SUPPLEMENTAL DATA

BcsK$_C$ is an essential protein for the Type VI secretion system activity in *Burkholderia cenocepacia* and forms an outer membrane complex with BcsL$_B$

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Table S1

Strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source and/or reference
B. cenocepacia		
K56-2	ET12 clone related to J2315, CF clinical Isolate	[b]BCRRC, (1)
K56-2 Δ*atsR*	Deletion of *atsR* in K56-2	This study
K56-2 Δ*atsR* Δ*T6SS*	Deletion of the T6SS cluster in K56-2 Δ*atsR*	This study
K56-2 Δ*atsR* Δ*TU1*	Deletion of the T6SS transcriptional unit #1 in K56-2 Δ*atsR*	This study
K56-2 Δ*atsR* Δ*TU2*	Deletion of the T6SS transcriptional unit #2 in K56-2 Δ*atsR*	This study
K56-2 Δ*atsR* Δ*TU3*	Deletion of the T6SS transcriptional unit #3 in K56-2 Δ*atsR*	This study
K56-2 Δ*atsR* Δ*bcsM*	Deletion of *bcsM* in K56-2 Δ*atsR*	This study
K56-2 Δ*atsR* $\Delta bcsL_B$	Deletion of $bcsL_B$ in K56-2 Δ*atsR*	This study
K56-2 Δ*atsR* $\Delta bcsK_C$	Deletion of $bcsK_C$ in K56-2 Δ*atsR*	This study
K56-2 Δ*atsR* Δ*hcp*	Deletion of *hcp* in K56-2 Δ*atsR*	This study
K56-2 Δ*atsR* $\Delta bcsI_E$	Deletion of $bcsI_E$ in K56-2 Δ*atsR*	This study
K56-2 Δ*atsR* $\Delta bcsH_F$	Deletion of $bcsH_F$ in K56-2 Δ*atsR*	This study
K56-2 Δ*atsR* $\Delta bcsG_G$	Deletion of $bcsG_G$ in K56-2 Δ*atsR*	This study
K56-2 Δ*atsR* $\Delta bcsF_H$	Deletion of $bcsF_H$ in K56-2 Δ*atsR*	This study
K56-2 Δ*atsR* $\Delta bcsE_A$	Deletion of $bcsE_A$ in K56-2 Δ*atsR*	This study
E. coli		
DH5α	F^- ϕ80*lacZ* M15 *endA1 recA1 supE44 hsdR*17(r_K^- m_K^+) *deoR* *thi-1 nupG supE44 gyrA96 relA1* Δ(*lacZYA-argF*)*U169*, λ–	Laboratory stock
SY327	*araD*, Δ*(lac pro) argE(Am) recA56 rif*^{R} *nalA*, λ *pir*	(2)
BL21(DE3)	F^- *ompT hsdSB*(r_B^- m_B^-) *gal dcm*(DE3)	Laboratory stock
KEM5	DH1 Δ*cyaA*, Cm^R	Kendra Maloney
Plasmids		
pBcsK$_C$	$bcsK_C$ in pME6000	This study
pBcsK$_{C\Delta 40-189}$	$bcsK_{C\Delta 40-189}$ in pME6000	This study
pBscK$_{C-FLAG}$	$bcsK_C$ in pEL-1, FLAG	This study
pBcsK$_{C\Delta 40-189-FLAG}$	$bcsK_{C\Delta 40-189}$ in pEL-1, FLAG	This study
pBcsK$_C$T18	$bcsK_C$ in pUT18 fused to N-terminus of T18 domain	This study
pBcsK$_{C\Delta 38-62}$T18	$bcsK_{C\Delta 38-62}$ in pUT18 fused to N-terminus of T18 domain	This study
pBcsK$_{C\Delta 40-189}$T18	$bcsK_{C\Delta 40-189}$ in pUT18 fused to N-terminus of T18 domain	This study
pBcsK$_{C\Delta 63-87}$T18	$bcsK_{C\Delta 63-87}$ in pUT18 fused to N-terminus of T18 domain	This study

