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Supporting Information

A Modular System for the Rapid Comparison of Different Membrane Anchors for Surface Display on *Escherichia coli*

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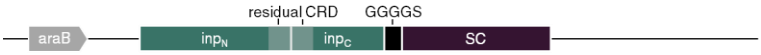
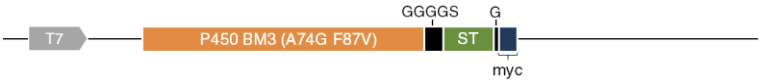
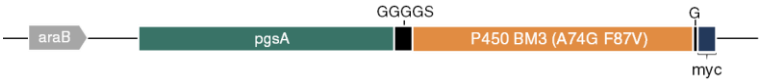
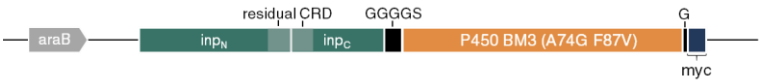
Contents

Materials and Methods

Plasmid construction. All plasmids used in this study, their detailed design and their sources are listed in Table S1. All plasmids used for surface display of cytochrome P450 BM3(A74G F87V) (BM3) via the S^{3}D or the conventional system where designed with a (glycine)$_4$-serine sequence (GGGGS) as a flexible linker between the gene-encoding sequences. To enable analyses by immunostaining, a Myc affinity tag was added to all fusion proteins containing BM3. The Myc tags were separated from the BM3 sequence by a single glycine. The amino acid sequences of all functional protein parts are listed in Table S2. The plasmids listed under "other plasmids" have been used to generate negative control cells with intracellular expression of SC (pET-EXPn1_his$_6$-SC, pET_SC-his$_6$) or to overexpress the mRFP-ST-his$_6$ probe (pET_mRFP-ST-his$_6$). The amino acids separating the his$_6$ tag and the SC sequence in plasmid pET-EXPn1_his$_6$-SC, derived from Peschke et al.[1], remained as scars after Gateway cloning.

Table S1: Plasmids used in this study. pTF16 based plasmids employed a p15A ori, a chloramphenicol resistance gene and the tight L-arabinose-inducible araB promoter. pET based plasmids employed a pBR322 ori, an ampicillin resistance gene and an IPTG inducible T7 promoter.

Plasmid	Design of genetic construct	Source
plasmids used for surface display via S^{3}D system		
pTF16_lpp-ompA-SC		Peschke et al.[1]
pTF16_pgsA-SC		this study
pTF16_inp$_{NC}$-SC		this study
pTF16_ctxB-SC-AIDA		this study
pET_BM3-ST-myc		Gallus et al.[2]
plasmids used for surface display via conventional system		
pTF16_lpp-ompA-BM3-myc		Gallus et al.[2]
pTF16_pgsA-BM3-myc		this study
pTF16_inp$_{NC}$-BM3-myc		this study
pTF16_ctxB-myc-BM3-AIDA		this study
plasmids used for expression of membrane anchors in absence of a passenger		
pTF16_lpp-ompA		this study
pTF16_pgsA		this study
pTF16_inp$_{NC}$		this study
pTF16_ctxB-AIDA		this study

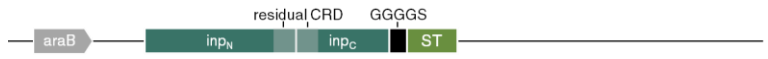
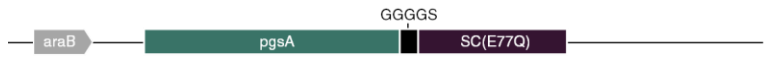
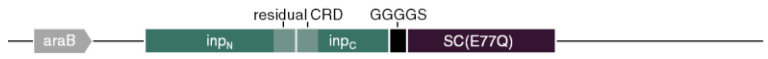
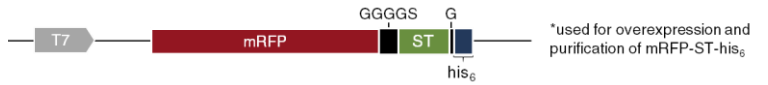
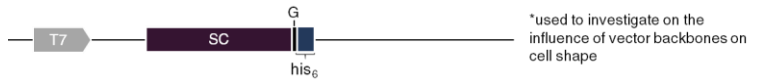
plasmids used for ST or SC_{EQ} controls		
pTF16_lpp-ompA-ST		Peschke et al.[1]
pTF16_pgsA-ST		this study
pTF16_inp_{NC}-ST		this study
pTF16_ctxB-ST-AIDA		this study
pTF16_lpp-ompA-SC_{EQ}		this study
pTF16_pgsA-SC_{EQ}		this study
pTF16_inp_{NC}-SC_{EQ}		this study
pTF16_ctxB-SC_{EQ}-AIDA		this study
other plasmids		
pET_mRFP-ST-his_6		Gallus et al.[2]
pET-EXPn1_his_6-SC		Peschke et al.[1]
pET_SC-his_6		this study

Table S2: Amino acid sequences of functional proteins parts used in this study.

