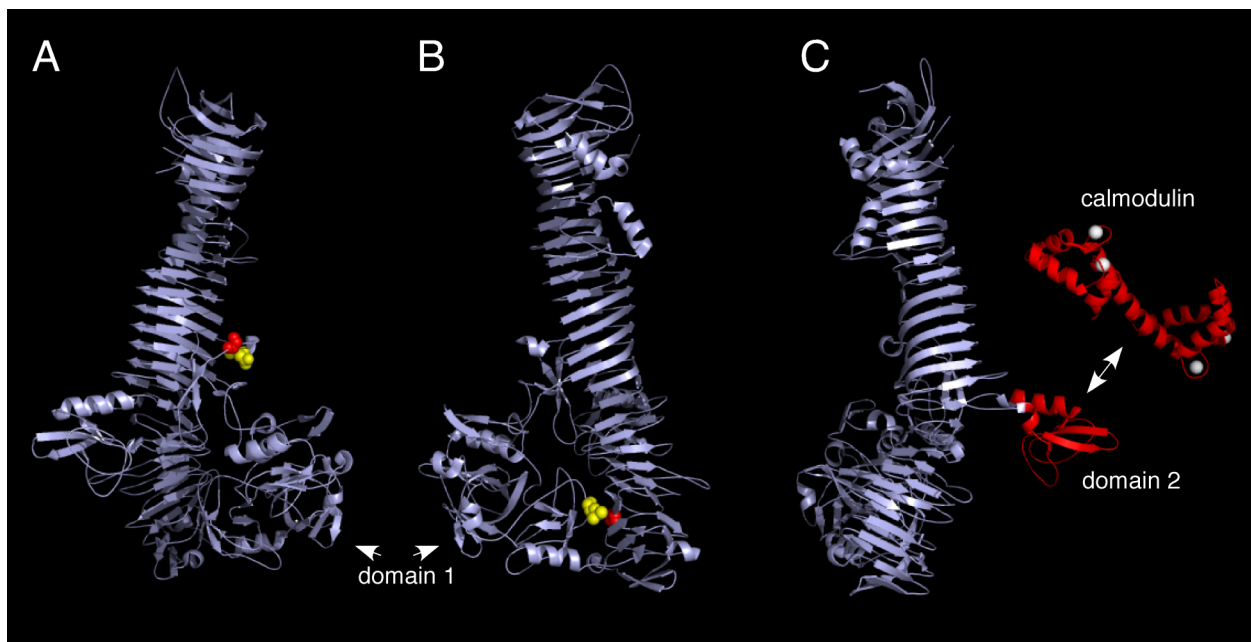


Fig. S1. Hbp derivatives used in this study. Hbp residues targeted for mutation are indicated in the crystal structure of the secreted Hbp passenger (1WXR[PDB]; Otto *et al*, 2005) (A) Hbp707C/712C: Ala⁷⁰⁷(red)/Asp⁷¹²(yellow) \rightarrow Cys⁷⁰⁷/Cys⁷¹². (B) Hbp110C/348C: Leu¹¹⁰(yellow)/Gly³⁴⁸(red) \rightarrow Cys¹¹⁰/Cys³⁴⁸. (C) Hbp(Calm): Hbp domain 2 (aa 483-555; red) substituted by the Ca²⁺-loaded form of human calmodulin (1CLL[PDB]; Chattopadhyaya *et al*, 1992). Ca²⁺-ions are represented by white spheres. The structure of Hbp has been rotated with respect to the vertical axis to provide the best view of the three mutants. Images were created using MacPyMOL.

