2 Materials and methods

2.1 Materials

2.1.1 Strains and Cell lines

Table 2.1: Name, genotype and source (reference) for the bacterial strains and cell lines used

NAME	GENOTYPE	SOURCE (REFERENCE)
***E. coli* strains**		
DH5α	F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	Invitrogen
GC10	F– mcrA Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 Δ(*ara*, *leu*) 7697 *ara*D139 *gal*U*gal*K *nup*G *rps*L	Biomol
JM109 (DE3)	*end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 (rK–, mK+), *rel*A1, *sup*E44, λ–, Δ(*lac-pro*AB), [F', *tra*D36, *pro*AB, *lac*IqZΔM15], IDE3	Promega
BL21(DE3) pLysS	F– *omp*T *hsd*SB(rB–, mB–) *gal dcm* (DE3) pLysS (CamR)	Novagen
BL21-CodonPlus(DE3)-RIL	F– *omp*T *hsd*SB(rB–, mB–) *gal dcm* (DE3) *endA* Hte *[argU ileY leuW* (CamR)	Stratagene
B834 (DE3)	F– *omp*T *hsd*SB(rB–, mB–) *gal dcm met*(DE3)	Novagen
***Shigella* strains**		
M90T	Wild-type strain of *S. flexneri*, serotype 5a	[5]
BS176	Plasmid cured derivative of M90T (avirulent)	[5]
SF619	Non-polar *ipgC* deletion mutant of M90T	[65]
SF620	Non-polar *ipaB* deletion mutant of M90T	[46]
BJ007	Non-polar *ipaB*, *ipgC* deletion mutant of M90T	This study
Cell lines		
HeLa	Epithelial-like cell line from human cervix carcinoma	Human
Macrophage	Murine bone marrow derived macrophage	Mouse

For cloning purpose DH5α, GC10 or JM109 (DE3) were used. For high yield protein expression, the plasmids were transformed in *E. coli* JM109 (DE3), BL21(DE3) pLysS and/or BL21-CodonPlus (DE3)- RIL. For Selenomethionine incorporation, a methionine auxotroph expression strain B834 (DE3) was employed. Functional complementation studies were performed in mutant *S. flexneri* 5a (M90T) using either constitutively active pUC19 or L-arabinose inducible pBAD vectors.

2.1.2 Plasmids

Table 2.2: Plasmids used in this study

NAME	INSERT/PROPERTY	VECTOR/ RESISTENCE	RESTRIC-TION SITES	REF
IpgC constructs				
I28-1	IpgC with cleavable 6x His tag	pET 28a/Kan	*Nhe*I-*Hind*III	This study
I44-1	IpgC with cleavable 6x His tag	pET44a/Amp	*Xma*I-*Hind*III	This study
R8	IpgC	pET21a/Amp	*Nhe*I-*Hind*III	This study
IpgCSDx	IpgC with cleavable 6x His tag with M1G mutation	pET 28a/Kan	-	This study
IgCStpN1	IpgC with N-ter non-cleavable Strep tag	pET 28a/Kan	-	This study
IpgCqd	IpgCSDx lacking C-ter 4 amino acids (DIKE)	pET 28a/Kan	*Nhe*I-*Hind*III	This study
IgC20CTH1	IpgCΔ1-21 having C-ter non-cleavable 6x His tag	pET 28a/Kan	*Xma*I-*Hind*III	This study
IgC30CTH	IpgCΔ1-32 having C-ter non-cleavable 6x His tag	pET 28a/Kan	*Nco*I-*Xho*I	This study
ipgCd21.1	IpgCΔ1-21	pBAD/Myc-HisA/Amp	*Nco*I-*Hind*III	This study
IpgCd32.1	IpgCΔ1-32	pBAD/Myc-HisA/Amp	*Nco*I-*Hind*III	This study
2qdBAD	IpgCSDx lacking C-ter 4 amino acids (DIKE)	pBAD/Myc-HisA/Amp	*Nco*I-*Hind*III	This study
2IgCFLBAD	IpgC	pBAD/Myc-HisA/Amp	*Nco*I-*Hind*III	This study
IpaC constructs				
ICCD1	IpaC full-length cloned in MCS1	pCDFDuet/Sm	*Nco*I-*Not*I	This study
ICCDB1	IpaC cloned in MCS1 with N-ter His tag	pCDFDuet/Sm	*BamH*I-*Not*I	This study
IC21D1	IpaC without tag	pET21d/Amp	*Nco*I/*Not*I	This study
IAC28a7	IpaC with cleavable 6x His tag	pET 28a/Kan	*Nde*I/*Not*I	This study
1-173.2	$IpaC^{1-173}$	pET21d/Amp	*Nco*I-*Not*I	This study
174-E.1	$IpaC^{174-363}$	pET21d/Amp	*Nco*I-*Not*I	This study
1-126.1	$IpaC^{1-126}$	pET21d/Amp	*Nco*I-*Not*I	This study
127-E.1	$IpaC^{126-363}$	pET21d/Amp	*Nco*I-*Not*I	This study
1-75.1	$IpaC^{1-75}$	pET21d/Amp	*Nco*I-*Not*I	This study
76-E.2	$IpaC^{76-363}$	pET21d/Amp	*Nco*I-*Not*I	This study
76-126.1	$IpaC^{75-126}$	pET21d/Amp	*Nco*I-*Not*I	This study
IC29.10	IC21D1with an amber mutation at K29	pET21d/Amp	*Nco*I-*Not*I	This study
IC33.2	IC21D1with an amber mutation at Y33	pET21d/Amp	*Nco*I-*Not*I	This study
IC36.2	IC21D1with an amber mutation at I36	pET21d/Amp	*Nco*I-*Not*I	This study
IC42.4	IC21D1with an amber mutation at L42	pET21d/Amp	*Nco*I-*Not*I	This study
IC50.4	IC21D1with an amber mutation at L50	pET21d/Amp	*Nco*I-*Not*I	This study
IC60.6	IC21D1with an amber mutation at L60	pET21d/Amp	*Nco*I-*Not*I	This study
IC63.2	IC21D1with an amber mutation at E64	pET21d/Amp	*Nco*I-*Not*I	This study
IC68.4	IC21D1with an amber mutation at H68	pET21d/Amp	*Nco*I-*Not*I	This study
IC73.4	IC21D1with an amber mutation at I73	pET21d/Amp	*Nco*I-*Not*I	This study
IC80.4	IC21D1with an amber mutation at K80	pET21d/Amp	*Nco*I-*Not*I	This study
IC84.2	IC21D1with an amber mutation at L84	pET21d/Amp	*Nco*I-*Not*I	This study
IC88.9	IC21D1with an amber mutation at S88	pET21d/Amp	*Nco*I-*Not*I	This study
IC99.7	IC21D1with an amber mutation at I99	pET21d/Amp	*Nco*I-*Not*I	This study
IC116.7	IC21D1with an amber mutation at L116	pET21d/Amp	*Nco*I-*Not*I	This study
IC120.3	IC21D1with an amber mutation at L120	pET21d/Amp	*Nco*I-*Not*I	This study
IC127.4	IC21D1with an amber mutation at L127	pET21d/Amp	*Nco*I-*Not*I	This study
IC146.4	IC21D1with an amber mutation at I146	pET21d/Amp	*Nco*I-*Not*I	This study
IC154.4	IC21D1with an amber mutation at L154	pET21d/Amp	*Nco*I-*Not*I	This study
IpaB constructs				
IB21a	IpaB without tag	pET21a/Amp	*Nde*I-*Xho*I	This study
IB28a11	IpaB with cleavable 6x His tag	pET 28a/Kan	*Nde*I-*Xho*I	This study
1IBCH	IpaB with non-cleavable C-ter 6x His tag	pET21b/Amp	*Nde*I-*Xho*I	This study
IB10	IB21a with an amber mutation at G10	pET21a/Amp	*Nde*I-*Xho*I	This study
IB15	IB21a with an amber mutation at K15	pET21a/Amp	*Nde*I-*Xho*I	This study
IB18	IB21a with an amber mutation at T18	pET21a/Amp	*Nde*I-*Xho*I	This study
IB22	IB21a with an amber mutation at L22	pET21a/Amp	*Nde*I-*Xho*I	This study
IB35	IB21a with an amber mutation at N35	pET21a/Amp	*Nde*I-*Xho*I	This study
IBn45	IB21a with an amber mutation at L45	pET21a/Amp	*Nde*I-*Xho*I	This study
IB48.7	IB21a with an amber mutation at N48	pET21a/Amp	*Nde*I-*Xho*I	This study
IB51.1	IB21a with an amber mutation at I51	pET21a/Amp	*Nde*I-*Xho*I	This study