Functional protein	Amino acid sequence
AIDA-I (linker region: **black bold**; β-barrel: *black italic*)	**LNPTKESAGNTLTVSNYTGTPGSVISLGGVLEGDNSLTDRLVVKGNTSGQSDIVYVNE DGSGGQTRDGINIISVEGNSDAEFSLKNRVVAGAYDYTLQKGNESGTDNKGWYLTS HLPTSDTRQYRPENGSYATNMALANSLFLMDLNERKQFRAMSDNTQPES** *ASVWMK ITGGISSGKLNDGQNKTTTNQFINQLGGDIYKFHAEQLGDFTLGIMGGYANAKGKTINY TSNKAARNTLDGYSVGVYGTWYQNGENATGLFAETWMQYNWFNASVKGDGLEEEK YNLNGLTASAGGGYNLNVHTWTSPEGITGEFWLQPHLQAVWMGVTPDTHQEDNGTV VQGAGKNNIQTKAGIRASWKVKSTLDKDTGRRFRPYIEANWIHNTHEFGVKMSDDSQ LLSGSRNQGEIKTGIEGVITQNLSVNGGVAYQAGGHGSNAISGALGIKYSF*
P450 BM3(A74G F87V)	TIKEMPQPKTFGELKNLPLLNTDKPVQALMKIADELGEIFKFEAPGRVTRYLSSQRLIKE ACDESRFDKNLSQGLKFVRDFAGDGLVTSWTHEKNWKKAHNILLPSFSQQAMKGYH AMMVDIAVQLVQKWERLNADEHIEVPEDMTRLTLDTIGLCGFNYRFNSFYRDQPHPFI TSMVRALDEAMNKLQRANPDDPAYDENKRQFQEDIKVMNDLVDKIIADRKASGEQSD DLLTHMLNGKDPETGEPLDDENIRYQIITFLIAGHETTSGLLSFALYFLVKNPHVLQKAA EEAARVLVDPVPSYKQVKQLKYVGMVLNEALRLWPTAPAFSLYAKEDTVLGGEYPLEK GDELMVLIPQLHRDKTIWGDDVEEFRPERFENPSAIPQHAFKPFGNGQRACIGQQFAL HEATLVLGMMLKHFDFEDHTNYELDIKETLTLKPEGFVVKAKSKKIPLGGIPSPSTEQSA KKVRKKAENAHNTPLLVLYGSNMGTAEGTARDLADIAMSKGFAPQVATLDSHAGNLP REGAVLIVTASYNGHPPDNAKQFVDWLDQASADEVKGVRYSVFGCGDKNWATTYQK VPAFIDETLAAKGAENIADRGEADASDDFEGTYEEWREHMWSDVAAYFNLDIENSEDN KSTLSLQFVDSAADMPLAKMHGAFSTNVVASKELQQPGSARSTRHLEIELPKEASYQE GDHLGVIPRNYEGIVNRVTARFGLDASQQIRLEAEEEKLAHLPLAKTVSVEELLQYVEL QDPVTRTQLRAMAAKTVCPPHKVELEALLEKQAYKEQVLAKRLTMLELLEKYPACEMK FSEFIALLPSIRPRYYSISSSPRVDEKQASITVSVVSGEAWSGYGEYKGIASNYLAELQE GDTITCFISTPQSEFTLPKDPETPLIMVGPGTGVAPFRGFVQARKQLKEQGQSLGEAHL

	YFGCRSPHEDYLYQEELENAQSEGIITLHTAFSRMPNQPKTYVQHVMEQDGKKLIELL DQGAHFYICGDGSQMAPAVEATLMKSYADVHQVSEADARLWLQQLEEKGRYAKDVW AG
ctxB SP	MIKLKFGVFFTVLLSSAYAHG
His_6	HHHHHH
INP_{NC} (INP_N: **black bold;** residual CRD: grey; INP_C: *black italic*)	**TLDKALVLRTCANNMADHCGLIWPASGTVESRYWQSTRRHENGLVGLLWGAGTSA FLSVHADARWIVCEVAVADIISLEEPGMVKFPRAEVVHVGDRISASHFISARQADPAS TSTSTSTSTLTPMPTAIPTPMPAVASVTLPVAEQARHEVFDVASVSAAAAPVNTLPVT TPQNLQT** ATYGSTLSGDNHSRLIAGYGSNETAGNHSDLIGGHDCTLMAGDQSRLTAG KNSVLTAGARSKLIGSEGSTLSAGEDSTLI *FRLWDGKRYRQLVARTGENGVEADIPYY VNEDDDIVDKPDEDDDWIEVK*
Lpp-OmpA (Lpp: **black bold**; OmpA: *black italic*)	**KATKLVLGAVILGSTLLAGCSSNAKIDQG** *INPYVGFEMGYDWLGRMPYKGSVENGA YKAQGVQLTAKLGYPITDDLDIYTRLGGMVWRADTKSNVYGKNHDTGVSPVFAGGVE YAITPEIATRLEYQWTNNIGDAHTIGTRPDNGV*
mRFP	ASSEDVIKEFMRFKVRMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKVTKGGPLPFAW DILSPQFQYGSKAYVKHPADIPDYLKLSFPEGFKWERVMNFEDGGVVTVTQDSSLQD GEFIYKVKLRGTNFPSDGPVMQKKTMGWEASTERMYPEDGALKGEIKMRLKLKDGGH YDAEVKTTYMAKKPVQLPGAYKTDIKLDITSHNEDYTIVEQYERAEGRHSTGA
Myc	EQKLISEEDL
PgsA	KKELSFHEKLLKLTKQQKKKTNKHVFIAIPIVFVLMFAFMWAGKAETPKVKTYSDDVLSA SFVGDIMMGRYVEKVTEQKGADSIFQYVEPIFRASDYVAGNFENPVTYQKNYKQADKE IHLQTNKESVKVLKDMNFTVLNSANNHAMDYGVQGMKDTLGEFAKQNLDIVGAGYSL SDAKKKISYQKVNGVTIATLGFTDVSGKGFAAKKNTPGVLPADPEIFIPMISEAKKHADI VVVQSHWGQEYDNDPNDRQRQLARAMSDAGADIIVGHHPHVLEPIEVYNGTVIFYSL GNFVFDQGWTRTRDSALVQYHLKKNGTGRFEVTPIDIHEATPAPVKKDSLKQKTIIREL TKDSNFAWKVEDGKLTFDIDHSDKLKSK
SpyTag	AHIVMVDAYKPTK
SpyCatcher	VDTLSGLSSEQGQSGDMTIEEDSATHIKFSKRDEDGKELAGATMELRDSSGKTISTWI SDGQVKDFYLYPGKYTFVETAAPDGYEVATAITFTVNEQGQVTVNGKATKGDAHI
SpyCatcher(E77Q)	VDTLSGLSSEQGQSGDMTIEEDSATHIKFSKRDEDGKELAGATMELRDSSGKTISTWI SDGQVKDFYLYPGKYTFVQTAAPDGYEVATAITFTVNEQGQVTVNGKATKGDAHI