pBcsK$_{C\Delta 88-112}$T18	*bcsK*$_{C\Delta 88-112}$ in pUT18 fused to N-terminus of T18 domain	This study
pBcsK$_{C\Delta 113-137}$T18	*bcsK*$_{C\Delta 113-137}$ in pUT18 fused to N-terminus of T18 domain	This study
pBcsK$_{C\Delta 138-162}$T18	*bcsK*$_{C\Delta 138-162}$ in pUT18 fused to N-terminus of T18 domain	This study
pBcsK$_{C\Delta 163-187}$T18	*bcsK*$_{C\Delta 163-187}$ in pUT18 fused to N-terminus of T18 domain	This study
pBcsK$_{C\Delta 188-212}$T18	*bcsK*$_{C\Delta 188-212}$ in pUT18 fused to N-terminus of T18 domain	This study
pBcsK$_{C\Delta 213-237}$T18	*bcsK*$_{C\Delta 213-237}$ in pUT18 fused to N-terminus of T18 domain	This study
pBcsK$_{C\Delta 213-496}$T18	*bcsK*$_{C\Delta 213-496}$ in pUT18 fused to N-terminus of T18 domain	This study
pDA45	pGPI-SceI with fragments flanking *hcp*	(3)
pDAI-SceI	*ori*$_{pBBR1}$, Tet^R, P_{dhfr}, *mob*$^+$, expressing I-SceI	(4)
pDelatsR	pGPI-SceI with fragments flanking the *atsR*	This study
pDelbcsE$_A$	pGPI-SceI with fragments flanking *bcsE*$_A$	This study
pDelbcsF$_H$	pGPI-SceI with fragments flanking *bcsF*$_H$	This study
pDelbcsG$_G$	pGPI-SceI with fragments flanking *bcsG*$_G$	This study
pDelbcsH$_F$	pGPI-SceI with fragments flanking *bcsH*$_F$	This study
pDelbcsI$_E$	pGPI-SceI with fragments flanking *bcsI*$_E$	This study
pDelbcsK$_C$	pGPI-SceI with fragments flanking *bcsK*$_C$	This study
pDelbcsL$_B$	pGPI-SceI with fragments flanking *bcsL*$_B$	This study
pDelbcsM	pGPI-SceI with fragments flanking *bcsM*	This study
pDelTU1	pGPI-SceI with fragments flanking the transcriptional unit #1	This study
pDelTU2	pGPI-SceI with fragments flanking the transcriptional unit #2	This study
pDelTU3	pGPI-SceI with fragments flanking the transcriptional unit #3	This study
pDelT6SS	pGPI-SceI with fragments flanking the T6SS cluster	This study
pEL-1	cloning vector, *ori*$_{pBBR1}$, Tet^R, *mob*$^+$, P_{dhfr}, FLAG(N) epitope	E. Lameignere
pGPI-SceI	*ori*$_{R6K,}$ ΩTp^R , *mob*$^+$, including an I-SceI restriction site	(4)
pKT25	pSU40 derivative for C-terminal fusion to T25, Kan^R, P_{Lac}	Euromedex
pME6000	*ori*$_{pBBR1}$, Tet^R, *lacZ, mob*$^+$	S. Heeb
pRK2013	*ori*$_{colE1,}$ RK2 derivative, Kan^R, *mob*$^+$, *tra*$^+$	(5)
pT18BcsK$_C$	*bcsK*$_C$ in pUT18C fused to C- terminus of T18 domain	This study
pT18BcsK$_{C\Delta 40-189}$	*bcsK*$_{C\Delta 40-189}$ in pUT18C fused to C- terminus of T18 domain	This study
pT25BcsL$_B$	*bcsL*$_B$ in pKT25 fused to C-terminus of T25 domain	This study
pUT18	pUC19 derivative for N-terminal fusion to T18, Amp^R, P_{Lac}	Euromedex
pUT18C	pUC19 derivative for C-terminal fusion to T18, Amp^R, P_{Lac}	Euromedex

[a] Amp^R, ampicillin resistance, Tp^R, trimethoprim resistance, Cm^R, chloramphenicol resistance, Kan^R, kanamycin resistance, Tet^R, tetracycline resistance.