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NAME	INSERT/PROPERTY	VECTOR/ RESISTENCE	RESTRIC-TION SITES	REF
IB55.4	IB21a with an amber mutation at N55	pET21a/Amp	*Nde*I-*Xho*I	This study
IB62.1	IB21a with an amber mutation at I62	pET21a/Amp	*Nde*I-*Xho*I	This study
IB65.3	IB21a with an amber mutation at P65	pET21a/Amp	*Nde*I-*Xho*I	This study
IB70n.1	IB21a with an amber mutation at K68	pET21a/Amp	*Nde*I-*Xho*I	This study
IBn74.1	IB21a with an amber mutation at N74	pET21a/Amp	*Nde*I-*Xho*I	This study
IBn79.3	IB21a with an amber mutation at L79	pET21a/Amp	*Nde*I-*Xho*I	This study
IB1-507	IpaB$^{1-507}$	pET21a/Amp	*Nde*I-*Xho*I	This study
1IB51t	IpaB$^{51-507}$	pET21a/Amp	*Nde*I-*Xho*I	This study
1IB51E	IpaB$^{51-580}$	pET21a/Amp	*Nde*I-*Xho*I	This study
1IB73t	IpaB$^{73-507}$	pET21a/Amp	*Nde*I-*Xho*I	This study
1IB73E	IpaB$^{73-580}$	pET21a/Amp	*Nde*I-*Xho*I	This study
1IBd51	IpaBΔ51-72	pET21a/Amp	*Nde*I-*Xho*I	This study
2IBd58	IpaBΔ58-72	pET21a/Amp	*Nde*I-*Xho*I	This study
IBPSIII1	IpaB with PreScission cleavage site in place of amino acids 51-72 cloned in pET21a	pET21a/Amp	MS	This study
IBd50	IpaBΔ1-50	pET21a/Amp	*Nde*I-*Xho*I	This study
IBd72	IpaBΔ1-72	pET21a/Amp	*Nde*I-*Xho*I	This study
3IBd51	IpaBΔ51-72	pET21a/Amp	*Nde*I-*Xho*I	This study
2IBd58	IpaBΔ58-72	pET21a/Amp	*Nde*I-*Xho*I	This study
IBpUC	IpaB	pUC19/Amp	*Nde*I-*Xho*I	This study
IBpUCd50	IpaBΔ1-50	pUC19/Amp	*Nde*I-*Xho*I	This study
IBpUCd72	IpaBΔ1-72	pUC19/Amp	*Nde*I-*Xho*I	This study
IBpUC5172	IpaBΔ51-72	pUC19/Amp	*Nde*I-*Xho*I	This study
IBpUC1650.8	IpaBΔ17-50	pUC19/Amp	*Nde*I-*Xho*I	This study
IBpUC1772	IpaBΔ17-72	pUC19/Amp	*Nde*I-*Xho*I	This study
IB28.1	IpaB$^{51-72}$	pET28a/Kan	*Nde*I-*Xho*I	This study
IB21.1	IpaB$^{51-72}$	pET21a/Amp	*Nde*I-*Xho*I	This study
1StrepIB1672	IpaB$^{16-72}$ having N-ter non-cleavable Strep tag	pET21a/Amp	*Nco*I-*Xho*I	This study
IpgC+IpaB construct				
IpgCIB272.1	IpaB$^{2-72}$ linked to C-ter of IpgCqd	pET28a/Kan	*Nco*I-*Xho*I	This study
IpgC+IpaC construct				
IpgCIC1668.1	IpaC$^{16-68}$ linked to C-ter of IpgCqd	pET28a/Kan	*Nco*I-*Xho*I	This study
IpaB+IpaC constructs				
IBICB3	IpaC cloned in MCS1 with N-ter His tag and IpaB cloned in MCS2	pCDFDuet-1/ Sm	MCS1: *BamH*I-*Not*I MCS2: *Nde*I-*Xho*I	This study
IBICD20	IpaC cloned in MCS1 and IpaB cloned in MCS2	pCDFDuet-1/ Sm	MCS1: *Nco*I-*Not*I MCS2: *Nde*I-*Xho*I	This study
Other constructs				
SA28a1	SicA with cleavable 6x His tag	pET 28a/Kan	*Nde*I-*Xho*I	This study
SycD	SycD with cleavable 6x His tag	pET 28a/Kan	*Nde*I-*Xho*I	This study
BicA	BicA with cleavable 6x His tag	pET 28a/Kan	*Nde*I-*Xho*I	This study
pSup-BpaRS-6TRN	For incorporation of p-benzoyl phenylalanine	Cm		[134]