Unless otherwise stated, plasmids constructed in this study were generated using the isothermal recombination as described by Gibson et al.[3] PCR-products or synthetic DNA-fragments were inserted into linearized vector backbones via 19-30 base pair homologous sequence overlaps. The sequences of all primers utilized for the PCR amplifications are listed in Table S3. The assembly reaction was performed at 50 °C for 1 h under continuous shaking. Subsequently, the reaction mixtures were treated with DpnI to remove any remaining vector from prior PCR reactions and transformed into chemical competent *E. coli* DH5α cells and selected on LB-agar plates containing 100 µg/ml ampicillin or 30 µg/ml chloramphenicol at 37 °C for at least 12 h. All plasmids were purified using ZR Plasmid Miniprep-Classic (Zymo Research) according to the manufacturer's instructions. Plasmid Sequences were verified by commercial sequencing (LGC genomics).

pTF16_pgsA-SC. A pTF16 backbone employing a C-terminal SC was amplified using primers SG42 and SG43 with pTF16_lpp-ompA-SC as the template. This backbone was then recombined with an insert encoding for PgsA, which was ordered as a codon-optimized gene fragment (GeneArt Gene Synthesis, Thermo Fisher Scientific).

pTF16_inp_{NC}-SC. A pTF16 backbone employing a C-terminal SC was amplified using primers SG42 and SG43 with pTF16_lpp-ompA-SC as the template. This backbone was then recombined with an insert encoding for INP_{NC}, which was ordered as a codon-optimized gene fragment (GeneArt Gene Synthesis, Thermo Fisher Scientific).

pTF16_ctxB-SC-AIDA. An empty pTF16 backbone was amplified using primers SG43 and SG44 with pTF16_lpp-ompA-SC as the template. This backbone was then recombined with an insert encoding for a fusion protein consisting of a ctxB signal peptide, SC and AIDA-I, which was ordered as a codon-optimized gene fragment (GeneArt Gene Synthesis, Thermo Fisher Scientific).

pTF16_pgsA-BM3-myc. Template pTF16_pgsA-SC was amplified using primers SG61 and SG98, generating a pTF16 backbone employing a N-terminal PgsA and a C-terminal partial Myc tag that was inserted with the SG61 primer. This backbone was then recombined with an insert encoding for P450 BM3(A74G F87V) fused to the missing part of the C-terminal Myc tag, which had been generated by amplification of the template pET_BM3-ST-his$_6$[2] using the primers SG99 and SG100 with SG100 inserting the partial Myc tag sequence.

pTF16_inp$_{NC}$-BM3-myc. Template pTF16_inp$_{NC}$-SC was amplified using primers SG61 and SG170, generating a pTF16 backbone employing a N-terminal INP$_{NC}$ and a C-terminal partial Myc tag that was inserted with the SG61 primer. This backbone was then recombined with an insert encoding for P450 BM3(A74G F87V) fused to the missing part of the C-terminal Myc tag, which had been generated by amplification of the template pET_BM3-ST-his$_6$[2] using the primers SG100 and SG171 with SG100 inserting the partial Myc tag sequence.

pTF16_ctxB-myc-BM3-AIDA. Template pTF16_ctxB-SC-AIDA was amplified using primers SG65 and SG83, generating a pTF16 backbone employing a C-terminal AIDA-I and a N-terminal ctxB signal peptide fused to a partial Myc tag that was inserted with the SG65 primer. This backbone was then recombined with an insert encoding for P450 BM3(A74G F87V) fused to the missing part of the N-terminal Myc tag, which had been generated by amplification of the template pET_BM3-ST-his$_6$[2] using the primers SG81 and SG82 with SG81 inserting the partial Myc tag sequence.

pTF16_lpp-ompA. Template pTF16_lpp-ompA-SC was amplified using primers KSR227-for and SG175 to delete the SC sequence.

pTF16_pgsA. Template pTF16_pgsA-SC was amplified using primers KSR227-for and SG174 to delete the SC sequence.

pTF16_inp$_{NC}$. Template pTF16_inp$_{NC}$-SC was amplified using primers KSR227-for and SG176 to delete the SC sequence.

pTF16_ctxB-AIDA. Template pTF16_ctxB-SC-AIDA was amplified using primers SG177 and SG178 to delete the SC sequence.

pTF16_pgsA-ST. Template pTF16_pgsA-SC was amplified using primers SG51 and SG52, which each contained partial ST sequences with homologous overlaps, to delete the SC sequence and replace it by the ST sequence.

pTF16_inp$_{NC}$-ST. Template pTF16_inp$_{NC}$-SC was amplified using primers SG51 and SG53, which each contained partial ST sequences with homologous overlaps, to delete the SC sequence and replace it by the ST sequence.

pTF16_ctxB-ST-AIDA. Template pTF16_ctxB-SC-AIDA was amplified using primers SG54 and SG55, which each contained partial ST sequences with homologous overlaps, to delete the SC sequence and replace it by the ST sequence.

pTF16_lpp-ompA-SC$_{EQ}$ / pTF16_pgsA-SC$_{EQ}$ / pTF16_inp$_{NC}$-SC$_{EQ}$ / pTF16_ctxB-SC$_{EQ}$-AIDA. Templates pTF16_lpp-ompA-SC, pTF16_pgsA-SC, pTF16_inp$_{NC}$-SC or pTF16_ctxB-SC-AIDA were amplified using primers SG172 and SG173 to introduce SC mutation E77Q by site-directed mutagenesis.

pET_SC-his$_6$. Template pET_PAD-SC-his$_6$[4] was amplified using primers SEB01 and KSR65-rev to delete the PAD sequence.

