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Optimization of recombinant membrane protein production in the engineered Escherichia coli strains SuptoxD and SuptoxR

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Abstract

Membrane proteins execute a wide variety of critical biological functions in all living organisms and constitute approximately half of current targets for drug discovery. As in the case of soluble proteins, the bacterium Escherichia coli has served as a very popular overexpression host for biochemical/structural studies of membrane proteins as well. Bacterial recombinant membrane proteins production, however, is typically hampered by poor cellular accumulation and severe toxicity for the host, which leads to low levels of final biomass and minute volumetric yields. In previous work, we generated the engineered E. coli strains SuptoxD and SuptoxR, which upon co-expression of the effector genes djlA or rraA, respectively, can suppress the cytotoxicity caused by membrane protein overexpression and produce enhanced membrane protein yields. Here, we systematically looked for gene overexpression and culturing conditions that maximize the accumulation of membrane-integrated and well-folded recombinant MPs in these strains. We have found that, under optimal conditions, SuptoxD and SuptoxR achieve greatly enhanced recombinant membrane protein production for a variety of membrane proteins, irrespective of their archaeal, eubacterial or eukaryotic origin. Furthermore, we demonstrate that the use of these engineered strains enables the production of well-folded recombinant MPs of high quality and at high yields, which are suitable for functional and structural studies. We anticipate that SuptoxD and SuptoxR will become broadly utilized expression hosts for recombinant membrane protein production in bacteria.

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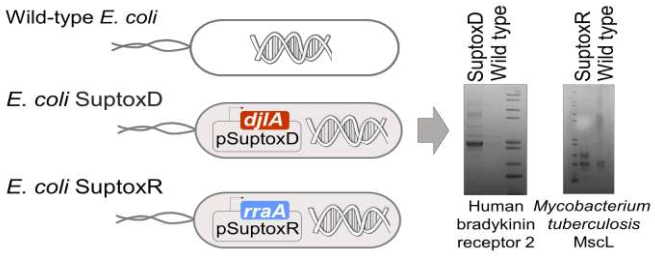


Keywords

Recombinant membrane protein production; toxicity; E.coli SuptoxD; E. coli SuptoxR; DjlA; RraA

Membrane proteins (MPs) play key functional roles in all living organisms[1] and hold a prominent position among current targets for drug discovery[2]. As they are typically encountered in their native cells and tissues only at very small quantities, acquiring sufficient amounts of isolated MPs for biochemical and structural studies relies on their recombinant production in heterologous hosts, such as bacteria, yeasts, insect cells, mammalian cells and transgenic animals[3].

As in the case of soluble proteins, the bacterium Escherichia coli has been one of the most popular overexpression hosts for production of recombinant MPs[4, 5]. Among its many advantages, E. coli offer great simplicity, speed and low cost; relatively good understanding of the cell host's physiology; numerous tools for genetic manipulation[5]; high transformation efficiency coupled with established technologies for engineering the properties of the target MP[6]; and ability to be propagated in chemically defined medium allowing for substitution with selenomethionine for X-ray crystal structure determination[7] or with isotopically labeled amino acids for nuclear magnetic resonance structural studies[8].

Bacterial MP production, however, is very often problematic, hampered by poor cellular accumulation and severe toxicity for the expression host[4]. MP-induced toxicity is a very frequent problem associated with recombinant MP production and it often leads to complete growth arrest, low levels of final biomass, and minute volumetric protein yields[9-11]. These unwanted phenomena occur most frequently for proteins of eukaryotic origin, which are also the ones of highest interest as targets for drug discovery.

In our previous work, we showed that we can engineer the bacterial protein synthesis machinery so as to generate modified E. coli strains with the ability to

withstand the toxicity caused by MP overexpression[12]. This allowed the development of specialized E. coli strains, which can be generally utilized for high-level recombinant MP production[12]. In order to achieve this, we sought single bacterial genes, whose co-expression can suppress MP overexpression-induced toxicity. After carrying out a genome-wide screen, we identified two highly potent suppressors: (i) djlA, the gene encoding for the membrane-bound DnaK co-chaperone DjlA[13], and (ii) rraA, the gene encoding for RraA, an inhibitor of the mRNA-degrading activity of the E. coli RNase E[14]. E. coli strains co-expressing either djlA or rraA, which were named SuptoxD and SuptoxR, respectively, were found capable of accumulating significantly higher levels of final biomass and of producing dramatically enhanced yields for a variety of recombinant MPs in properly membrane-embedded form[12, 15].

In the present work, we systematically looked for gene overexpression and culturing conditions that maximize the accumulation of membrane-integrated and well-folded recombinant MPs in E. coli SuptoxD and SuptoxR. We report that, under optimal conditions, SuptoxD and SuptoxR achieve greatly enhanced recombinant membrane protein production compared to wild-type E. coli, for a variety of both prokaryotic and eukaryotic MPs. Furthermore, we demonstrate that the use of these engineered strains enables the production of well-folded recombinant MPs at high quality and yields, which are suitable for functional and structural studies. Based on these results, we anticipate that SuptoxD and SuptoxR will become broadly utilized expression hosts for recombinant MP production in bacteria.