References

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Supplementary Fig. S2

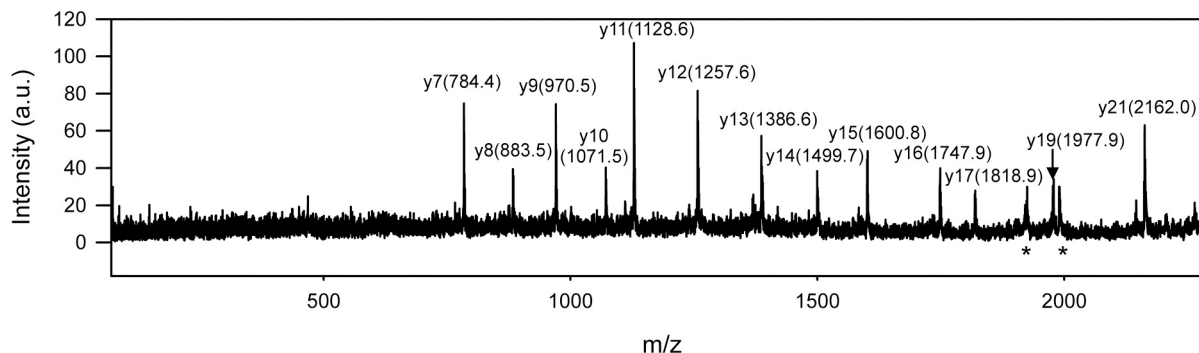


Fig. S2. Secreted Hbp707C/712C contains a disulphide bridge. Cultures of DHB4 cells expressing either Hbp or Hbp707C/712C were treated with 100 mM iodoacetamide 2h and 45min after induction with IPTG. Cultures were directly chilled on ice after which spent medium was isolated and subjected to TCA precipitation. TCA pellets were suspended in non-reducing SDS-PAGE sample buffer containing 50 mM iodoacetamide, boiled for 5 min and resolved by non-reducing 12% NuPAGE/MOPS (Invitrogen). After staining with SimplyBlue SafeStain (Invitrogen), the appropriate protein bands were cut out of gel and treated with trypsin. The generated peptides were mixed with α -cyano-4-hydroxy-cinnamic acid matrix and spotted on a target for MALDI-TOF peptide mass fingerprinting. An intense peak with an m/z value of 2391 was detected in trypsin digest of the mutant, but not in the digest of the wild type protein (not shown). We hypothesized that the peak corresponded to the tryptic peptide 691NDGQGTAFTLEEGTSVCTKDACK713 (m/z 2374) in which trypsin had also cleaved between positions 709 and 710, but the two resulting peptides were covalently connected by a disulphide bridge between the two cysteines. The m/z value of the resulting molecule is $2374 + 18$ (hydrolysis by trypsin at position 709) $- 2$ (disulphide formation) $+ 1$ (proton addition, single charge) = 2391. This was unambiguously confirmed by MALDI-TOF-TOF tandem mass spectrometry (see figure). A large series of y-ions was found (ranging from y7 to y21) confirming the presence of the amino acid sequence 691NDGQGTAFTLEEGTSV706. y-ions below y7 were not found, likely because the disulphide bond caused anomalous/reduced fragmentation. The smallest y-ion (y7) corresponds to the C-terminus of the molecule, consisting of the two peptides 707CTK709 and 710DACK713 linked by the disulphide bridge (m/z 784.4 for the protonated molecule). The two peaks labeled with the asterisk were not assigned.

Supplementary Fig. S3

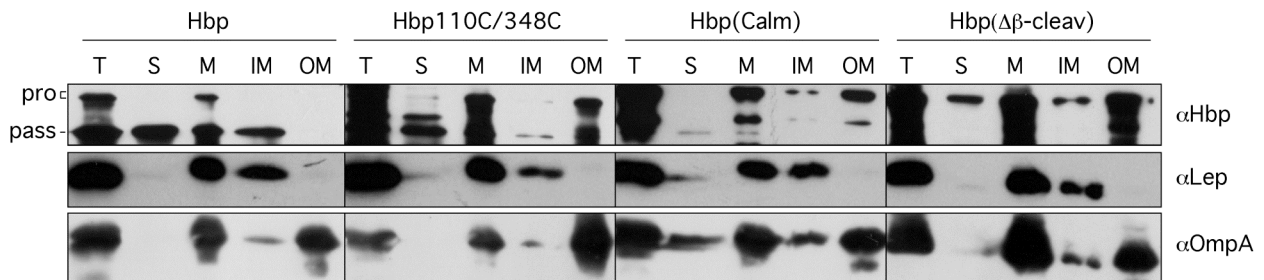


Fig. S3. Subcellular localization of secretion incompetent Hbp derivatives. KS476 cells expressing either Hbp, Hbp110C/348C, Hbp(Calm) or Hbp($\Delta\beta$ -cleav) were fractionated. The following fractions were obtained: total cells (T), soluble fraction (S), total membranes (M), inner membranes (IM) and outer membranes (OM). Cells were chilled on ice 1h after induction with 1 mM IPTG, collected by centrifugation and broken in a French pressure cell to obtain a total cell lysate. Unbroken cells and debris were removed by centrifugation at 5,000xg for 5 min. The total membrane fraction was collected by ultracentrifugation. The resulting supernatant fraction was taken as the soluble protein fraction. To separate the inner membranes from the outer membranes, the total membrane fraction was treated with 0.5% Sarkosyl as described previously (Filip *et al*, 1973). Samples of the fractions, derived from equal amounts of cell material, were analyzed by SDS-PAGE and immunoblotting using antiserum against Hbp (*top panel*). To verify the membrane separation, both the inner membrane protein Leader peptidase (Lep) (*center panel*) and the outer membrane protein A (OmpA) (*bottom panel*) were detected in the fractions by immunoblotting. The pro-form (*pro*) and passenger (*pass*) of the Hbp derivatives are indicated.

Reference

Filip, C., Fletcher, G., Wulff, J.L. and Earhart, C.F. (1973) Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate. *J Bacteriol* **115**: 717-722.