Table S3: Sequences of primers used in this study.

Primer	Sequence (5´→ 3´)
KSR65-rev	ATGTATATCTCCTTCTTAAAGTTAAAC
KSR227-for	TGAGATCCGGCTGCTAACAAAGCCC
SEB01	AAGAAGGAGATATACATATGGTTGATACCCTGAGCGGTCTGAGC
SG42	GGTGGTGGTGGTAGCGTTGATAC
SG43	ATGTATATCTCCTTCTTAAAGTTAAACAAAATTC
SG44	TGAGATCCGGCTGCTAACAAAGC
SG51	TTATGGTTGATGCCTATAAACCGACCAAATAATGAGATCCGGCTGCTAACAAAGCCC
SG52	TTATAGGCATCAACCATAACAATATGTGCGCTACCACCACCACCTTTGCTTTTC
SG53	TTATAGGCATCAACCATAACAATATGTGCGCTACCACCACCACCTTTCACCTC
SG54	TTATGGTTGATGCCTATAAACCGACCAAAGGTGGTGGTGGTAGCCTGAATCC
SG55	TTATAGGCATCAACCATAACAATATGTGCACCATGTGCATATGCGCTGCTCAG
SG61	CAGAAACTGATCAGCGAAGAAGATCTGTAATGAGATCCGGCTGCTAACAAAGC
SG65	CAGATCTTCTTCGCTGATCAGTTTCTGTTCACCATGTGCATATGCGCTGCTC
SG66	GTTGCAGAAAATCAAGGTCATCATCATCACCATCACTAAG
SG81	CAGAAACTGATCAGCGAAGAAGATCTGGGCACAATTAAAGAAATGCCTCAGCCAAAAAC
SG82	TTTGGTCGGATTCAGGCTACCACCACCACCCCCAGCCCACACGTCTTTTGC
SG83	GGTGGTGGTGGTAGCCTGAATC
SG98	GCTACCACCACCACCTTTGCTTTTC
SG99	AAACTGAAAAGCAAAGGTGGTGGTGGTAGCACAATTAAAGAAATGCCTCAGCCAAAAAC
SG100	CTTCGCTGATCAGTTTCTGTTCACCCCCAGCCCACACGTCTTTTGC
SG170	GCTACCACCACCACCTTTCACC
SG171	TGGATCGAGGTGAAAGGTGGTGGTGGTAGCACAATTAAAGAAATGCCTCAGCCAAAAAC
SG172	GCAAATACACCTTTGTTCAGACCGCAGC
SG173	CAAAGGTGTATTTGCCAGGGTACAG
SG174	CAGCCGGATCTCATTTGCTTTTCAGTTTATCGCTATGATCGATATCAAAG
SG175	CAGCCGGATCTCACACACCATTATCCGGACGGGTGC
SG176	CAGCCGGATCTCATTTCACCTCGATCCAATCATCATCTTCATCC
SG177	GCATATGCACATGGTCTGAATCCGACCAAAGAAAGCGCAG
SG178	ACCATGTGCATATGCGCTGCTCAGC

Supplementary Figures

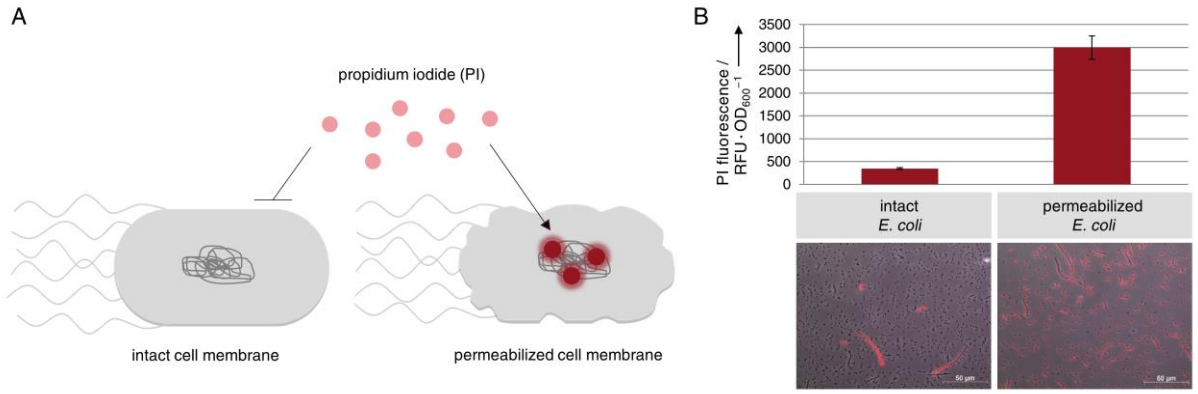


Figure S1. Investigation of membrane permeability by propidium iodide (PI) staining. **(A)** PI is a fluorescent dye not able to penetrate into the cell membrane of intact *E. coli* cells. However, if the cell membrane is permeabilized, the dye can penetrate the cell and intercalate into the DNA, shifting its excitation and emission wavelengths and increasing the fluorescence intensity by 20 to 30 times. The fluorescence of the DNA bound PI can be quantified with a microplate reader (λ_{Ex}=535 nm, λ_{Em}=617 nm) or visualized using a rhodamine filter set on a fluorescence microscope. **(B)** Exemplary PI fluorescence quantification and corresponding microscopy images of intact and permeabilized *E. coli* cells. *E. coli* BL21 were cultured for 4 h at 37 °C and stained with PI directly (intact *E. coli*) of after permeabilization by incubation with 2% toluene for 30 min at room temperature (permeabilized *E. coli*). Error bars were obtained from at least two independent experiments.