Results and Discussion

Characteristics of the E. coli strains SuptoxD and SuptoxR. E. coli SuptoxD and SuptoxR are two specialized strains for achieving high-level recombinant MP production in bacteria[12]. Their use has a dual positive effect on recombinant MP production: (i) it suppresses the toxicity that is frequently associated with MP overexpression, thus resulting in enhanced levels of final bacterial biomass, and (ii) it markedly enhances the cellular accumulation of membrane-incorporated and well-folded protein for a variety of recombinant MPs of both prokaryotic and eukaryotic origin[12, 15]. The combination of these two positive effects of MP production, results in greatly enhanced volumetric accumulation of recombinant MPs compared to wild-type E. coli [12, 15].

The toxicity-suppressing and cellular production-promoting capabilities of SuptoxD and SuptoxR are based on the overexpression of either one of the effector genes djlA or rraA, respectively. In these strains, djlA and rraA are overexpressed from the vectors pSuptoxD and pSuptoxR, respectively, under the control of the araBAD promoter and its inducer L(+)-arabinose (Figure 1; Table 1)[12]. For the production of recombinant MPs in these strains, we typically use the vector pASK75[16] under the control of a tet promoter and its inducer anhydrotetracycline (aTc) (Figure 1; Table 1), although the enhanced MP productivity SuptoxD and SuptoxR is not promoter-dependent[12, 15].

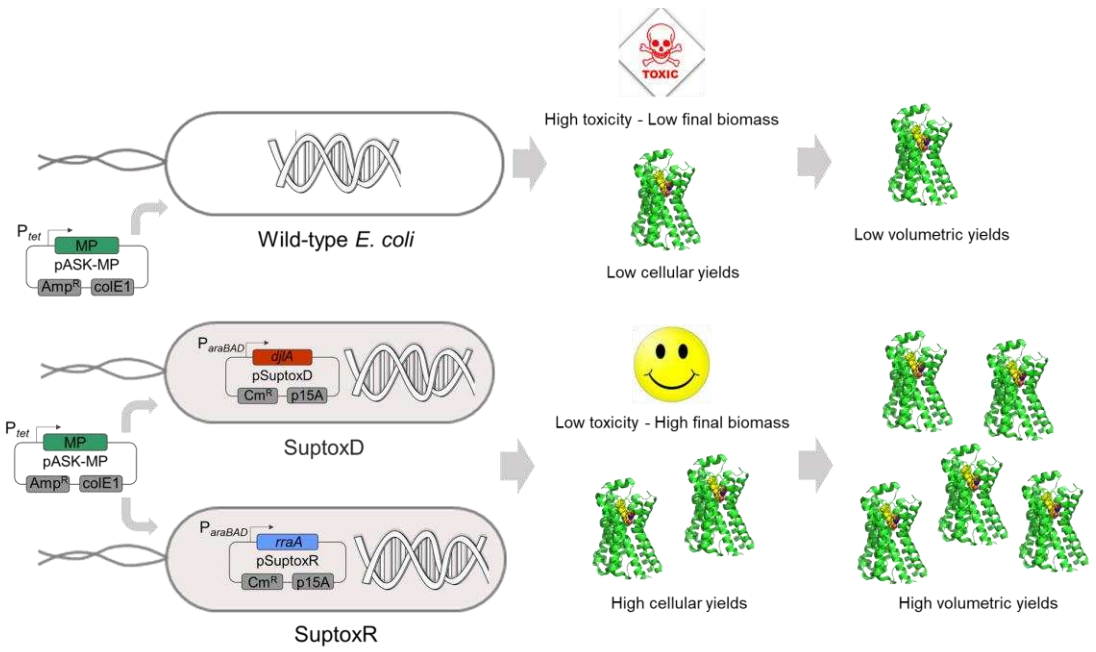


Figure 1. Characteristics of the specialized MP-producing E. coli strains SuptoxD and SuptoxR. The toxicity-suppressing and cellular production-promoting capabilities of SuptoxD and SuptoxR are based on the overexpression of either one of the effector genes djlA or rraA, respectively. djlA and rraA are overexpressed from the vectors pSuptoxD and pSuptoxR, respectively, under the control of the araBAD promoter and its inducer L(+)-arabinose. For the production of recombinant MPs in these strains, we typically use pASK75-based plasmids under the control of a tet promoter and its inducer aTc (pASK-MP vector).

Optimization of expression conditions for recombinant MP production in SuptoxD and SuptoxR. In order to determine optimal conditions for recombinant MP production in SuptoxD and SuptoxR, we tested a range of different expression parameters and looked for combinations that maximize volumetric accumulation of membrane-integrated and folded MP. For fast expression monitoring, we utilized C-terminal MP fusions with the green fluorescent protein (GFP) and recorded the levels of cellular MP-GFP fluorescence corresponding to equal culture volumes (volumetric accumulation) for each condition. This choice was based on the fact that the fluorescence of E. coli cells expressing MP-GFP fusions has previously been found to

correlate well with the amount of membrane-integrated recombinant MP[17] and has been used extensively for facile monitoring of the accumulation levels of membrane-incorporated recombinant MPs, for optimization of overexpression parameters and for strain development, by us[12, 15, 18-20] and many others groups[21-26].