MS: Multiple cloning strategies employed

Amp: Ampicillin; Cm: Chloramphenicol; Kan: Kanamycin; Sm: Streptomycin

2.1.3 DNA oligos for cloning and site-directed mutagenesis

Table 2.3: Name, sequence and application of the used oligonucleotides

NAME	SEQUENCE 5' TO 3'	APPLICATION
NheI-28a-lpgC-fw HindIII-28a/44a-lpgC-bw	*cgctttGCTAGC*atgtctttaaatatcaccgaaaat *gcaata*AAGCTTatcattactccttgatatcctgaatt	Cloning of lpgC wt into pET-28a
XmaI-44a-lpgC-fw HindIII-28a/44a-lpgC-bw	*cgctttCCCGGGGCAGC*atgtctttaaatatcaccgaaaat *gcaata*AAGCTTatcattactccttgatatcctgaatt	Cloning of lpgC wt into pET-44a
lpgC-NheI-new-fw HindIII-28a/44a-lpgC-bw	*cgctttGCTAGC*ttaaatatcaccgaaaatgaaag *gcaata*AAGCTTatcattactccttgatatcctgaatt	Cloning of lpgC wt into pET-21a
lpgC-SD28-fw	gtgccgcgcggcagcTTAAATATCACCGAA	Substitution of Met 1 by Gly
QCMlpgC-StrepNter-f	ataccatgggcagcTGGAGCCACCCGCAGTTCGAAAAAggcagc ttaaatatcacc	Substitution of N-ter cleavable 6x His with non-cleavable Strep tag
lgcqd-f	cttggacgcaattcagTAATGATAAGCTTGC	Deletion of C-ter. 4 amino acids (DIKE)
lgCd21-NcoI-bad-f lpgC-XhoI-28aCTH-r	*cgctttCCATGG*gcgctacactgaaagatatta *cgctttCTCGAG*ggcctccttgatatcctgaattgc	Cloning of lpgCΔ1-21 having non-cleavable C-ter 6x His into pET-28a
lgCd32-NcoI-bad-f lpgC-XhoI-28aCTH-r	*cgctttCCATGG*atgatatgatggatgacatttat *cgctttCTCGAG*ggcctccttgatatcctgaattgc	Cloning of lpgCΔ1-32 having non-cleavable C-ter 6x His into pET-28a
lgCd21-NcoI-bad-f HindIII-28a/44a-lpgC-bw	*cgctttCCATGG*gcgctacactgaaagatatta *gcaata*AAGCTTatcattactccttgatatcctgaatt	Cloning of lpgCΔ1-21 into pBAD/Myc-HisA
lgCd32-NcoI-bad-f HindIII-28a/44a-lpgC-bw	*cgctttCCATGG*atgatatgatggatgacatttat *gcaata*AAGCTTatcattactccttgatatcctgaatt	Cloning of lpgCΔ1-32 into pBAD/Myc-HisA
lpgC-NcoI-28aCTH-f lpgCqd-HindIII-bw	*cgctttCCATGG*gctctttaaatatcaccgaaaatga *gcaata*AAGCTTatcactgaattgcgtccaagtatg	Cloning of lpgC C-ter 4 aa into pBAD/Myc-HisA
lpgC-NcoI-28aCTH-f HindIII-28a/44a-lpgC-bw	*cgctttCCATGG*gctctttaaatatcaccgaaaatga *gcaata*AAGCTTatcattactccttgatatcctgaatt	Cloning of lpgC wt into pBAD/Myc-HisA
lpaC-21d/CDFD-NcoI-fw lpaC-21d/CDFD-NotI-bw	*cgctttCCATGG*aaattcaaaacacaaaacc *ctcagttaaa*GCGGCCGCtcattaagctcgaatgttaccagca	Cloning of lpaC in pET21d and in MCS1 of pCDFDuet-1
lpaC-CDFD-BamHI-fw lpaC-21d/CDFD-NotI-bw	*cgctttGGATCC*aatggaaattcaaaacacaaaacc *ctcagttaaa*GCGGCCGCtcattaagctcgaatgttaccagca	Cloning of lpaC in MCS1 of pCDFDuet-1 with N-ter His tag
lpaC-p28-NdeI-fw lpaC-21d/CDFD-NotI-bw	*gggaattcCATATG*gaaattcaaaacacaaaacc *ctcagttaaa*GCGGCCGCtcattaagctcgaatgttaccagca	Cloning of lpaC wt into pET-28a
lpaC-21d/CDFD-NcoI-fw lpaC173-p21d-Not-r	*cgctttCCATGG*aaattcaaaacacaaaacc *ctcagttaaa*GCGGCCGCtcagcgagcaatgatttctgaatc	Cloning lpaC$^{1-173}$ in pET-21d
lpaC174-p21d-NcoI-f lpaC-21d/CDFD-NotI-bw	*cgctttCCATGG*caaaaacgcattcagggattagcga *ctcagttaaa*GCGGCCGCtcattaagctcgaatgttaccagca	Cloning lpaC$^{174-363}$ in pET-21d
lpaC-21d/CDFD-NcoI-fw lpaC126-p21d-Not-r	*cgctttCCATGG*aaattcaaaacacaaaacc *ctcagttaaa*GCGGCCGCtcatttagtttctgcagtgcgga	Cloning lpaC$^{1-126}$ in pET-21d
lpaC127-p21d-NcoI-f lpaC-21d/CDFD-NotI-bw	*cgctttCCATGG*gctctcaattgtcattgattg *ctcagttaaa*GCGGCCGCtcattaagctcgaatgttaccagca	Cloning lpaC$^{126-363}$ in pET-21d
lpaC-21d/CDFD-NcoI-fw lpaC75-p21d-Not-r	*cgctttCCATGG*aaattcaaaacacaaaacc *ctcagttaaa*GCGGCCGCtcagcgagcaatgatttctgaatc	Cloning lpaC$^{1-75}$ in pET-21d
lpaC76-p21d-NcoI-f lpaC-21d/CDFD-NotI-bw	*cgctttCCATGG*cacttactgacaaaaagatgaaag *ctcagttaaa*GCGGCCGCtcattaagctcgaatgttaccagca	Cloning lpaC$^{76-363}$ in pET-21d
lpaC76-p21d-NcoI-f lpaC126-p21d-Not-r	*cgctttCCATGG*cacttactgacaaaaagatgaaag *ctcagttaaa*GCGGCCGCtcatttagtttctgcagtgcgga	Cloning lpaC$^{75-126}$ in pET-21d
lpaC29A-f	tcttccgaaacacaa**tag**tcacaaaattatcag	lpaC with an amber mutation at K29 in pET-21d
lpaC33A-f	caaaaatcacaaaat**tag**cagcagattgcagcg	lpaC with an amber mutation at Y33 in pET-21d
lpaC36A-f	caaaattatcagcag**tag**gcagcgcatattcca	lpaC with an amber mutation at I36 in pET-21d
lpaC42A-f	gcagcgcatattcca**tag**aatgtcggtaaaaat	lpaC with an amber mutation at L42 in pET-21d
lpaC50A-f	ggtaaaaatcccgta**tag**acaaccacattaaat	lpaC with an amber mutation at L50 in pET-21d
lpaC60A-f	aatgatgatcaactt**tag**aagttatcagagcag	lpaC with an amber mutation at L60 in pET-21d
lpaC64A-f	cttttaaagttatca**tag**caggttcagcatgat	lpaC with an amber mutation at E64 in pET-21d
lpaC68A-f	tcagagcaggttcag**tag**gattcagaaatcatt	lpaC with an amber mutation at H68 in pET-21d
lpaC73A-f	catgattcagaaatc**tag**gctcgccttactgac	lpaC with an amber mutation at I73 in pET-21d
lpaC80A-f	cgccttactgacaaa**tag**atgaaagatctttca	lpaC with an amber mutation at K80 in pET-21d