[b] BCRRC, *B. cepacia* Research and Referral Repository for Canadian CF Clinics.

Table S2

Oligonucleotide primers

Primer No.	5'-3' Primer sequence	Restriction enzyme*
2115	TTTCTAGAGTTCTGGGACACCTTCCACC	XbaI
3060	TTTTCTCGAGCGAGGACCAGGTGATTTTT	XhoI
3061	TTTTGAATTCCGCGATATCGAACGCTATTT	EcoRI
3062	TACGTCTAGAAAAAGCCTGCTGACAACCTG	XbaI
3071	TTTTCTCGAGGTGCTCGATCTCGAACTGC	XhoI
3393	TTTTGAGCTCTTATGAATTTGCCGGTTTG	SacI
3401	TTTTGGTACCGACGGAGTGTTGAGATG	KpnI
3456	AAAAGCGGCCGCCGTTTGCTGGTTCATCTCAA	NotI
3457	TTTTGAATTCGCCGGTTTAGTATCGGACAG	EcoRI
3466	AAAAGAATTCCGAGATGGCCAAGAAAGAAA	EcoRI
3467	TTTTGAATTCTGGATCGAACTGAAATCGTG	EcoRI
3468	TTAGCGGCCGCCGTGGAGACTTTCCTCAACC	NotI
3469	TTTTGCGGCCGCCTGGGTTCGAATCGTTTCAT	NotI
3470	TTAATCTAGAAAATGGACCGTCACTTCGTC	XbaI
3483	AAAATCTAGATATAGCGTGCTTCCGAGATG	XbaI
3485	AAAAGCGGCCGCGCACGCATGCAGTAAATCAA	NotI
3700	TTTTAAGCTTTATGAATTTGCCGGTTTG	HindIII
3701	TTATGGATCCTAACCAGCAAACGGCTGCGG	BamHI
3716	TTTTCTGCAGGAACCAGCAAACGGCTGC	PstI
3717	AAAAGGATCCCGCCAAGAAAGAAAGCATT	BamHI
3718	AAAAGAATTCCGTCGTCATTCGCCGTCT	EcoRI
3719	TTTTTCTAGAGTTGAATTTGCCGGTTTGGG	XbaI
3778	TTTTCTCGAGGGACCTGTTCGACTTCCTTG	XhoI
3779	TTTTTCTAGAGCGTCTCATTGAATCCTGGT	XbaI
3780	GGTCAGGAGACACGAACCAT	NA
3781	TGATCAAGACGTCGAACCTG	NA
3785	TTTTTCTAGAGATCGTCAGGCGAAGAGC	XbaI
3786	TTTTCTCGAGTTTCTTTCTTGGCCATCTC	XhoI
3787	ACGACAAGCTCGAGGATCTG	XhoI
3788	AAAAGAATTCGAAAGCTGCGTGGAAATCAG	EcoRI
3944	TTTTGCGGCCGCGGACCTGTTCGACTTCCTTG	NotI
3945	TTTTGCGGCCGCCGGTGAAGGGCTACCTGAT	NotI
3947	TTTTCTCGAGTAGCGAATCATGGACGATCT	XhoI
3948	TTTTTCTAGATGTTGAACACTTCCGCAGTC	XbaI
3949	TTTTGAATTCTAGCGAATCATGGACGATCT	EcoRI
3950	TTTTCTCGAGTGTTGAACACTTCCGCAGTC	XhoI
3951	TTATCTCGAGGAGCAGTCGTAAACGCAAGA	XhoI
3952	TTTATCTAGACCGACGTACACGATCAAGAA	XbaI
3966	TAAATCTAGACTCGACCTGTTCGACGACTT	XbaI
3967	TAAACTCGAGGCAACTGAAACCGCAACTGAC	XhoI
3968	TTTTGAATTCGCGCATTCATCTGCAAGTC	EcoRI
3969	TCTTCTCGAGAAACGATAATTCGCGTTCGT	XhoI
3970	TCTTCTCGAGATGCTGACGCTGTACGACCT	XhoI
3971	TTTTTCTAGAGCCTGGAAGAACTCGAAGC	XbaI
3972	TTTTGAATTCATGCTGACGCTGTACGACCT	EcoRI
3973	TTTTCTCGAGGCCTGGAAGAACTCGAAGC	XhoI