We first evaluated the effect of varying the expression levels of the effector gene djlA in SuptoxD by changing the concentration of the inducer L(+)-arabinose in the growth medium. Arabinose concentrations in the range 0.0025-0.02% yielded maximal increases in volumetric cellular MP-GFP fluorescence compared to wild-type E. coli for two recombinant MPs tested: the human bradykinin receptor 2 (BR2) and the D03 variant of the rat neurotensin receptor 1 (NTR1(D03))[6] (Table 1; Figures. 2a and b). Both MPs are members of the G protein-coupled receptor (GPCR) superfamily and are of high interest as targets for drug discovery[27, 28]. The three-dimensional structure of NTR1 has been solved recently[27], while the structure of BR2 remains undetermined. Arabinose concentrations higher than 0.02% did not result in higher MP productivity, presumably due to the toxicity that is associated with strong djlA overexpression[13, 29]. Western blot and in-gel fluorescence analyses[30] of isolated total membrane fractions of wild type and SuptoxD cells producing NTR1(D03)-GFP verified that the enhanced MP-GFP fluorescence phenotypes observed occur due to increased production of full-length, membrane-embedded and well-folded recombinant MP (Figure 2c). Thus, addition of 0.0025-0.02% arabinose results in high-level production of recombinant MPs in E. coli SuptoxD, with an apparent optimum at 0.01%.

We next performed a similar analysis for the effector gene rraA in SuptoxR using again two model MPs: BR2 and the large conductance mechanosensitive ion channel (MscL) from Mycobacterium tuberculosis (Table 1). In this case, higher arabi-

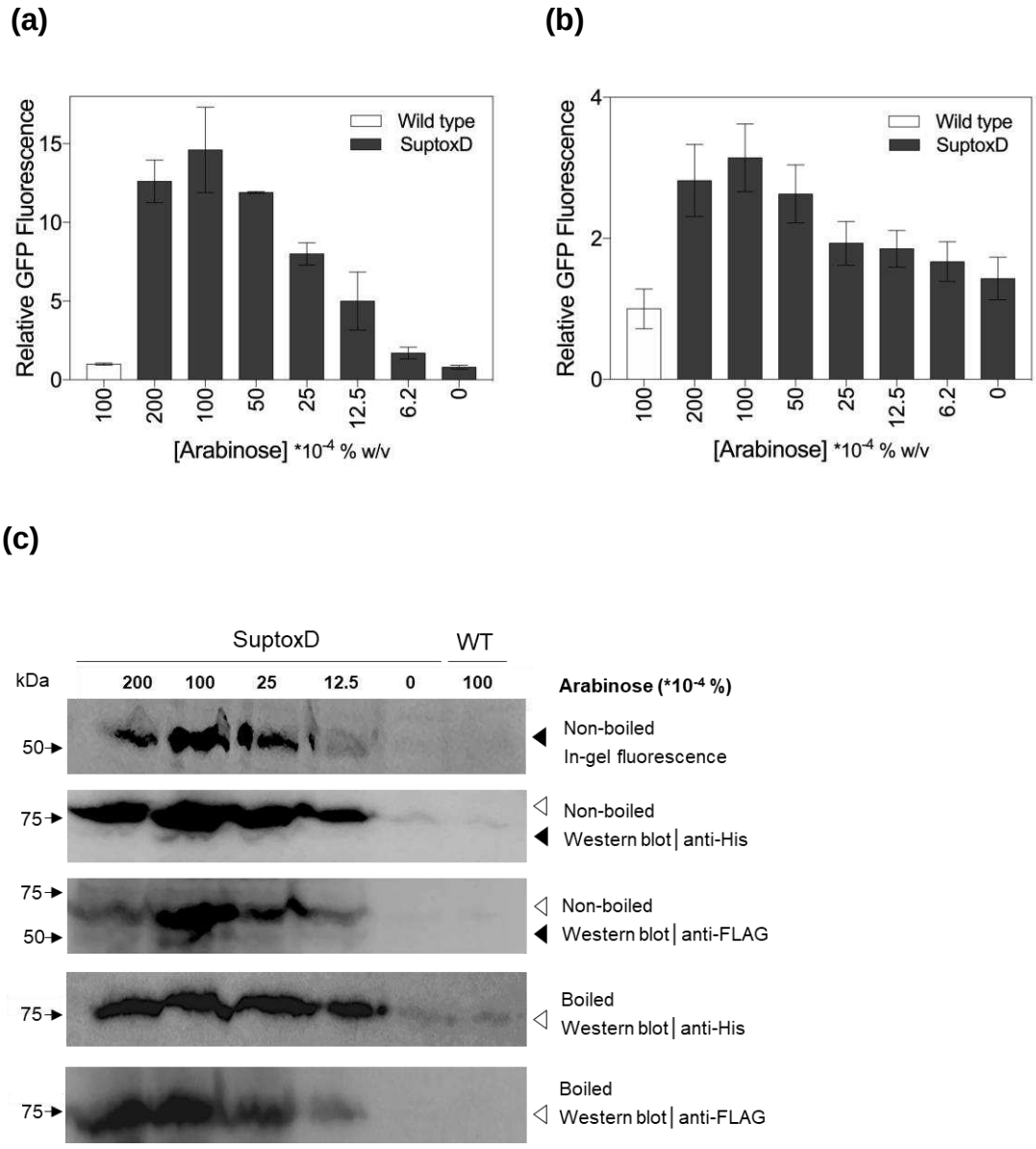


Figure 2. Determination of the optimal levels of djlA co-expression for maximal recombinant MP production in E. coli SuptoxD cells by arabinose titration. **(a).** Fluorescence of equal culture volumes of E. coli MC1061 (Wild type) and SuptoxD cells producing BR2-GFP by the addition of 0.2 µg/ml aTc and the indicated arabinose concentrations for 16 h at 25 °C. The fluorescence of BR2-producing MC1061 cells was arbitrarily set to one. **(b).** For NTR1(D03)-GFP as in (a). **(c).** Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)/western blot analysis of isolated total membrane fractions of equal culture volumes of MC1061 (Wild type, WT) and SuptoxD cells producing NTR1(D03)-GFP as described in (b), visualization of the produced fusion by in-gel fluorescence and western blotting using a C-terminal anti-polyhistidine or a N-terminal anti-FLAG antibody without (non-boiled) or with boiling (boiled) of the samples prior to loading as indicated. The black and white arrows indicate the positions of the fluorescent and non-fluorescent NTR1(D03)-GFP bands, respectively. In (a) and (b), experiments were carried out in replica triplicates and the error bars represent one standard deviation from the mean value.