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NAME	SEQUENCE 5' TO 3'	APPLICATION
IpaC84a-f	aaaaagatgaaagat**tag**tcagagatgagtcac	IpaC with an amber mutation at L84 in pET-21d
IpaC88a-f	gatctttcagagatg**tag**cacacccttactcca	IpaC with an amber mutation at S88 in pET-21d
IpaC99a-f	gagaacactctggat**tag**tccagtctttcttct	IpaC with an amber mutation at I99 in pET-21d
IpaC116a-f	attagtgtagccgtt**tag**ctttctgctctccgc	IpaC with an amber mutation at L116 in pET-21d
IpaC120A-f	gttctactttctgct**tag**cgcactgcagaaact	IpaC with an amber mutation at L120 in pET-21d
IpaC127A-f	actgcagaaactaaa**tag**ggctctcaattgtca	IpaC with an amber mutation at L127 in pET-21d
IpaC146A-f	tcagctgcagagaac**tag**gttcggcaaggcctg	IpaC with an amber mutation at I146 in pET-21d
IpaC154A-f	caaggcctggcagcc**tag**tcatcaagcattact	IpaC with an amber mutation at L154 in pET-21d
IpaB-21a/CDFD-NdeI-fw IpaB-21a/CDFD-XhoI-bw	*gggaattc*CATATGcataatgtaagcaccacaac *cgctttCTCGAG*ttatcaagcagtagtttgttgcaaaa	IpaB without tag in pET-21a and with cleavable 6x His in pET-28a. Also in MCS II of pCDFDuet-1.
IpaB-21a/CDFD-NdeI-fw IpaB-21b-XhoI-bw	*gggaattc*CATATGcataatgtaagcaccacaac *cgctttCTCGAG*agcagtagtttgttgcaaaattg	IpaB with non-cleavable C-ter 6x His tag in pET-21b
Ipabn10tag-f	agcaccacaaccact**tag**tttcctcttgccaaaat	IpaB a with an amber mutation at G10 in pET-21a
IpaBn15tag-f	ggttttcctcttgcc**tag**atattgacttccact	IpaB a with an amber mutation at K15 in pET-21a
Ipabn18tag-f	cttgccaaaatattg**tag**tccactgagcttgga	IpaB a with an amber mutation at T18 in pET-21a
Ipabn22tag-f	ttgacttccactgag**tag**ggagacaatactatc	IpaB a with an amber mutation at L22 in pET-21a
IpaBn35tag-f	gcaaatgatgcagct**tag**aaattattttctctt	IpaB a with an amber mutation at N35 in pET-21a
IpaBn45tag-f	cttacaattgctgat**tag**actgctaaccaaaat	IpaB a with an amber mutation at L45 in pET-21a
IpaBn48tag-f	gctgatcttactgct**tag**caaaatattaataca	IpaB a with an amber mutation at N48 in pET-21a
IpaBn51tag-f	actgctaaccaaaat**tag**aatacaactaatgca	IpaB a with an amber mutation at I51 in pET-21a
IpaBn55tag-f	aatattaatacaact**tag**gcacactcaacttca	IpaB a with an amber mutation at N55 in pET-21a
IpaBn62tag-f	cactcaacttcaaat**tag**ttaatccctgaactt	IpaB a with an amber mutation at I62 in pET-21a
IpaBn65tag-f	tcaaatatattaatc**tag**gaacttaaagcacca	IpaB a with an amber mutation at P65 in pET-21a
IpaBn68tag-f	ttaatccctgaactt**tag**gcaccaaagtcatta	IpaB a with an amber mutation at K68 in pET-21a
IpaBn74tag-f	gcaccaaagtcatta**tag**gcaagttcccaacta	IpaB a with an amber mutation at N74 in pET-21a
IpaBn79tag-f	aatgcaagttcccaa**tag**acgcttttaattgga	IpaB a with an amber mutation at L79 in pET-21a
IpaB-21a73-HdIII-f IpaB-21a/CDFD-XhoI-bw IpaB-21a/CDFD-NdeI-fw IpaB-21a50-SacI-r IpaBPS5172f	*cgctttAAGCTT*taaatgcaagttcccaactaa *cgctttCTCGAG*ttatcaagcagtagtttgttgcaaaa *gggaattc*CATATGcataatgtaagcaccacaac *cgctttGAGCTC*attttggttagcagtaagatca CTGCTAACCAAAAT**tttctgcatagcctggaagttctgtt** **ccaggggcccgaaattatgaaa**TTAAATGCAAGTTC	IpaB with PreScission cleavage site in lieu of amino acids 51-72 cloned in pET21a
IpaB51-21a-fw IpaB-21b-XhoI-bw	*gggaattc*CATATGattaatacaactaatgcacactc *cgctttCTCGAG*agcagtagtttgttgcaaaattg	IpaBΔ1-50 cloned in sites of pET21a
IpaB73-21a-fw IpaB-21b-XhoI-bw	*gggaattc*CATATGttaaatgcaagttcccaactaa *cgctttCTCGAG*agcagtagtttgttgcaaaattg	IpaBΔ1-72 cloned in NdeI-XhoI sites of pET21a
IpaBd51-72-F	actgctaaccaaaatTTAAATGCAAGTTCC	IB21a with a deletion from amino acids 51-72 in IpaB
IpaBd58-72-fw	acaactaatgcacacTTAAATGCAAGTTCC	IB21a with a deletion from amino acids 58-72 in IpaB
Ipa-F Ipa-R	*gggg*AAGCTTgatgcataatgtaagcaccacaac *gggg*CTGCAGtccttatttgtatcaagcagtagt	IpaB wt in pUC19
IpaB51-pUC-fw Ipa-R	*gggg*AAGCTTattaatacaactaatgcacactc *gggg*CTGCAGtccttatttgtatcaagcagtagt	IpaBΔ1-50 cloned in pUC19
IpaB73-pUC-fw Ipa-R	*gggg*AAGCTTttaaatgcaagttcccaactaa *gggg*CTGCAGtccttatttgtatcaagcagtagt	IpaBΔ1-72 cloned in pUC19
IpaBd51-72-F	actgctaaccaaaatTTAAATGCAAGTTCC	IpaBΔ51-72 cloned in pUC19
IB1650del-f	gttttcctcttgccaaaATTAATACAACTAATG	IpaBΔ17-50 cloned in pUC19
IB1672del-f	gttttcctcttgccaaaTTAAATGCAAGTTCCCA	IpaBΔ17-72 cloned in pUC19

continued on next page

NAME	SEQUENCE 5' TO 3'	APPLICATION
IpaB51-21a-fw	*gggaattc*CATATGattaatacaactaatgcacactc	IpaB$^{51-72}$ without tag in pET-21a and with cleavable 6x His in pET-28a
IpaB72-Xho-21a-b	*cgctttCTCGAG*ttatcatgactttggtgctttaagttc	
IB16strep-Nco-28-f	*gcttCCATGG*gcTGGAGCCACCCGCAGTTCGAAAAAggtgcaAT ATTGACTTCCACTG	IpaB$^{16-72}$ having N-ter non-cleavable Strep tag
IpaB16-Nde-21a-f	*gggaattcCATATG*atattgacttccactgag	IpaB$^{16-72}$ in pET-21a without any tag and in pET-28a with cleavable 6x His tag
IpaB72-Xho-21a-b	*cgctttCTCGAG*ttatcatgactttggtgctttaagttc	
IpgCqd-Nco-f	*cgctttCCATGG*gcgctacactgaaagatatta	Cloning fusion of IpgCqd and IpaB2-72 in pET-28a
IpaB72-Xho-21a-b	*cgctttCTCGAG*ttatcatgactttggtgctttaagttc	
ipgC-IpaB-oB	ggttgtggtgcttacattatgcTGAATTGCGTCCAAGTATG	
ipgC-IpaB-oC	catacttggacgcaattcaGCATAATGTAAGCACCACAACC	
IpgCqd-Nco-f	*cgctttCCATGG*gcgctacactgaaagatatta	Cloning fusion of IpgCqd and IpaB16-68 in pET-28a
IpaC68-Xho-28-r	*cgctttCTCGAG*ttatcaatgctgaacctgctctgataa	
ipgC-IpaC-oB	ctttgagtttgttttgtggatatCTGAATTGCGTCCAAGTATG	
ipgC-IpaC-oC	catacttggacgcaattcagATATCCACAAAACAAACTCAAAG	
bicA-21a-NdeI-fw	*gggaattcCATATG*acgcaacgcgacgtgaaca	BicA wt in pET-28a
bicA-21a-XhoI-bw	*cgctttCTCGAG*ttatcagtggtcgttcccggcgg	
sicA-21a-NdeI-fw	*gggaattcCATATG*gattatcaaaataatgtcagcga	SicA wt in pET-28a
sicA-21a-XhoI-bw	*cgctttCTCGAG*ttatcattccttttcttgttcactgtgc	
SycD-28a-NdeI-f	*gggaattcCATATG*caacaagagacgacagacac	SycD wt in pET-28a
SycD-28a-XhoI-r	*cgctttCTCGAG*ttatcatgggttatcaacgcactcat	

Notes:
Complementary oligonucleotides for site-directed mutagenesis were also used but are not listed.
Primers were purchased from Eurofins MWG operon, synthesis scale: 0.01 – 0.05 µmol.
Changes from wild-type *ipgC* sequence (including additionally inserted nucleotides) are indicated in bold.
Restriction endonuclease target sites are underlined.

2.1.4 Peptides

Table 2.4: Name, sequence, residue numbers and modifications of the peptides used

NAME	SEQUENCE	RESIDUES
IpaB18-35	NH_2-TSTELGDNTIQAANDAAN-COOH	18-35
IpaB51-72	NH_2-INTTNAHSTSNILIPELKAPKS-COOH	51-72
IpaB65-70	Ac-PELKAP-$CONH_2$	65-70
IpaB-P1mutant	Ac-**R**ELKAP-$CONH_2$	65-70
IpaB-P2 mutant	Ac-PE**N**KAP-$CONH_2$	65-70
IpaB-P3 mutant	Ac-PELKA**D**-$CONH_2$	65-70
IpaB-SB mutant	Ac-PEL**A**AP-$CONH_2$	65-70
BipB-pep	Ac-PALRAP-$CONH_2$	74-79
YopB-pep	Ac-VQLPAP-$CONH_2$	50-55
YopD-pep	Ac-PELIKP-$CONH_2$	58-63
SipB-pep	Ac-VGLKPP-$CONH_2$	66-71
IpaC36-68	NH_2-IAAHIPLNVGKNPVLTTTLNDDQLLKLSE-COOH	75-80

Notes:
Peptide synthesis was performed by Peter Henklein, Charité-Universitätsmedizin Berlin, Insitut für Biochemie.
Changes from wild-type IpgC primary sequence are indicated in bold.