3974	TTTTCTCGAGGCACGTGATTCACTGAGACC	XhoI
3975	TTTTTCTAGACAGCCATGCTTGCTCGAA	XbaI
3976	TTTTGAATTCCTGGGATGCGTTCCTGTG	EcoRI
3977	TTTTCTCGAGTGTTGCCGGTCTTCAGG	XhoI
3978	TTTTCTCGAGACGTCGACCACATCCTGAAC	XhoI
3979	TTTTTCTAGACAGTCGGCCTCCTTGATCT	XbaI
3980	TTAACTCGAGGTTCGAGAACAGCAGGTCGT	XhoI
3989	TTTTCCCGGGCCGAGATCATCGAGCTGAA	SmaI
4056	**CTCGGAATCGGATTT**CGCGAC	NA
4057	**AAATCCGATTCCGAG**CTGTCGGCCACGATCGATGC	NA
4058	**GTTGTCCGACACGAT**CACCGT	NA
4059	**ATCGTGTCGGACAAC**GCGCCGGAATTCCAGCGCCTC	NA
4060	**GTGCATCACCGCGGA**AAGCTC	NA
4061	**TCCGCGGTGATGCAC**ACGATCAAGATCAAGGCGCTG	NA
4062	**CTGGCCCGTGTTGCT**TTCCTT	NA
4063	**AGCAACACGGGCCAG**CAGAGCGCGCTGTTCAAGAAG	NA
4064	**GTCGAACTCGCTCGC**GCCCTT	NA
4065	**GCGAGCGAGTTCGAC**GGCGATTACGAGATCTCGCGC	NA
4066	**TTCGGTGCGCTGATC**CATGCGCCGTTCATCGCGTCG	NA
4067	**GATCAGCGCACCGAA**CGGCGA	NA
4213	**CGCGGCCGCCGCGAC**GTGCGAC	NA
4214	**GTCGCGGCGGCCGCG**GACCTCGGCAAGGTGTTCGAC	NA
4215	**GCGCGGCTTGCCGAG**GTCGGAG	NA
4216	**CTCGGCAAGCCGCGC**GTCGGCCTGACGCTGCCGCGCTT	NA
4302	TTTTTCTAGAGGATCCCCGGGTAC	XbaI
4303	TTTTTCTAGAGTGCGCGGCTTGCCGAGGTC	XbaI

*****Restriction endonuclease sites incorporated in the oligonucleotide sequences are underlined.
N/A indicates absence of restriction site
Complementary sequences are bolded.

Plasmids constructions details

Mutagenesis of B. cenocepacia *K56-2 and complementing plasmids*—Unmarked and non-polar deletions were performed as described previously (4). To delete *atsR* (*BCAM0379*) PCR amplifications of regions flanking *atsR* were performed using 3061-3071 and 3060-3062 primer pairs. The amplicons were digested with the restriction enzymes EcoRI-XhoI and XhoI-XbaI, respectively, and cloned into the mutagenic plasmid pGPI-SceI digested with EcoRI and XbaI giving rise to pDelatsR. The deletion of *atsR* was first analyzed by PCR and also confirmed by Southern blot hybridization.