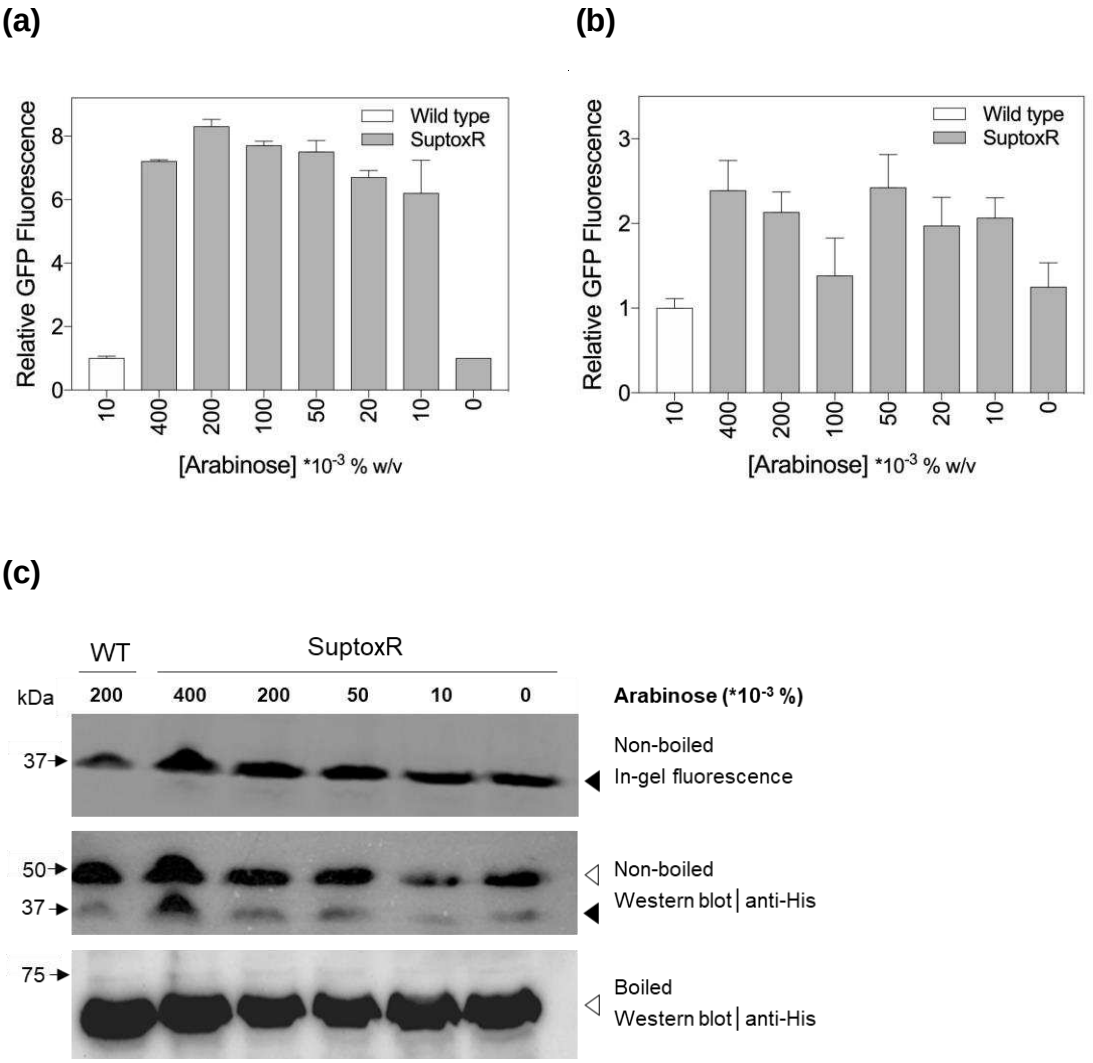


Figure 3. Determination of the optimal levels of rraA co-expression for maximal recombinant MP production in E. coli SuptoxR cells by arabinose titration. (a). Fluorescence of equal culture volumes of E. coli MC1061 (Wild type) and SuptoxR cells producing BR2-GFP by the addition of 0.2 µg/ml aTc and the indicated arabinose concentrations for 16 h at 25 °C. The fluorescence of BR2-producing MC1061 cells was arbitrarily set to one. Experiments were carried out in replica triplicates and the error bars represent one standard deviation from the mean value. **(b).** For MscL-GFP as in (a). Mean values ± s.d. are presented (n=3 independent experiments, each one performed in replica triplicates). **(c).** SDS-PAGE/western blot analysis of isolated total membrane fractions of equal culture volumes of MC1061 (Wild type, WT) and SuptoxR cells producing MscL-GFP as described in (b), visualization of the produced fusion by in-gel fluorescence and western blotting using a C-terminal anti-polyhistidine antibody without (non-boiled) or with (boiled) boiling of the samples prior to loading as indicated. The black and white arrows indicate the positions of the fluorescent and non-fluorescent MscL-GFP bands, respectively.

nose concentrations in the range 0.01-0.4% were required for maximal recombinant MP production, with an apparent optimum at 0.2-0.4% (Figure 3).