2.1.5 Culture media and antibiotics

Culture media were autoclaved and antibiotic stock solutions sterile-filtrated after preparation. 50 µg/ml of ampicillin (Amp), 100 µg/ml of chloramphenicol (Cm), 30 µg/ml of kanamycin (Kan) and/or 50 µg/ml streptomycin (Sm) were added to each medium before use. For agar plates, 15 g of agar were dissolved in 1 L milipore H_2O and autoclaved. For protein expression using the pET-system, isopropyl-thiogalactoside (IPTG) was added to cultures to a final concentration of 0.5 mM. L-arabinose at a final concentration of 10 mM was used to induce protein expression in *Shigella* strains transformed with pBAD based constructs. Media were stored at 4 °C, if not indicated specifically, antibiotic stock solutions at -20 °C.

Table 2.5: Culture media

NAME	AMOUNT	CHEMICAL
LB medium	10 g	Tryptone
	5 g	Yeast extract
	10 g	NaCl
	up to 1 l	ddH_2O
DMEM media	10%	Fetal Calf Serum (FCS) (heat inactivated: 56° C, 30 min)
	1%	Na pyruvate
	100 units/ml	Penicillin
	100 µg/ml	Streptomycin
	2 mM	L-glutamine
	500 ml	Dulbecco's modified Eagle's medium
Serum free media	1%	Na pyruvate
	2 mM	L-glutamine
	500 ml	Dulbecco's modified Eagle's medium
RPMI 1640 media	10%	Fetal Calf Serum (FCS) (heat inactivated: 56° C, 30 min)
	100 units/ml	Penicillin
	100 µg/ml	Streptomycin
	2 mM	L-glutamine
	500 ml	Dulbecco's modified Eagle's medium

2.1.6 Buffers and solutions

Table 2.6: Buffers and solution

APPLICATION	NAME	AMOUNT	CHEMICAL
Affinity chromatography (HisTrap)	Washing buffer A	20 mM 500 mM 40 mM	Sodium phosphate pH 7.4 NaCl Imidazole
	Elution buffer B	20 mM 500 mM 500 mM	Sodium phosphate pH 7.4 NaCl Imidazole
Affinity chromatography (Strep tag)	Buffer W	100 mM 150 mM 1 mM	Tris, pH 8.0 NaCl EDTA
	Buffer E	2.5 mM	Desthiobiotin in Buffer W
	Buffer R	1 mM	HABA (hydroxy-azophenyl-benzoic acid) in Buffer W
Cell lysis buffer		5 mM 1 tablet/50 ml	β-Mercaptoethanol Complete, EDTA-free protease inhibitor cocktail (Roche Applied Science) in Washing buffer A or Buffer W
Gel filtration buffer		20 mM 50 - 150 mM	HEPES pH 7.5 NaCl

All other common buffers and media for molecular biology and protein visualization used in this work are adapted from the molecular cloning laboratory manual [135]

2.1.7 Enzymes and chemicals

Table 2.6: Enzymes and chemicals

ENZYME/CHEMICAL	COMPANY
Benzonase, DNAseI, Lysozyme	Roche
Bradford reagent	Bio-Rad
BugBuster protein extraction reagent	Novagen
Complete EDTA-free Protease Inhibitor Cocktail Tablets, Pefabloc SC (AEBSF)	Roche
GenTherm DNA polymerase	Rapidozym
Kaleidoscope Protein Standard, Polypeptide SDS-PAGE Standard	Bio-Rad
L-Selenomethionine	Acros
Overnight Express Autoinduction System 2	Novagen
PageRuler protein ladder	Fermentas
p-benzoyl phenylalanine [H-p-Bz-Phe-OH (F-2800)]	Bachem
PfuUltra High Fidelity DNA polymerase	Stratagene
QIAGEN Plasmid Midi Kit, QIAprep Spin Miniprep Kit, QIAquick Gel Extraction Kit, QIAquick PCR Purification Kit, QuikChange site-directed mutagenesis kit	QIAGEN
SureClean Plus	Bioline
Thrombin CleanCleave Kit [RECOMT]	Sigma-Aldrich

Enzymes not listed above were all purchased from New England Biolabs. All other fine-chemicals used in this work were purchased in analytical grade from Merck, Roth or Sigma-Aldrich.

2.1.8 Instrumentation

Table 2.8: Instrumentation

NAME	IDENTIFIER/COMPANY
Balances	Sartorius B3100 S, Sartorius AC 210 S
Centrifuges and rotors	Avanti J-20 XP, Avanti J-25, Beckman Coulter JLA 8.100, JLA 16.250, JA 25.50, Beckman Coulter Micro Centrifuges 5415R, Eppendorf Heraeus Biofuge Primo, Kendro MLA-130 (130 K RPM), Beckman Coulter Optima MAX Ultracentrifuge, Beckman Coulter
Cell lysis	French Press, Slm Aminco Spectronic Instruments
CO_2 incubators	Forma Series II 3110 Water Jacketed CO_2 Incubator, Thermo Forma
Concentrators	Amicon NMWL 10 kDa, Millipore Microcon Ultracel YM-3, Millipore
Crystallization	Screens, Hampton Research and Jena Bioscience Crystallisation plates for hanging drop crystallisation, Molecular Dimensions Limited Coverslides, Menzel-Gläser Goniometer Heads, Capillary Mounts and Supplies, Hampton Research 18° C incubators, Thermo electron Corporation and Liebherr
Dialysis	Slide-A-Lyzer Dialysis cassette, 3.5 K MWCO, Thermo Scientific
Electrophoresis	Power Pac 300, Bio-Rad Polyacrylamide gel electrophoresis, Criterion Precast Gel System, Bio-Rad Agarose gel electrophoresis, Mini-Sub Cell GT, Bio-Rad
Electroporation	Gene Pulser, Bio-Rad
Filtering	Sterile filter 0.05 µm, Wyatt Technology Corp. Sterile filters 0.1, 0.2 and 0.4 µm, Millipore Membrane filters 0.025 µm, Millipore
FPLC	AKTA Explorer, pH/C-900, UV-900, P-900, Frac-950, GE Healthcare HisTrap HP Columns, 1 ml/5ml, GE Healthcare HiPrep 26/10 Desalting Column, GE Healtcare HiLoad Superdex 75 prep grade, 200 prep grade 16/60, GE Healthcare HiPrep Sephacryl S-100 26/60, GE Healthcare Superose 6 and 12, GE Healthcare Strep-Tactin Superflow column, IBA GmbH TALON, Clontech
Incubators	INP 500, Memmert
Incubator Shakers	Innova 44, New Brunswick Scientific
Isothermal titration calorimetry	VP-ITC, MicroCal
LoBind tubes (DNA, protein)	Eppendorf
Mass spectrometry	MALDI TOF/TOF, 4700 Proteomics Analyzer, Applied Biosystems
Microscope	Olympus SZX12
Mixer	Thermomixer compact, Eppendorf Assistant 348, RM5, Karl Hecht KG
Multi-Angle Light Scattering	miniDAWN Tristar system, Wyatt Technology Corp.
PCR	Stratagene RoboCycler 40 PCR Machine
Protein transfer	Semi-dry, Carl Roth
pH-meter	PB11 PY-P10, Sartorius
Quartz cuvettes	Hellma GmbH & Co KG
Spectroscopy	UV-VIS Spectrophotometer, GE Healtcare NanoDrop ND-1000, Peqlab Biophotometer, Eppendorf
Speed Vac	Concentrator 5301, Eppendorf
Surface Plasmon Resonance	BIAcore, GE Healthcare
UV crosslinker	UV Stratalinker 2400, Stratagene