Several deletion plasmids were created using a similar approach and deletions were subsequently performed in *atsR* deleted mutant, *B. cenocepacia* K56-2 Δ*atsR*. To create pDelT6SS (to delete the entire T6SS gene cluster), amplicons were generated using primers 3778-3779 and 3780-3781 and were digested with XhoI-XbaI and XhoI-EcoRI. To create pDelTU1, pDelTU2 and pDelTU3 (to delete the T6SS Transcriptional Unit #1, #2 and #3, respectively; see *Results*), PCR products were amplified using primers 3457-3944 and 3945-2115; 3949-3950 and 3951-3952; 3947-3948 and 3780-3781, respectively. Amplicons were digested with the appropriate restriction enzymes and cloned into plasmid pGPI-SceI. Each gene within the transcriptional unit #2 was individually deleted. Plasmids pDelbcsM (to delete *BCAL0340*), pDelbcsL$_B$ (to delete *BCAL0341*), pDelbcsK$_C$ (to delete *BCAL0342*), pDelbcsI$_E$ (to delete *BCAL0344*), pDelbcsH$_F$ (to delete *BCAL0345*), pDelbcsG$_G$ (to delete *BCAL0346*), pDelbcsF$_H$ (to delete *BCAL0347*) and pDelbcsE$_A$ (to delete *BCAL0348*) were created using amplicons generated with primers 3949-3950 and 3966-3967; 3785-3786 and 3787-3788; 3466-3456 and 3468-3470; 3467-3469 and 3483-3485; 3968-3969 and 3970-3971; 3972-3973 and 3974-3975; 3976-3977 and 3978-3979; 3951-3952 and 3980-3989, respectively. Plasmid pDA45 was used to delete *BCAL0343.*

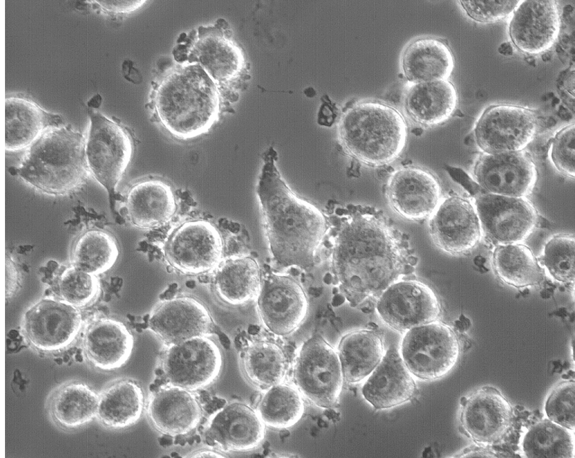
Bacterial two-hybrid system, β-galactosidase assay and plasmid constructions—In vivo interaction between BcsK$_C$ and BcsL$_B$ was investigated using the Bacterial Adenylate Cyclase Two-Hybrid (BACTH) System Kit (Euromedex). This system is based on the interaction-mediated reconstitution of the adenylate cyclase activity in an adenylate cyclase deficient *E. coli* reporter strain (6). The catalytic domain of adenylate cyclase (CyaA) from *Bordetella pertussis* consists of two complementary fragments, T25 and T18 that are not active when physically separated. When these two fragments are fused to interacting polypeptides and co-expressed in *E. coli*, heterodimerization of the hybrid proteins restores the activity CyaA, leading to cyclic AMP synthesis and transcriptional activation of the *lac* operon. Therefore, interaction between two hybrid proteins in *E. coli* will generate high levels of ß-galactosidase activity, which can be readily scored on X-gal containing media and quantified.