Having established optimal cellular levels for DjlA and RraA, we searched for aTc concentrations that maximize overexpression of recombinant MPs in SuptoxD and SuptoxR. For both strains, 50-400 μg aTc per L of shake flask culture resulted in the highest accumulation of membrane-embedded, full-length and well-folded recombinant MP for all tested targets (Figures 4 and 5).

The incubation temperature, at which MP production occurs, had a strong impact on the final volumetric accumulation of recombinant MP for both SuptoxD and SuptoxR (Figure 6a). This is consistent with what has been observed previously with other strains and for other recombinant MPs[22]. Induction of protein production at 25 ^{o}C resulted in maximized productivity of recombinant MP for both SuptoxD and SuptoxR (Figure 6a).

Under optimal production conditions (incubation temperature and inducer concentrations), the volumetric production of recombinant MP increased for both SuptoxD and SuptoxR until 12-14 h after aTc addition to the medium and leveled-off after that (Figure 6b). Importantly, even after 16 h continuous production of MPs, whose strong overexpression is severely toxic for E. coli and typically causes complete growth arrest following induction, such as BR2 and NTR1[12, 20, 31], SuptoxD and SuptoxR cultures were homogeneous as revealed by flow cytometry analysis, thus demonstrating MP genetic stability in these strains (Figure 6c).