2.1.9 Software for X-ray structure determination

Table 2.9: Software

PROGRAM	NAME/REFERENCE
Data processing	XDS Program Package [136], HKL2000
Phasing	autoSHARP [137], SHELXC/D [138], SHARP [139], Phaser [140]
Solvent flattening	SOLOMON [141]
Model building	ARP/wARP [142]
Structure refinement	CNS [143], Coot [144]
Structure evaluation and validation	Procheck [145]
Visualization	Pymol [146], Coot [144]

2.2 Methods

2.2.1 Molecular biology methods

Construction of plasmids

The plasmids used in this study are listed in Table 2.2 and the primers used are listed in Table 2.3. Most of the plasmids were generated by employing standard molecular biology technique. For amplification the cycling conditions consisted of an initial denaturation step of 2 min at 94 °C, followed by 25 cycles of 45 s at 94 °C, 1 min at 55 - 58 °C and 1.5 min/ Kb at 72 °C. Amplification reactions were concluded with a final extension step of 10 min at 72 °C. The amplification condition was standardized for RoboCycler, Stratagene which consists of 4 blocks preheated to the temperatures described. Colony-PCR in 15 µl was used to verify cloning. Cells from a bacterial colony were directly transferred into tubes containing PCR mix with pipette tips. The following guideline for used for setting up the reaction mixture:

Component	Final concentration
10x Ultra *Pfu* buffer	1x
Primers	0.5 µM each
Template DNA	10-30 ng
dNTPs	200 µM each
Ultra *Pfu* polymerase	2.5 U
H_2O	to 50 µl

Table 2.10: Standard PCR

Component	Final concentration
10x *Taq* buffer	1x
Primers	0.25 µM each
Template DNA	colony
dNTPs	200 µM each
Taq polymerase	0.5 U
H_2O	to 15 µl

Table 2.11: Colony PCR

The PCR amplicon was gel extracted and cloned into plasmid by ligation to the restriction endonuclease sites of the polylinker.

Site-directed mutagenesis

Site-directed mutations and internal deletions were created using the QuikChange site-directed mutagenesis kit. To maximize mutagenesis efficiency and minimize the incidence of primer dimer formation, a two-stage protocol described by Wang and Malcolm [147] was used. Two single-primer PCR reactions preceding the standard QCM procedure was performed (see below).

Component	Final conc.
10x Ultra *Pfu* buffer	1x
one Primer (forward or reverse	0.2 µM
Template DNA	50-200 ng
dNTPs	200 µM each
Ultra *Pfu* polymerase	2.5 U
H_2O	to 50 µl

Table 2.12: Two-stage QCM PCR

segment	cycle	Temp [°C]	Time
1	1	95	30 s
2	3	95 55 68	30 s 30 s 10 min
mix the contents of both the reactions			
3	16	run segment 2	
4	hold	4	forever

Table 2.13: Cycling parameter

Following temperature cycling, 10 units of *Dpn*I restriction enzyme was directly added to the PCR reaction mixture and incubated for 1 h at 37 °C. 1 - 3 µl of the reaction mix were transformed into *E. coli* DH5α. The clones for correct introduction of the desired mutation were screened by sequencing.

Gene splicing by PCR-driven overlap extension

Chimeric IpgC-IpaB$^{2-72}$ and IpgC-IpaC$^{16-68}$ fusions were generated by splicing together *ipaB*$^{2-72}$ or *ipaC*$^{16-68}$ and *ipgC* using the strategy described by Heckman and Pease [148] (Fig. 1). Internal primers (b and c) were designed to contain nucleotide sequences from either *ipaB*$^{2-72}$ or *ipaC*$^{16-68}$ and ipgC. Overlapping segments created by the initial PCR (a+b, c+d) served as templates in the second PCR. The second PCR amplified by primers a and d yielded a hybrid product AD. The final product containing either *ipgC-ipaB*$^{2-72}$ or *ipgC-ipaC*$^{16-68}$ was inserted into pET28a

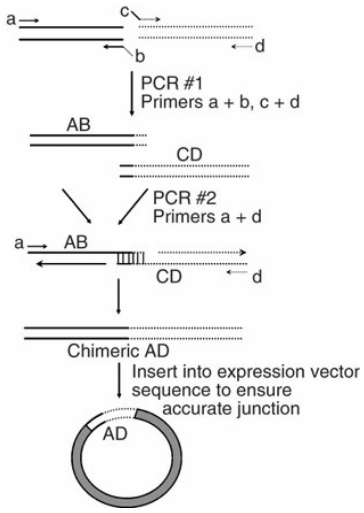

Fig. 2.1: Chimeric gene product generation by overlap extension method. Adapted from [20]

Generation of chimeric IpaB

For generating pIBPS (IpaB having PreScission cleavage site in place of amino acids 51-72), first, *ipaB* comprising amino acids 73-580 was cloned in the sites of *Hind*III and *Xho*I of pET21a; next, *ipaB* comprising amino acids 1-50 was cloned in the sites of *Nde*I and *Sac*I; finally, dsDNA obtained by annealing complementary ssDNA comprising PreScission cleavage site flanked by 4 amino acid linker on either side and having *Sac*I and *Hind*III was cloned into the respective sites to create pIBPS.

2.2.2 Cell based methods

HeLa invasion assay

Infections of HeLa cells were performed as previously described [5, 101] using a multiplicity of infection of 100. To test for epithelial cell invasion, the number of intracellular bacteria in infected HeLa cells was determined using a gentamicin protection assay as reported before [17]. By this method, various *ipgC* and *ipaB* mutants were analyzed for their functionality in the SF619 (Δ*ipaB*), SF620 (Δ*ipgC*) and BJ007 (Δ*ipaB*/ Δ*ipgC*) background. Briefly, HeLa cells infected for 20 min were incubated in the presence of gentamicin (50 mg/ml) for an additional 1 h and 2 h. Intracellular bacteria were determined after lysing the infected cells, plating dilutions of the lysates, and counting the CFU. In the assays described above, the standard error was calculated based on at least three independent determinations.

Secretion assay

Cultures of exponentially growing *Shigellae* were standardized by measuring the optical density at 600 nm and harvested by centrifugation at 10,000 x *g* for 10 min. Crude bacterial extracts were obtained from the pellets, and proteins of filtered (0.2-µm pore size) culture supernatants were precipitated with 10% trichloroacetic acid. Protein expression and secretion was analyzed in basal conditions from pellets and supernatants respectively of cultures grown with specific inducers [45]. Protein samples were analyzed by 12% SDS-PAGE. Immunoblotting procedures were carried out with either rabbit anti-IpaB polyclonal antibody or mouse anti-IpaB monoclonal antibody (Mab) H16, rabbit anti-IpgC polyclonal antibody, anti-IpaC Mab J22, mouse anti-DnaK monoclonal antibody. Sheep anti-mouse or goat anti-rabbit antibodies were used as secondary antibodies and visualized by addition of SuperSignal West Dura Substrate (Pierce) enhanced chemiluminescence.

LDH release assay

Cytotoxicity assay was performed as previously described [149]. Murine macrophages were grown in 96-well plates and infected in serum-free medium for 2 h. Gentamicin (100 µg/ml) was added at 30 min postinfection. Cytotoxicity was quantified by measuring the release of lactate dehydrogenase enzyme from infected cells using the CytoTox 96 kit (Promega) following the manufacturer's instructions.

2.2.3 Protein methods

2.2.3.1 Protein expression

BL21(DE3) RIL harboring plasmid/s containing the gene/s were used for expression supplemented with appropriate antibiotics. Cell cultures were grown at 37 °C with a rotary shaker until an A_{600} of ~0.6 was reached. Induction was initiated by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells harvested 4 h later were spun down, flash frozen and stored at -80 °C.