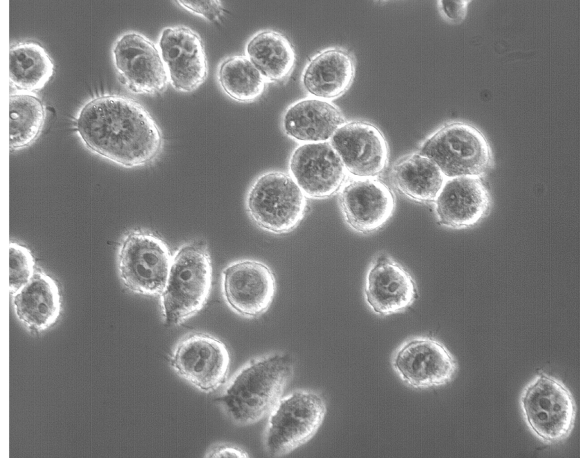
BcsL$_B$ and BcsK$_C$ were fused to the T25 and T18, respectively as follows. The *bcsL$_B$* gene was PCR amplified using primer pair 3717-3718 primer pair. The amplicon was digested with BamHI and EcoRI and ligated into pKT25 plasmid similarly digested. The resulting plasmid pT25BcsL$_B$ encodes a hybrid protein made of BcsL$_B$ fused to the C-terminus of the T25 fragment (T25-BcsL$_B$). *BcsK$_C$* and *bcsK$_{C\Delta40\text{-}189}$* genes were first PCR amplified using primer pair 3716-3393. The amplicons were digested with PstI-SacI and ligated into similarly digested pUT18C. The resulting plasmids pT18BcsK$_C$ and pT18BcsK$_{C\Delta40\text{-}189}$ encode hybrid proteins made of BcsK$_C$ or a truncated variant fused to the C-terminal end of T18. *BcsK$_C$* and *bcsK$_{C\Delta40\text{-}189}$* were also PCR amplified using primer pair 3716-3719 primer pair. The amplicons were digested with PstI and XbaI and ligated into similarly digested pUT18. The resulting plasmids pBcsK$_C$T18, pBcsK$_{C\Delta40\text{-}189}$T18, encode hybrid proteins made of BcsK$_C$ or a truncated variant fused to the N-terminal end of T18.

Sequential 25 amino acids deletions were performed within BcsK$_C$. Deletion of amino acids 38 to 62 was performed as follows: DNA sequences encoding amino-acids 2 to 38 and amino acids 63 to 495 were PCR amplified using primer pairs 3716-4056 and 4057-3719, respectively. Primers 4056 and 4057 contain complementary sequences at their 5' extremity. Amplicons were purified using the QiaQuick PCR Purification Kit (Qiagen) according to the manufacturer's recommendations. Fusion PCR was performed using 2 µl of each product in a HotStar HiFidelity DNA polymerase containing-reaction mix (Qiagen) with primers 3716-3719. The PCR product was purified, digested with PstI and XbaI, and

ligated into pUT18 plasmid similarly digested giving rise to plasmid pBcs$K_{C\Delta 38\text{-}62}$T18. Using a similar approach deletions of amino acids 63 to 87, 88 to 112, 113 to 137, 138 to 162, 163 to 187, 188 to 212, 213 to 237 were performed by fusion PCR using amplicons generated with primers 3716-4058 and 4059-3719, 3716-4060 and 4061-3719, 3716-4062 and 4063-3719, 3716-4064 and 4065-3719, 3716-4067 and 4066-3719, 3716-4213 and 4214-3719, 3716-4215 and 4216-3719, respectively, giving rise to plasmids pBcs$K_{C\Delta 63\text{-}87}$T18, pBcs$K_{C\Delta 88\text{-}112}$T18, pBcs$K_{C\Delta 113\text{-}137}$T18, pBcs$K_{C\Delta 138\text{-}162}$T18, pBcs$K_{C\Delta 163\text{-}187}$T18, pBcs$K_{C\Delta 188\text{-}212}$T18 and pBcs$K_{C\Delta 213\text{-}237}$T18, respectively. Deletions of amino acids 213 to 496 were performed with the Expand long template PCR system (Roche) using primers 4302-4303 and pBcsK_CT18 as template. PCR products were digested with XbaI and intramolecular ligation was performed giving rise to plasmid pBcs$K_{C\Delta 213\text{-}496}$T18 expressing the first 212 amino acids from BcsK_C fused to the N-terminus of T18.