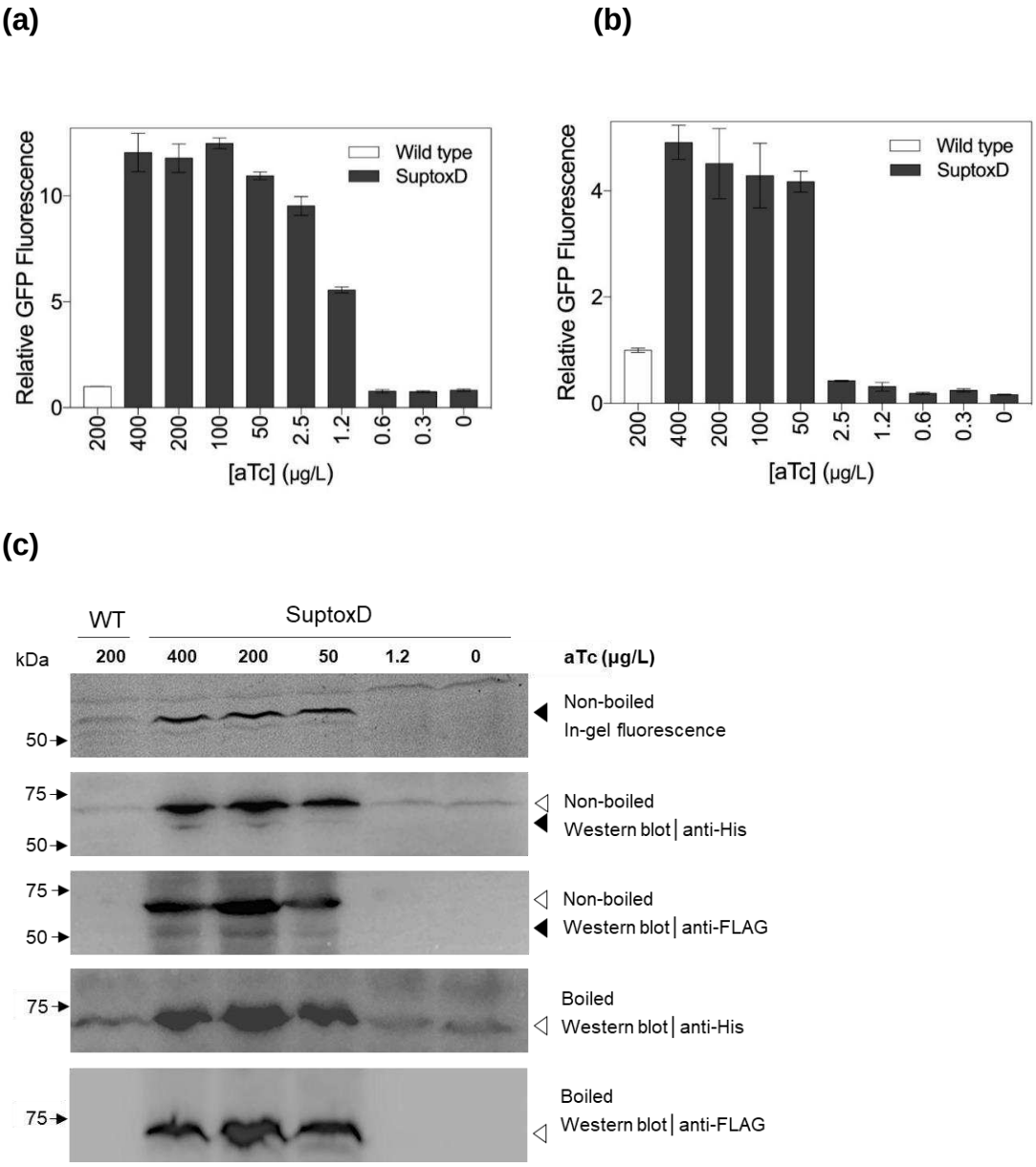


Figure 4. Optimization of recombinant MP production in E. coli SuptoxD cells by aTc titration. (a). Fluorescence of equal culture volumes of E. coli MC1061 (Wild type) and SuptoxD cells producing BR2-GFP by the addition of 0.01% arabinose for 16 h at 25 °C. The fluorescence of BR2-producing MC1061 cells was arbitrarily set to one. **(b).** For NTR1(D03)-GFP as in (a). **(c).** SDS-PAGE/western blot analysis of isolated total membrane fractions of equal culture volumes of MC1061 (Wild type, WT) and SuptoxD cells producing NTR1(D03)-GFP as described in (b), visualization of the produced fusion by in-gel fluorescence and western blotting using a C-terminal anti-polyhistidine or a N-terminal anti-FLAG antibody without (non-boiled) or with boiling (boiled) of the samples prior to loading as indicated. The black and white arrows indicate the positions of the fluorescent and non-fluorescent NTR1(D03)-GFP bands, respectively. In (a) and (b), experiments were carried out in replica triplicates and the error bars represent one standard deviation from the mean value.

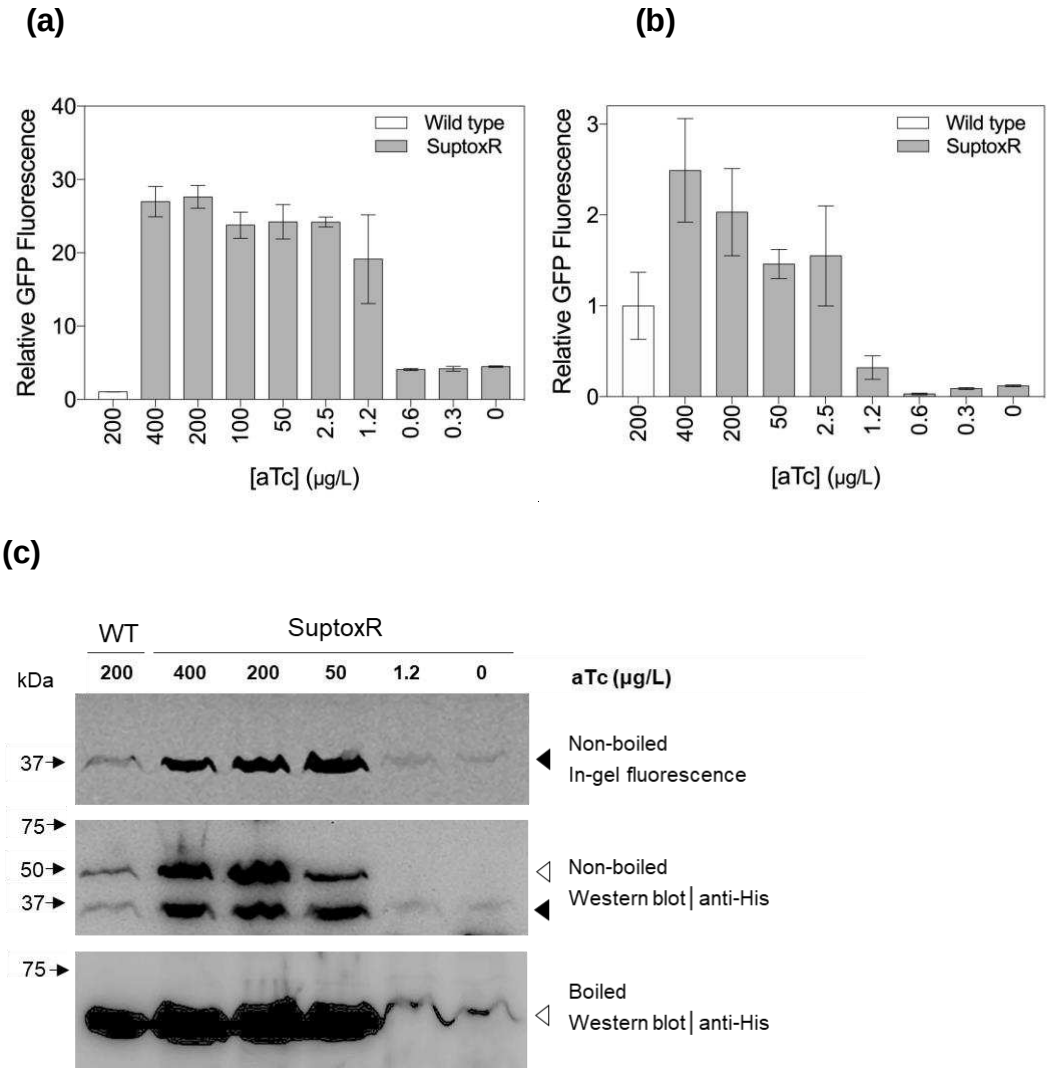


Figure 5. Optimization of recombinant MP production in E.coli SuptoxR cells by aTc titration. (a). Fluorescence of equal culture volumes of E. coli MC1061 (Wild type) and SuptoxR cells producing BR2-GFP by the addition of 0.2% arabinose for 16 h at 25 °C. The fluorescence of BR2-producing MC1061 cells was arbitrarily set to one. **(b).** For MscL-GFP as in (a). **(c).** SDS-PAGE/western blot analysis of isolated total membrane fractions of equal culture volumes of MC1061 (wild type, WT) and SuptoxR cells producing MscL-GFP as described in (b), visualization of the produced fusion by in-gel fluorescence and western blotting using a C-terminal anti-polyhistidine antibody without (non-boiled) or with (boiled) boiling of the samples prior to loading as indicated. The black and white arrows indicate the positions of the fluorescent and non-fluorescent MscL-GFP bands, respectively. In (a) and (b), experiments were carried out in replica triplicates and the error bars represent one standard deviation from the mean value.