For the SeMet labeling, the protein was expressed in *E.coli* B834 (DE3). 50 ml inoculum containing appropriate antibiotic was generated by multistaging according to the manufacture's protocol to inoculate 1 L of Overnight Express System 2 (Novagen) medium in 5 L Erlenmeyer flask supplemented with 30 mg/L kanamycin, 125 mg/L L-seleno-methionine and 100 nM vitamin B12. After incubating the culture for 16 h at 37 °C with shaking at 300 rpm, the cells are harvested, spun down, flash frozen and stored at -80 °C.

2.2.3.2 Protein purification

Cell lysis

All steps were carried out at 4 °C, unless stated otherwise. Cell pellets were thawed on ice and suspended in ice-cold lysis buffer (5 ml per g of cell pellet) containing one tablet of Complete EDTA-free Protease Inhibitor Cocktail and *Dnase*I. The suspension was passed 3 times through the French Press at a pressure of 1,000 psi. The lysate was centrifuged for 30 min at 22,000 rpm and 4 °C and the supernatant passed through a 0.2 µm filter.
All steps of protein purification were carried out at 4 °C unless stated otherwise. Fast protein liquid chromatography (FPLC) was carried out on an Äkta Explorer low pressure system. All columns were equilibrated in corresponding buffers before use and filled with 20% ethanol for long time storage.

Immobilized metal affinity chromatography

Affinity chromatography using Ni Sepharose was the first step in the chromatographic purification. It was performed to remove most cellular proteins from recombinantly expressed 6x His tagged proteins. The supernatant of the cell lysis was applied to HisTrap HP columns and then washed with 5 column volumes of washing buffer A. Further removal of non-specifically bound proteins was increased by washing with 5-10% of elution buffer B (in total 65-90 mM imidazole concentration). Finally, bound protein was eluted by washing with 100% of elution buffer B.

Purification with *Strep*-Tactin

The *Strep*-tag purification system is based on the highly selective binding of engineered streptavidin, *Strep*-Tactin [150], to *Step*-tag II fusion proteins. The elution of the protein is achieved by the addition of the biotin analog desthiobiotin, which is a reversible binding specific competitor. The fusion protein having *Strep*-tag II was loaded on *Strep*-Tactin Superflow cartridge equilibrated with buffer W. The column was washed extensively till the absorbance at 280 nm showed a baseline, and subsequently the protein was eluted with buffer E.

Buffer exchange

In order to exchange the Na phosphate buffer which is incompatible with the downstream thrombin cleavage buffer requiring calcium, the buffer was exchanged using a HiPrep 26/10 Desalting column to gel filtration buffer (20 mM HEPES, pH 7.4, 150 mM NaCl). Proteins differ greatly in size from salts and other small molecules. In this process, separation of the two groups is achieved as high molecular weight substances (>5 kDa) are excluded from the column resin and thus elute first, whereas low molecular weight substances enter the pores of the resin freely and thus elute later.

Thrombin cleavage

N-terminal 6x His fusion tag was removed by recombinant thrombin using the RECOMT Thrombin CleanCleave Kit according to user instructions. The thrombin is covalently bound to agarose beads for easy removal. The buffer is supplemented with 10 mM $CaCl_2$. The cleavage reaction was performed overnight at 4 °C with slow agitation on a shaker to ensure homogenous distribution of the thrombin beads in the protein solution. Once the digestion is completed, as monitored by SDS-PAGE, the beads were sedimented by low speed centrifugation (600 x g, 5 min, 4 °C). The protein solution was filtered through 0.2 µM filter to rid the protein of any residual thrombin beads. Additionally, 1 mM EDTA and Pefabloc were added to the protein solution.

Size exclusion chromatography

The last step of protein purification involved polishing by gel filtration chromatography using either HiLoad Superdex 75 prep grade 16/60 or HiLoad Superdex 200 prep grade 26/60 column having separation ranges of 3-70 kDa and 10-600 kDa respectively. The molecules in the sample are separated on the basis of size and shape. Fractionation of the molecules is achieved as they move through a bed of porous beads, diffusing into the beads to greater or lesser degrees. Smaller molecules diffuse further into the pores of the beads and therefore move through the bed more quickly. This step was required to separate aggregates from the protein which is difficult to achieve by any other technique.

2.2.3.3 Protein analysis

Photocrosslinking

Plasmid pSup-BpaRS-6TRN [134] (Fig. 2) contains the mutant *M. jannaschii* tyrosyl-tRNA synthetase (*Mj*TyrRS(BpaRS)) gene that specifically aminoacylates the photocrosslinking amino acid, *p*-benzoyl-L-phenylalanine expressed under control of a mutant *glnS* (*glnS'*) promoter. Three copies each of mutant *M. jannaschii* tyrosyl-tRNA (*Mj*tRNATyrCUA) gene, encoding an amber suppressor tRNA is expressed under the control of two *proK* promoters. The plasmid has chloramphenicol resistance. It was co-transformed with either mutant *ipaB* or *ipaC* gene with an amber codon at specific position having ampicillin resistance and *ipgC* having a N-terminal 6x His tag and kanamycin resistance into BL21(DE3). Bacteria were incubated at 37 ℃ in LB containing 50 µg/ml ampicillin, 100 µg/ml Chloramphenicol, 30 µg/ml kanamycin and 1 mM pBpa. At OD_{600}=0.6, cells were induced by the addition of 0.5 mM IPTG and incubated for 4 h. Cells were harvested by centrifugation and lysed with French press, and protein complex was purified exploiting the N-terminus 6x His tag on IpgC. The protein complex was exchanged to 1x PBS using Amicon concentrators of 10 KDa cut off. Purified protein concentration was measured by the Bradford method. Crosslinking reactions were performed on 300 µl of protein complex in a 24-well microtiter plate (Nunclon, Nalge Nunc, Denmark) on ice. Samples were irradiated at 365 nm using a UV Stratalinker 2400 (Stratagene, 15 W), for 0, 15 and 30 min. Samples were removed from the wells and boiled with SDS loading buffer before resolution of products by SDS-PAGE on a 12% gel.

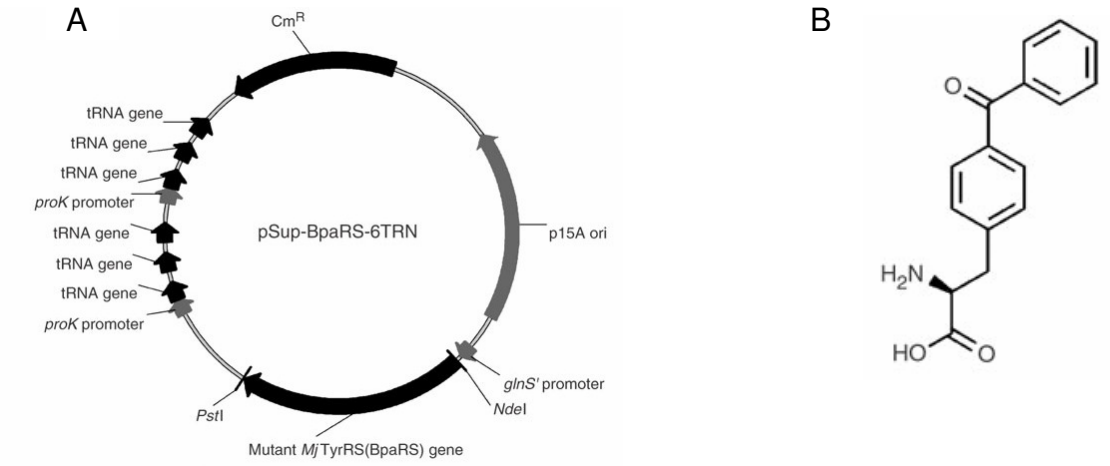

Fig. 2: A, Plasmid map of pSup-BpaRS-6TRN. The plasmid contains 3 copies of amber suppressor tRNA under the control of two *proK* promoter each and contains a copy of tRNA synthetase. **B**, structure of photocrosslinking amino acid *p*-benzoyl-L-phenylalanine. From [134].