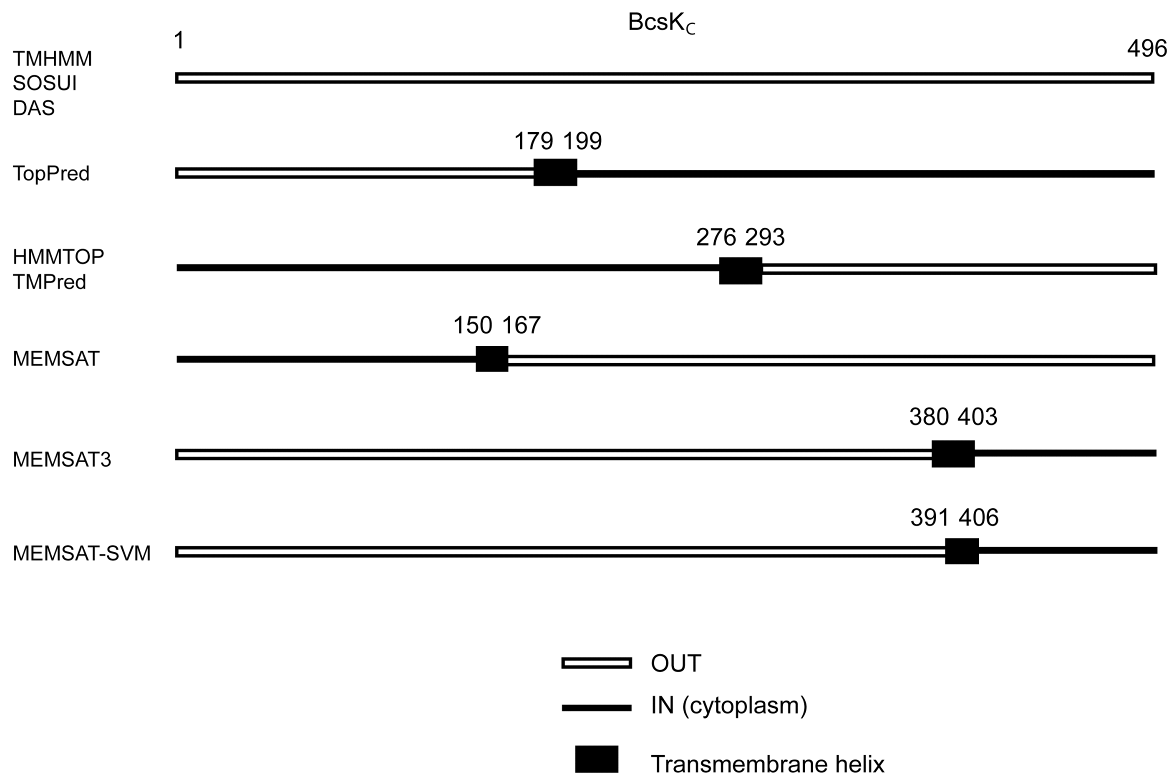
Supplemental Figures





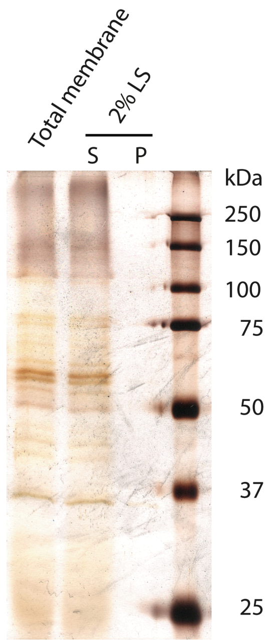
Aubert *et al.*, Figure S1

Fusion of a FLAG epitope to the N-terminus of $BcsK_C$ does not alter the protein function. Phase-contrast microscopy of infected ANA-1 macrophages. The infections were performed at a MOI of 50:1 for 4 h with the following strains: *B. cenocepacia* K56-2 Δ*atsR* Δ$bcsK_C$ ($pBcsK_{C\text{-}FLAG}$) and K56-2 Δ*atsR* Δ$bcsK_C$ ($pBcsK_{C\Delta 40\text{-}189\text{-}FLAG}$).