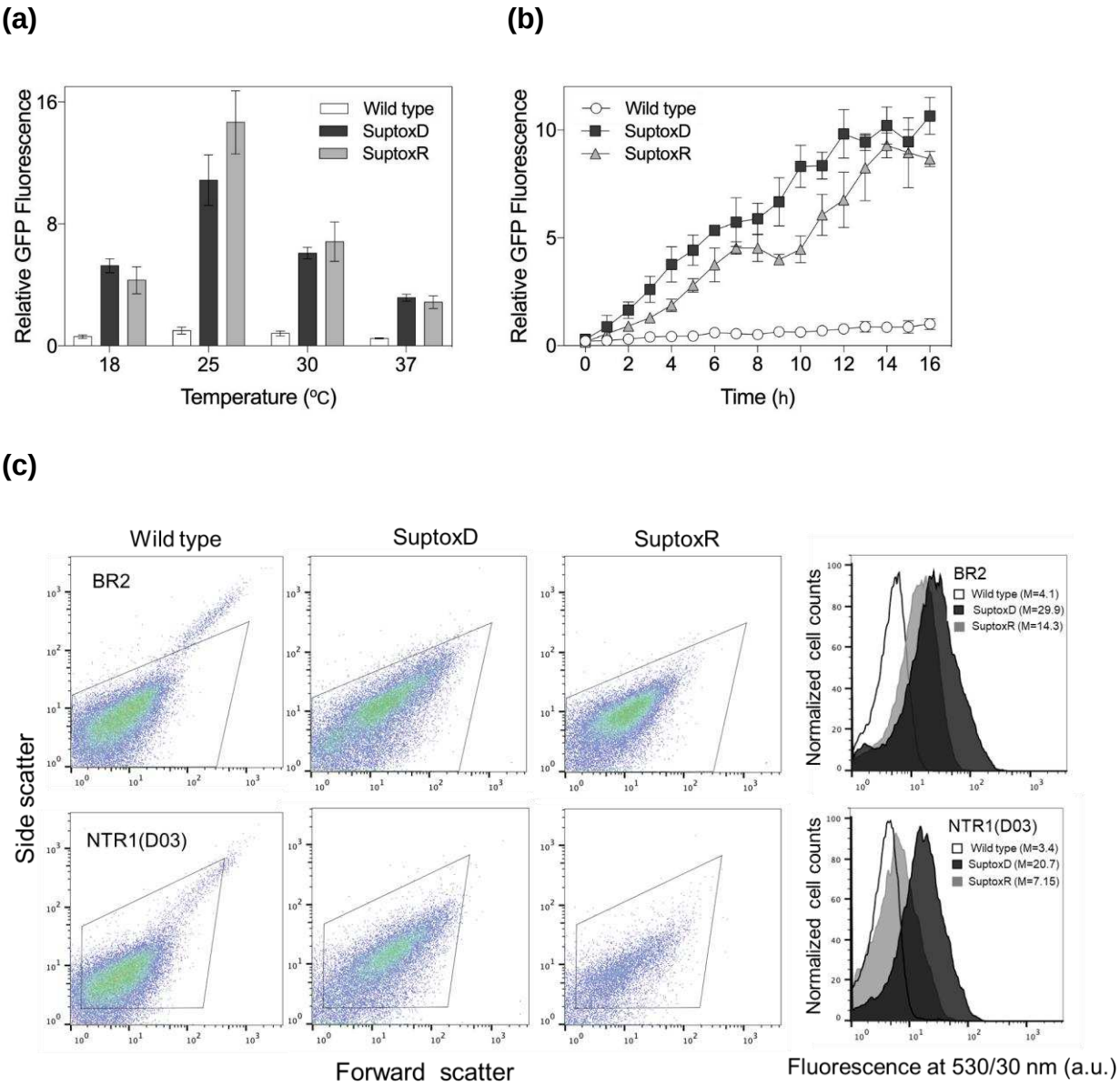
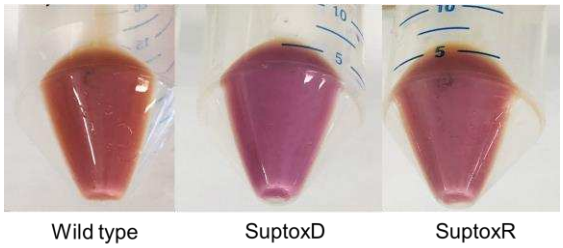


Figure 6. Determination of optimal culturing temperature and time for recombinant MP production in E. coli SuptoxD and SuptoxR. (a). Fluorescence of E. coli MC1061 (Wild type), SuptoxD and SuptoxR cells producing BR2-GFP by the addition of 0.2 µg/ml aTc and 0.01% (SuptoxD) or 0.2% (SuptoxR) arabinose at the indicated temperatures overnight. Mean values ± s.d. are presented (n=3 independent experiments, each one performed in replica triplicates). The fluorescence of BR2-producing MC1061 cells at 25 ºC was arbitrarily set to one. **(b).** Comparison of the fluorescence of equal culture volumes of E. coli MC1061 (wild type), SuptoxD and SuptoxR cells producing BR2-GFP by the addition of 0.2 µg/mL aTc and 0.01% (SuptoxD) or 0.2% (SuptoxR) arabinose overnight at 25 °C at different time points after aTc addition to the medium. The fluorescence of BR2-producing MC1061 cells at 16 h was arbitrarily set to one. Experiments were carried out in replica triplicates and the error bars represent one standard deviation from the mean value. **(c).** (Left) Forward versus side scatter plots as determined by flow cytometry analysis of E. coli MC1061