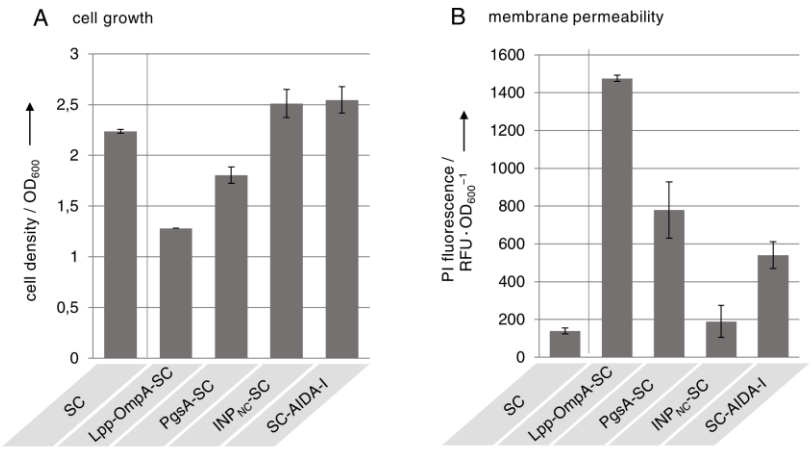


Figure S2. Growth and membrane permeability of *E. coli* cells expressing SC intracellularly or fused to the membrane anchors Lpp-OmpA, PgsA, INP_{NC} or AIDA after 4h of induction. **(A)** Density of cell cultures determined by measuring the OD_{600}. **(B)** Permeability of the cell membrane determined by incubation with the membrane impermeable DNA intercalating dye propidium iodide (PI). A high PI fluorescence signal is related to increased membrane permability. Details about the staining procedure are given in Figure S1. Error bars were obtained from at least two independent experiments.

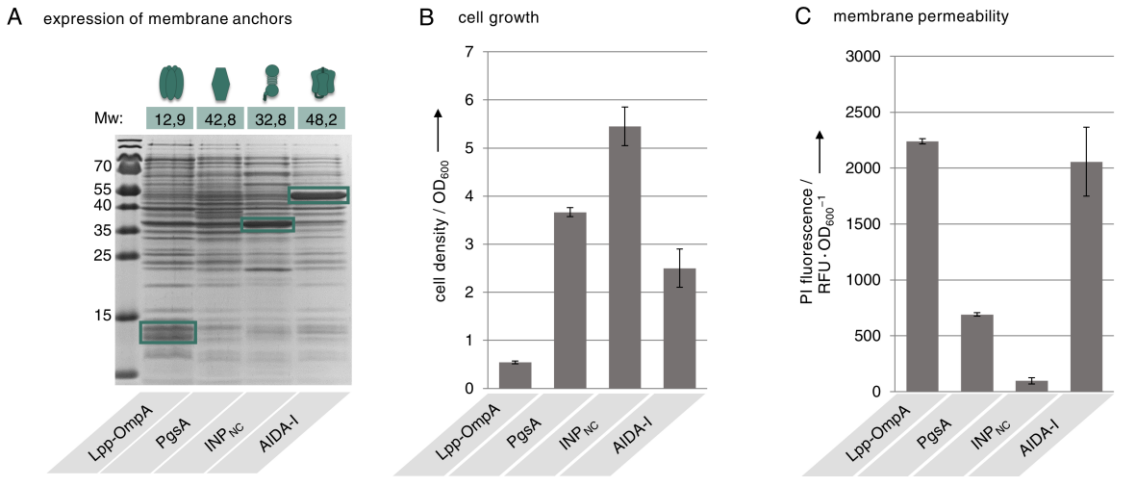


Figure S3. Protein expression, growth and membrane permeability of *E. coli* cells expressing the different membrane anchors in absence of any passenger domain. Cells were analyzed 20 h after of induction of protein expression. **(A)** Total protein fractions separated by Coomassie stained SDS-PAGE (15% (w/v) acrylamide). The expected molecular weight (Mw) of each protein as well as the molecular weight of marker bands is given in kDa. The bands of the detected proteins are outlined in green. Similar to the SC modified membrane anchors (see Figure 2), expression of PgsA was lowest among all tested membrane anchors and appeared to be too low to detect a clear protein band. **(B)** Density of cell cultures determined by measuring the OD_{600}. **(C)** Permeability of the cell membrane determined by incubation with the membrane impermeable DNA intercalating dye propidium iodide (PI). A high PI fluorescence signal is related to increased membrane permability. Details about the staining procedure are given in Figure S1. Error bars were obtained from at least two independent experiments.

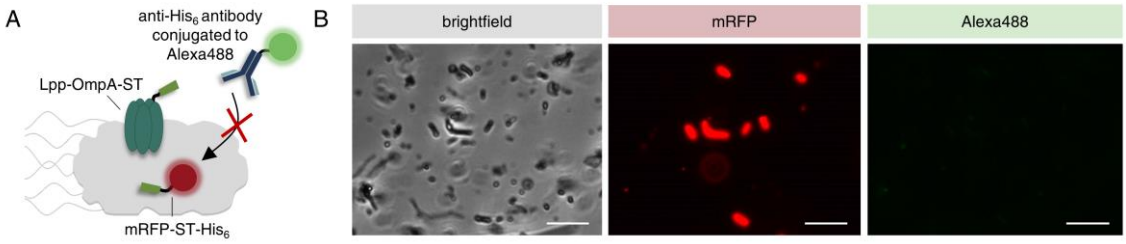


Figure S4. Verification of the membrane-impermeability of the anti-His_6 antibody conjugated to Alexa488. **(A)** *E. coli* cells were transformed with plasmids pTF16_lpp-ompA-ST and pET_mRFP-ST-his_6 and were incubated for 20 h after induction to express membrane integrated Lpp-OmpA-ST and cytosolic mRFP-ST-his_6. The cells were incubated with the anti-his_6 antibody conjugated to Alexa488 for 1 h at room temperature, washed three times with PBS buffer and subsequently analyzed using fluorescence microscopy. **(B)** A clear mRFP fluorescence was detected indicating that the cells express a high amount of cytosolic mRFP-ST-His_6. However, the cells did not exhibit any Alexa488 fluorescence, demonstrating that the antibody cannot bind to the cytosolic mRFP-ST-his_6, even though expression of the Lpp-OmpA membrane anchor was shown to cause a high degree of membrane permeabilization (see Figure 2C). Scale bars correspond to 10 µm.