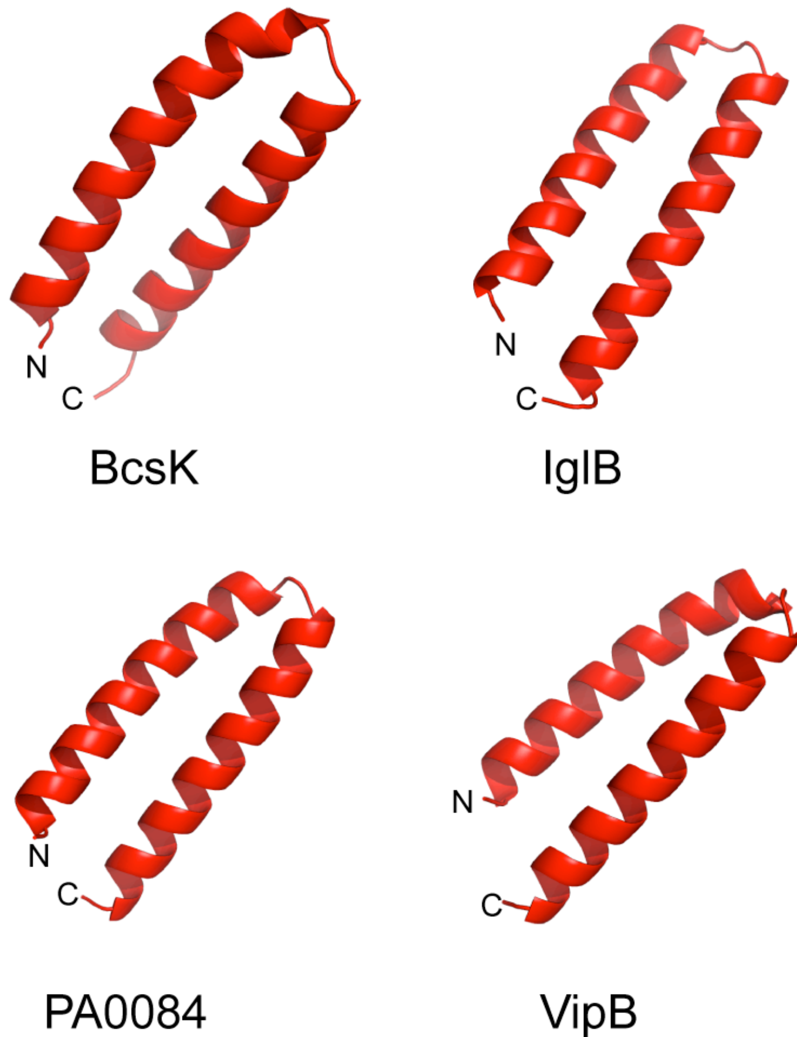
Aubert *et al.*, Figure S2

***In silico* analysis of the BcsK$_C$ protein.** Analyses were done using different transmembrane helices prediction programs available at the ExPASY Proteomics (http://www.expasy.ch) and PSIPRED servers (http://bioinf4.cs.ucl.ac.uk:3000/psipred/) (7,8).



Aubert *et al.*, Figure S3

Protein solubilization with 2% *N*-lauroylsarcosine (LS). Insoluble (Pellet, P) and soluble (Supernatant, S) fractions were loaded on a 12% SDS-PAGE gel and silver stained. The protein profiles from untreated total membrane and from soluble fraction are identical while no protein can be detected in the pellet fraction, suggesting that treatment of *B. cenocepacia* membrane with 2% *N*-lauroylsarcosine for 1h in ice leads to the solubilization of both inner and outer membrane.



Aubert *et al.*, Figure S4

***Ab initio* models of the region required for binding to $BcsL_B$ (residues 63-112 in $BcsK_C$) in $BcsK_C$ homologs.** These include IglB from *F. novicida*, VipB from *V. cholerae*, and PA0084 from *P. aeruginosa*. Structural modeling was performed with the I-TASSER server (http://zhang.bioinformatics.ku.edu/I-TASSER/). A similar fold consisting of paired α-helices is predicted in all cases.

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