Supplementary Materials and Methods

Plasmid construction

To create pEH3-Hbp, the *hbp* gene was amplified by PCR using pACYC-Hbp (van Dooren *et al.*, 2001) as a template. The primers used were pEH-*Xba*I-Hbp_fw (5'-**taactttctagatt**acaaaacttaggaggggtttttaccatgaacagaattattctcttcg-3'; *Xba*I site in boldface, pEH3 Shine-Dalgarno sequence underlined, and *hbp* initiation codon in italics) and Hbp-*Eco*RI-rv (5'-**tcgagaattc**cagaatgaataacgaatattag-3'; *Eco*RI site in boldface). Subsequently, the amplified product was cloned into the *Xba*I/*Eco*RI sites of a modified version of pEH3 (Hashemzadeh-Bonehi *et al.*, 1998) that contains an *Xba*I recognition sequence 24 bp upstream of the *Nco*I recognition sequence, resulting in pEH3-Hbp.

To generate the cysteine mutants pEH3-Hbp110C, pEH3-Hbp348C, pEH3-Hbp110C/348C and pEH3-Hbp707C/712C, plasmid pEH3-Hbp was mutated using nested PCR, essentially as described (Scotti *et al.*, 2000). For introduction of Cys¹¹⁰, the primers used were HbpL110C_fw (5'-ggctggataaatgcgtagacagagg-3'; Cys¹¹⁰ codon underlined) and HbpL110C_rv (5'-cctctgtcacgcatttatccagcc-3'; Cys¹¹⁰ codon underlined). For introduction of Cys³⁴⁸, the primers used were HbpG348C_fw (5'-ggctggataaatgcgtagacagagg-3'; Cys³⁴⁸ codon underlined) and HbpG348C_rv (5'-cctctgtcacgcatttatccagcc-3'; Cys³⁴⁸ codon underlined). Mutations to introduce Cys⁷⁰⁷ and Cys⁷¹² were incorporated in one single primer pair: Hbp707C/712C_fw (5'-gcacatctgtttgcactaaagatgcatgcaaaagtgtcttc-3'; Cys⁷⁰⁷ codon underlined, Cys⁷¹² codon in boldface) and Hbp707C/712C_rv (5'-gaagacacttttgcattcatcttttagtgcaaacagatgtgc-3'; Cys⁷⁰⁷ codon underlined, Cys⁷¹² codon in boldface).

Plasmid pEH3-Hbp(Δ dom2) was created by substitution of the Hbp-domain-2-coding sequence for a *Xho*I-*Sac*I-*Hind*III-*Nco*I linker sequence via nested PCR using pEH3-Hbp as a template and HbpGDELpoly_fw (5'-ctcaggagctcaagcttccatggaatactgcagggtatctgttcc-3', linker sequence underlined) and HbpGDELpoly_rv (5'-ccatggagcttgagctcctcggggcatagtcaccgctgatagtg-3'; linker sequence undelined) as mutagenesis primers. Subsequently, pEH3-Hbp(Calm) was constructed as follows. First, a PCR fragment was generated encoding human calmodulin 3 without its stop-codon, flanked by Pro-Gly-Gly spacers and restriction sites. In this reaction, plasmid pASKInt100-Cal (Adams *et al.*, 2005) served as a template and the primers used were *Xho*I/*Sac*I/PGG-Calm_fw (5'-gtactctagactcgaggagctcggcgggatggctgatcagctgaccgaagaac-3'; *Sac*I site underlined, pro-gly-gly spacer in italics) and *Nco*I/*Hind*III/PGG-Calm_rv (5'-gtacaggcctccatggaagcttggggcccccttgcagtcacatctgtacaaac-3'; *Hind*III site underlined, pro-gly-gly spacer in italics). This fragment was cloned into the *Sac*I/*Hind*III sites of pEH3-Hbp(Δ dom2), giving pEH3-Hbp(Calm).

Plasmid pEH3-Hbp(Δ β -cleav) was generated via nested PCR replacing residues Asn¹¹⁰⁰ and Asn¹¹⁰¹ (calculated from Meth¹ of the pre-proHbp), constituting the cleavage site between the Hbp passenger and b-domain, by a Gly and Ser residue respectively. In this reaction pEH3-Hbp served as the template and primers used were Hbp-del β cleav_fw (5'-catcactgaagttggatccctgaacaaacg-3', Gly-Ser codons underlined) and Hbp-del β cleav_rv (cgtttgttcaggatccaacttcagtgatg-3', Gly-Ser codons underlined).

Plasmid pEH3-Hbp(Pass) expressing a truncated version of Hbp lacking the β -domain was constructed as follows: First, to introduce a stop-codon and *Hind*III restriction site downstream of the Hbp passenger coding sequence, a PCR fragment was generated using pEH3-Hbp as a template and the primers Hbp2734-2751_fw (5'-cagatgtgttacagcctg-3') and *Hind*III-

Hbp_{rv} (5'-ctagaagctt**cag**taacttcagtgatgaagttg-3', *Hind*III site underlined, stop-codon in boldface). Subsequently, the fragment was cloned into a modified version of pEH3-Hbp carrying a *Hind*III site downstream of *hbp*, using the *Kpn*I/*Hind*III restriction sites, thereby removing the Hbp β-domain coding sequence from pEH3-Hbp. Nucleotide sequences were all confirmed by semi-automated DNA sequencing.

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