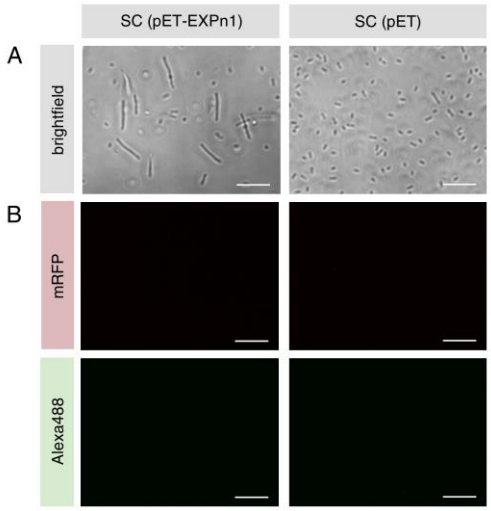


Figure S5. Comparison of *E. coli* cells expressing intracellular SC encoded on a pET-EXPn1 or a pET vector. In this study the intracellular SC was expressed using our previously described plasmid pET-EXPn1_his$_6$-SC[1], based on pET-DEST42 (Thermo Fisher Scientific), to ensure comparability with previous studies. In order to investigate on the impact of an alternative plasmid backbone, the pET22b(+) (Novagen) based vector pET_SC-his$_6$ was constructed and analyzed. **(A)** Microscopic images of cells expressing intracellular SC encoded on the pET-EXPn1 plasmid (left side, elongated cell shape) or on the pET plasmid (right side, round cell shape) indicate that the plasmid backbone has an influence on cell shape. **(B)** However, as expected, incubation of the cells with mRFP-ST-his$_6$ and subsequent staining with the membrane-impermeable Alexa488 conjugated anti-his$_6$ antibody, as conducted in Figure 3 for detection of functional and surface presented SC domains, resulted in no detectable mRFP or Alexa488 fluorescence for both plasmids. This demonstrates that the plasmids are equally suited to produce control cells with intracellular expression of SC as shown in Figure 3. Scale bars correspond to 10 µm.