Sodium dodecylsulfate polyacrylamide gel electroporesis (SDS-PAGE)

Samples of bacterial cultures and aliquots of protein preparations were analyzed by discontinuous SDS-PAGE. For constructs <15 kDa, 16.5% precast Criterion Tris-HCl gels from Bio-Rad was employed which gave similar results compared to Tris-Tricine PAGE developed by Schägger & Jagow [151]. SDS, an anionic detergent denatures the secondary and non-disulfide-linked tertiary structures. SDS binds in a ratio of approximatey 1.4:1 (w/w) to protein giving a uniform mass:charge ratio for most proteins.

The IpaB, IpgC were run on 12 and 15% acrylamide SDS gels respectively. Samples were dissolved in Laemmli buffer [152], denatured at 95 °C for 5 min and loaded into individual wells of the gel. The electrophoresis was carried out at 50 V, until the samples stacked onto the separation gel and was switched to 90 V.

Staining of PAGE gels

For regular staining Coomassie Brilliant Blue R-250 based staining solution was used. For silver staining, a rapid protocol by Nesterenko *et al*. [153] was adopted. The procedure is described below.

Steps	Solution	Time
Fixation	60 ml acetone stock; 1.5 ml TCA stock; 25 µl 37% HCHO	5 min
Rinse	H_2O	3 x 5 s
Wash	H_2O	5 min
Rinse	H_2O	3 x 5 s
Pretreat	60 ml acetone stock	5 min
Pretreat	100 µl $Na_2S_2O_3.5H_2O$ stock in 60 ml H_2O	1 min
Impregnate	0.8 ml $AgNO_3$ stock; 0.6 ml 37% HCHO; 60 ml H_2O	8 min
Rinse	H_2O	3 x 5 s
Develop	1.2 g Na_2CO_3; 25 µl 37% HCHO; 25 µl $Na_2S_2O_3.5H_2O$ stock; 60 ml H_2O	10-20 s
Stop	1% glacial acetic acid in H_2O	30 s

Stock solutions: 50% acetone in H_2O; 50% TCA in H_2O; 20% $AgNO_3$ in H_2O (store in dark); 10% $Na_2S_2O_3.5H_2O$ in H_2O

Determination of protein concentration

For colorimetric determination of protein concentrations a Protein dye reagent concentrate (Bio-Rad) was used according to the manufacturer's instructions. Absorption at 595 nm was measured and compared to BSA standard values.

Limited proteolysis

For limited proteolysis purified IpaB-IpgC and IpaC-IpgC complexes were digested with thermolysin and trypsin on ice and at 25°C with protein at a concentration of 1 mg/ml in 20 mM HEPES pH 7.4, 125 mM NaCl for digestion with thermolysin and similar buffer at pH 8.0 in case of digestion with trypsin. The E:S was 1:100 (by weight) in each case. At different time intervals, aliquots (15 µl) were sampled and boiled immediately in 10 µl 2x SDS sample buffer for 5 min to stop the proteolysis and stored at -20 °C. The protein samples were analyzed on 16.5% Criterion peptide gel (Bio-Rad).

Western transfer

Proteins that were separated by SDS-PAGE were transferred onto polyvinylidene fluoride (PVDF) membranes (Western Blotting) for immunodetection. PVDF membranes were activated by soaking in methanol. SDS gel and activated membrane were soaked in transfer buffer (50 mM Tris-HCl pH 8.3, 40 mM glycine, 20% (v/v) methanol) and placed between presoaked filter paper. Electrotransfer of the proteins was performed for 1 h at a constant current of 1 mA/cm^2 in a semi-dry blotting chamber. Immunodetection by relevant antibodies is described in 2.2.5.

Mass spectrometry

Proteins were identified by mass spectrometry in the core facility for protein analysis at Max-Planck Institute for Infection Biology, Berlin. Bands of interest were excised from polyacrylamide gels, digested with trypsin and the resulting peptides analyzed by MALDI-MS peptide-mass 'fingerprinting' under reducing conditions. The identity of the peptides was confirmed by MALDI-TOF-tandem mass spectrometry as described [154].

2.2.4 Spectroscopic methods

Crystallization

The constructs were crystallized according to the hanging-drop vapor-diffusion method. Initial crystallization conditions were obtained by sparse-matrix screening [155]. Drops were prepared by mixing 1 and 2 µl protein solution (10-15 mg/ml, 20 mM Hepes pH 7.5, 150 mM NaCl) with 1 µl reservoir solution and were equilibrated against 800 µl reservoir solution at 291.15 K. Co-crystallization trials were set up by mixing the appropriate IpaB or IpaC peptide/s with IpgC at a molar ratio of 2:1. Depending on the construct the crystals took overnight to two months to grow. This initial condition was optimized by varying the pH and precipitant concentration until crystals of larger size were obtained.

Crystal freezing

For cryoprotection, crystals were incubated for 1-5 min in reservoir solution containing 30% glycerol, removed by nylon loops on copper pins (Hampton Research) and submerged into liquid nitrogen. For data collection crystals were mounted and flash-cooled in the cryostream during the diffraction experiment.

Data collection and processing

Single-wavelength X-ray data collection was performed at the ESRF beamline ID23.1, Grenoble; BESSY beamlines BL14.1 and BL14.2, Berlin and DESY beamline BW6, Hamburg. Diffraction data from SeMet labeled were collected at BESSY beamline BL14.2, Berlin. Data were indexed, merged and scaled using XDS [136].

Crystal structure determination

Initial phases have been obtained using MAD with one peak, one inflection and two remote data sets. The software package autoSHARP [137] has been used in order to manage the following programs. SHELXC/D [138] found the position of 6 selenium atoms in the SeMet labeled crystal. Phases were calculated by SHARP [139], and improved by solvent flattening in SOLOMON [141]. An initial helical model was built with ARP/wARP helix building module [142]. Cycles of refinement using CNS [156] against data collected from the native crystal, and manual rebuilding with Coot [157], led to the final model. The crystal structure of the IpgC/IpaB$_{51-72}$ complex has been

solved by molecular replacement with Phaser [158] using as template a truncated apo structure. Figures were prepared either with the program Pymol [159] or Bioedit [160].

Multi-angle laser light scattering (MALLS)

For mass determination a combined setup consisting of size exclusion chromatography (SEC) and subsequent online detection by UV absorption, (three angle) static laser light scattering and differential refractive index measurement was used [161]. SEC was performed with a Tricorn Superdex 200 10/300 GL column (GE Healthcare) connected to an ÄKTA purifier chromatography system (GE Healthcare) equilibrated with 20 mM HEPES (pH 7.5), 50 mM NaCl. For static light scattering and differential refractive index measurements a linear coupled miniDAWN™ Tristar (Wyatt Technology) system and a differential refractive index detector (RI-101, Shodex), respectively, was used. All calculations were done with the software ASTRA (Wyatt Technology) on the basis of the detected signals for the UV absorption (280 nm), the refractive index [162] and three different angles of scattered laser light. All MALLS experiments were performed at 4-6 °C. Each experiment was repeated at least in triplicate. Effects of interdetector band broadening between instruments in the online experiment were corrected by using the ASTRA software.

2.2.5 Isothermal Titration Calorimetry (ITC)

Titration experiments were carried out using a VP–ITC calorimeter from MicroCal (Northampton, MA, USA). Titrations were performed by injecting consecutive 15 µl aliquots of peptide solution (15 mM) into 1.36 ml lpgC solution (700 µM) in the chamber. Peptides were dissolved in and the proteins dialyzed against 20 mM HEPES pH 7.5, 150 mM NaCl. Two independent titration experiments were performed at 25 °C. Binding stoichiometry, enthalpy, and equilibrium association constants were determined by fitting the corrected data to a bimolecular interaction model using the evaluation software provided by the manufacturer.