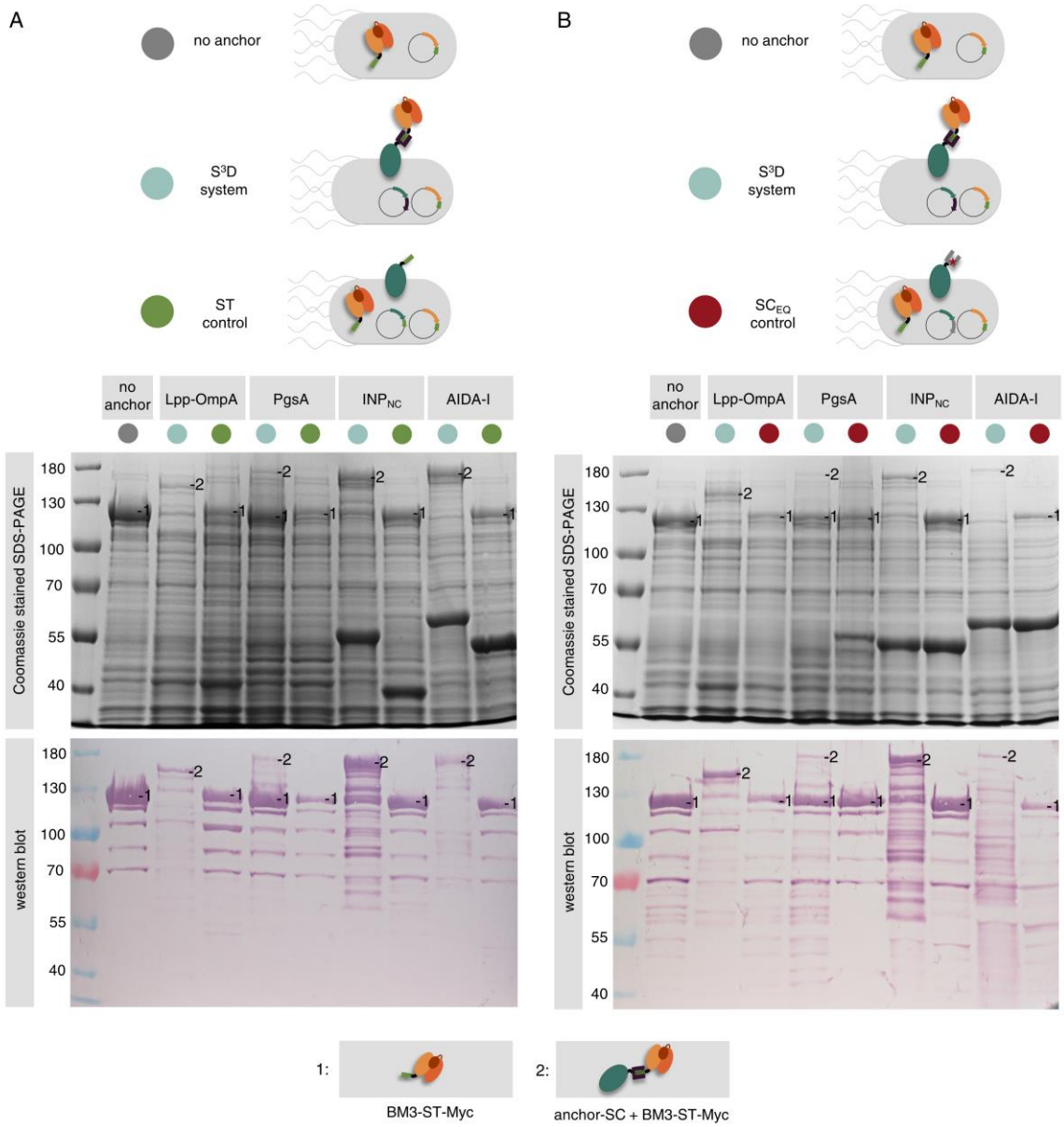


Figure S6. Western blot analysis to verify the covalent coupling of BM3-ST to different SC modified membrane anchors. Cells expressing BM3-ST intracellularly (no anchor, grey dots) were compared to cells expressing BM3-ST together with the different SC modified membrane anchors (S_3D system, light blue dots). As controls, we analyzed cells expressing BM3-ST together with each membrane anchor fused to either ST (**A**, ST control, green dots) or to an inactivated SC_{EQ}, in which formation of the covalent isopeptide bond is prevented by the mutation E77Q (**B**, SC_{EQ} control, red dots). All cells were analyzed 20 h after induction. Total protein fractions were separated by SDS-PAGE (8% (w/v) acrylamide) and stained with Coomassie or transferred to a PVDF membrane for western blot detection of a terminal Myc Tag on BM3-ST using primary mouse anti-Myc antibodies and secondary anti-mouse antibodies conjugated to alkaline phosphatase. For all control cells (grey, green or red dots) only one protein band (1) corresponding to the full-length BM3-ST was detected. In contrast, a higher molecular weight band (2) was detected for all cells employing the S^3D system (light blue dots), thereby confirming covalent conjugation of BM3-ST to all SC modified membrane anchors Lpp-OmpA, PgsA, INP_{NC} and AIDA-I. Note that several lower molecular weight bands were detected for all samples presumably caused by degradation of the BM3-ST passenger and/or its corresponding conjugates. Expected molecular weight of proteins bands: BM3-ST (1): 120 kDa; anchor-SC + BM3-ST (2): 148 kDa (for Lpp-OmpA anchor), 175 kDa (for PgsA anchor), 165 kDa (for INP_{NC} anchor) or 180 kDa (for AIDA-I anchor)**.** Molecular weight of marker bands is given in kDa.

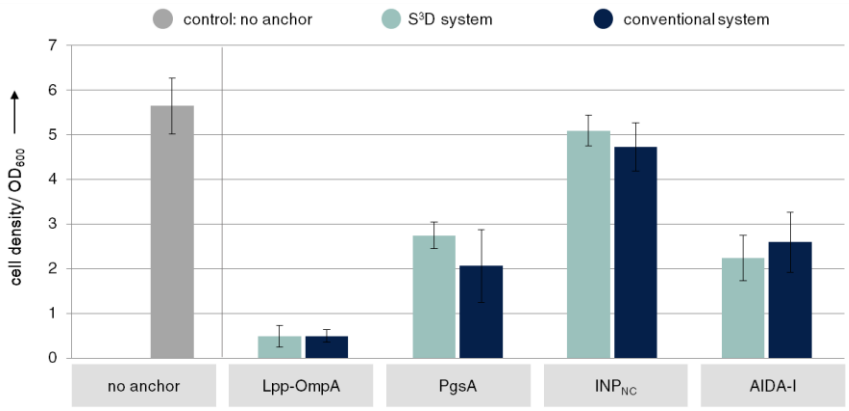


Figure S7. Growth of *E. coli* cells expressing BM3-ST intracellularly (no anchor control, grey) compared to cells displaying BM3 with the S^{3}D system (light blue) or with the conventional system (dark blue) employing different membrane anchors. Error bars were obtained from at least two independent experiments.

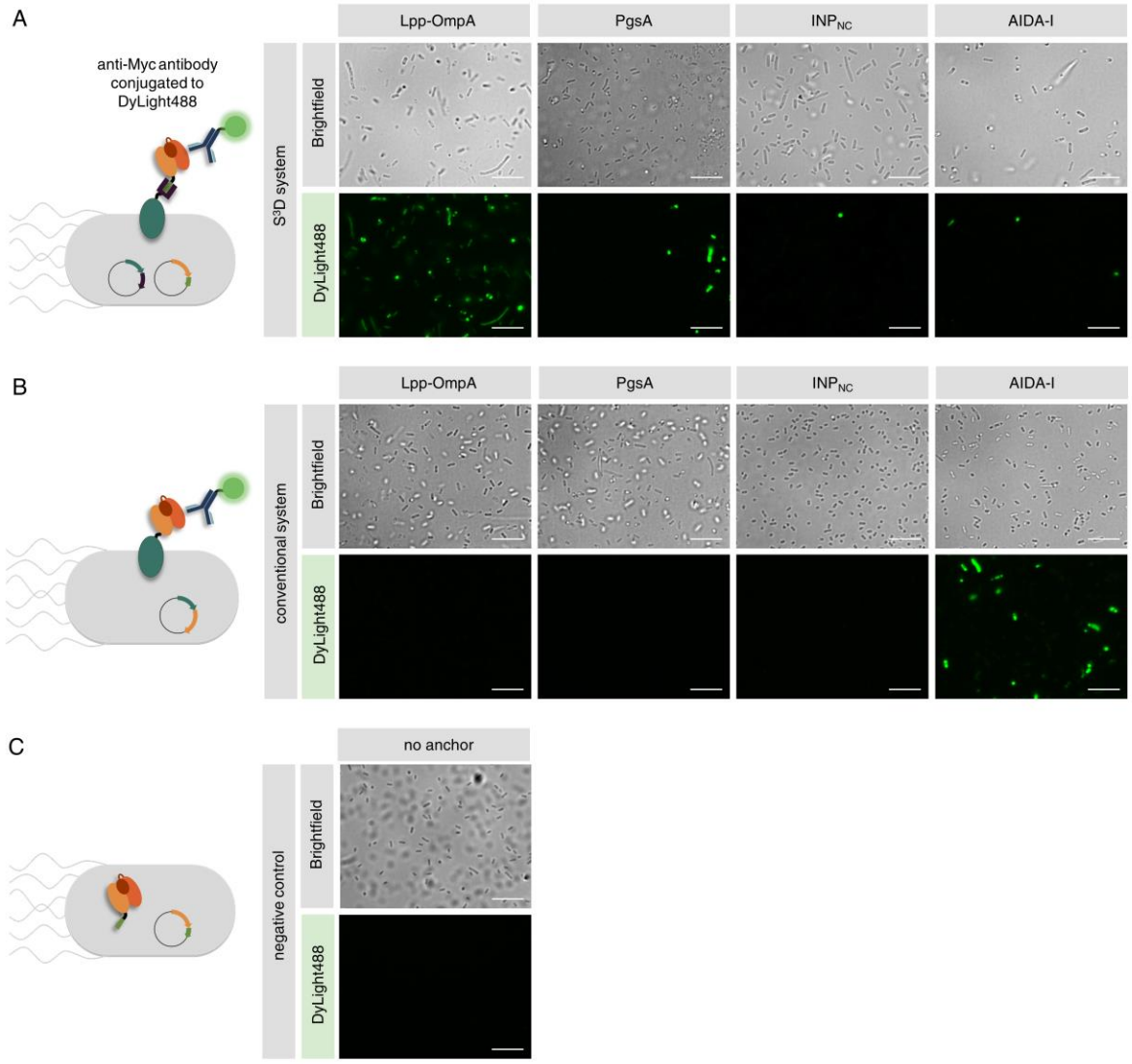


Figure S8. Microscopy images of immunofluorescence staining used to investigate on the BM3 surface exposure employing different membrane anchors for the S^3D system **(A)** or for the conventional system **(B)**. As a negative control cells expressing intracellular BM3-ST were analyzed **(C)**. BM3 was modified with a terminal Myc tag and detected using an anti-Myc antibody conjugated to DyLight488. Scale bars correspond to 10 µm.

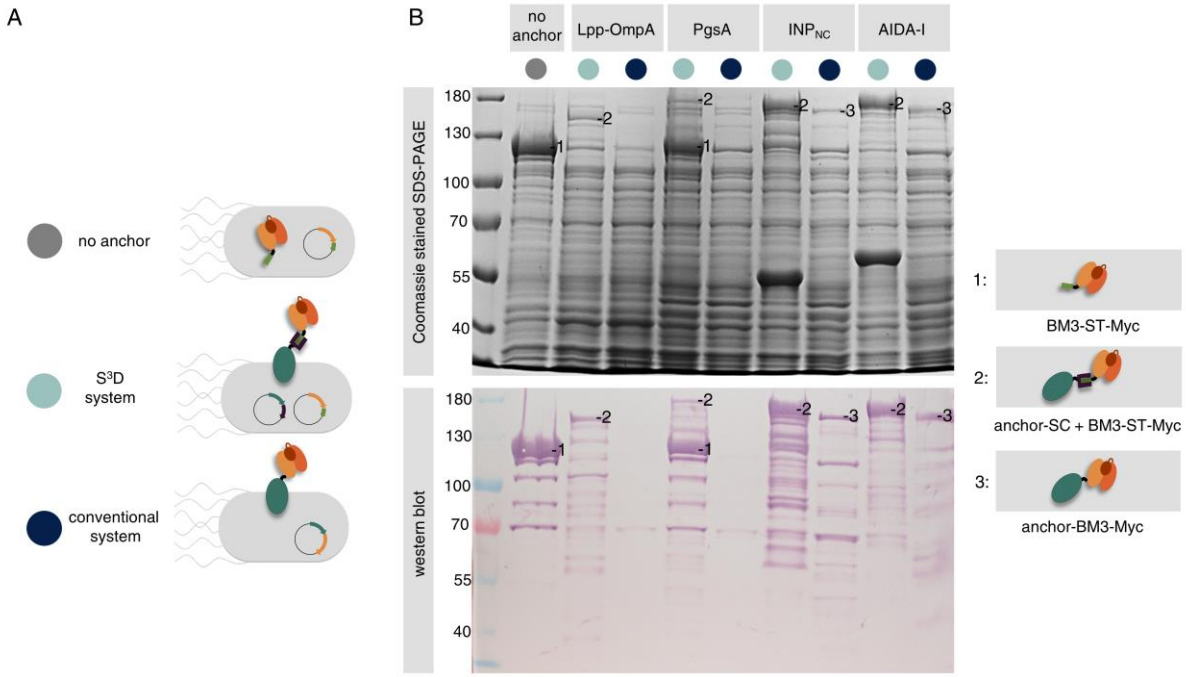


Figure S9. Comparison of BM3 expression levels by western blot. **(A)** Control cells expressing BM3-ST intracellularly (grey) were compared to cells displaying BM3 with the S^3D system (light blue) or with the conventional system (dark blue) employing different membrane anchors. All cells were analyzed 20 h after induction. **(B)** Total protein fractions were separated by SDS-PAGE (8% (w/v) acrylamide) and stained with Coomassie or transferred to a PVDF membrane for western blot detection of a terminal Myc Tag on BM3 fusion proteins using primary mouse anti-Myc antibodies and secondary anti-mouse antibodies conjugated to alkaline phosphatase. Note that using the conventional system no expression was observed for the fusion proteins Lpp-OmpA-BM3 or PgsA-BM3. Expected molecular weights of proteins bands: BM3-ST (1): 120 kDa; anchor-SC + BM3-ST (2): 148 kDa (for Lpp-OmpA), 175 kDa (for PgsA), 165 kDa (for INP_{NC}) or 180 kDa (for AIDA-I); anchor-BM3 (3): 134 kDa (for Lpp-OmpA), 162 kDa (for PgsA), 152 kDa (for INP_{NC}) or 167 kDa (for AIDA-I). Several lower molecular weight bands were detected presumably caused by degradation of the BM3 fusion proteins. Molecular weight of marker bands is given in kDa.

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