(Wild type), SuptoxD and SuptoxR cells producing BR2-GFP or NTR1(D03)-GFP by the addition of 0.2 μg/mL aTc and 0.01% (SuptoxD) or 0.2% (SuptoxR) arabinose for 16 h at 25 °C. (Right) Comparison of the levels of individual cell fluorescence of E. coli MC1061 (Wild type), SuptoxD and SuptoxR cells producing BR2-GFP or NTR1(D03)-GFP(bottom) as measured by flow cytometry. Cells were gated as indicated by the grey line (left). Fluorescence measurements correspond to the mean value (M) of replica experiments performed in triplicate.

SuptoxD and SuptoxR achieve enhanced production of detergent-extractable and functional recombinant MP. Our results indicate that, under optimal conditions, SuptoxD and SuptoxR achieve greatly enhanced production of full-length, membrane-embedded and well-folded recombinant MPs compared to wild-type E. coli. In order to test whether these enhanced amounts contain biologically active protein and, thus, would be suitable for functional studies, we compared the accumulation of the archaeal deltarhodopsin from Haloterrigena turkmenica (HtdR) in wild-type E. coli and SuptoxD/SuptoxR. HtdR is a light-driven outwards proton pump, which when properly folded, can bind the chromophore all-trans-retinal and acquires a characteristic purple color with an adsorption maximum at ~550 nm[32]. Apart from model proteins for convenient monitoring of functional protein production[33], microbial rhodopsins are also invaluable tools for optogenetic regulation applications[30]. E. coli has served as a cell factory for recombinant production of this type of MPs for biochemical and structural studies[34, 35], as well as for the engineering of protein variants with new properties[36, 37]. Under optimal MP production conditions, our strains were found to accumulate significantly enhanced levels of both total and detergent-extractable functional HtdR (Figure 7).

(a)



(b)

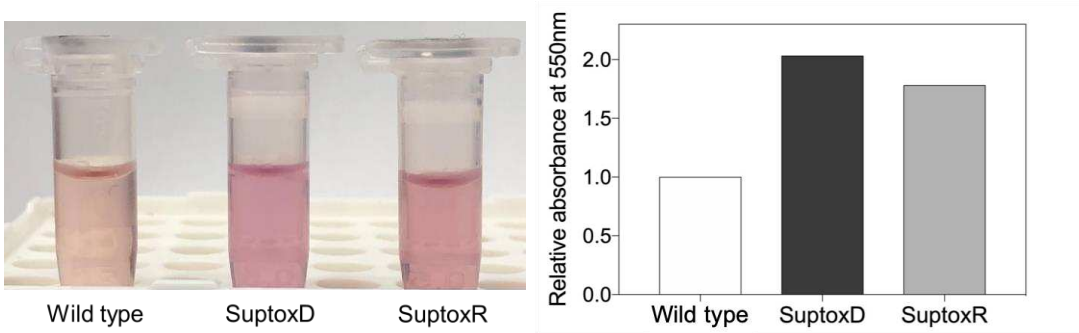


Figure 7. SuptoxD and SuptoxR produce enhanced amounts of functional recombinant MP. (a). Photographs of purple-colored pellets derived from equal culture volumes of MC1061 (Wild type), SuptoxD and SuptoxR cells producing HtdR by the addition of 0.2 μg/ mL aTc and 0.01% (SuptoxD) or 0.2% (SuptoxR) arabinose for 16 h at 25 °C in the presence of 10 μM all-trans-retinal. **(b).** (Left) Photographs of DDM-extracted HtdR acquired from the total lysates of equal culture volumes of the MC1061 (Wild type), SuptoxD and SuptoxR cells shown in (a). (Right) Relative absorbance at 550 nm of the samples shown in (b), left.

SuptoxD and SuptoxR achieve greatly enhanced production of purified recombinant MPs. Structural and biophysical studies of MPs typically require the availability of mg quantities of detergent-extracted and isolated protein in a folded state. The required yields of purified protein from an MP-overexpression strain for such purposes should be in the range 0.2-1 mg per L of cell culture[3]. To determine the yields of purified recombinant MPs expressed in our strains, we produced polyhistidine-tagged versions of BR2 in SuptoxD and the MscL variant F88C (see below) in SuptoxR under the optimal expression conditions determined above. Both MPs were solubilized

from isolated total membranes in detergent and were purified using immobilized metal affinity chromatography (IMAC), followed by size-exclusion chromatography (SEC). BR2 was extracted using fos-choline-14 (Fos14), while MscL using n-dodecyl β-D-maltoside (DDM). For both MPs, we performed control purification experiments from wild-type E. coli under identical conditions. The yield of isolated BR2 from SuptoxD was found to be ~1 mg per L of shake flask culture. This is approximately 14-fold higher than the corresponding yield from wild-type E. coli (~47.4 mAU absorption for SuptoxD compared to ~3.5 mAU for wild type) (Figure 8a). BR2 isolated from SuptoxD eluted as a single peak in SEC, indicating that purified protein in DDM solution is folded and non-aggregated (Figure 8a).

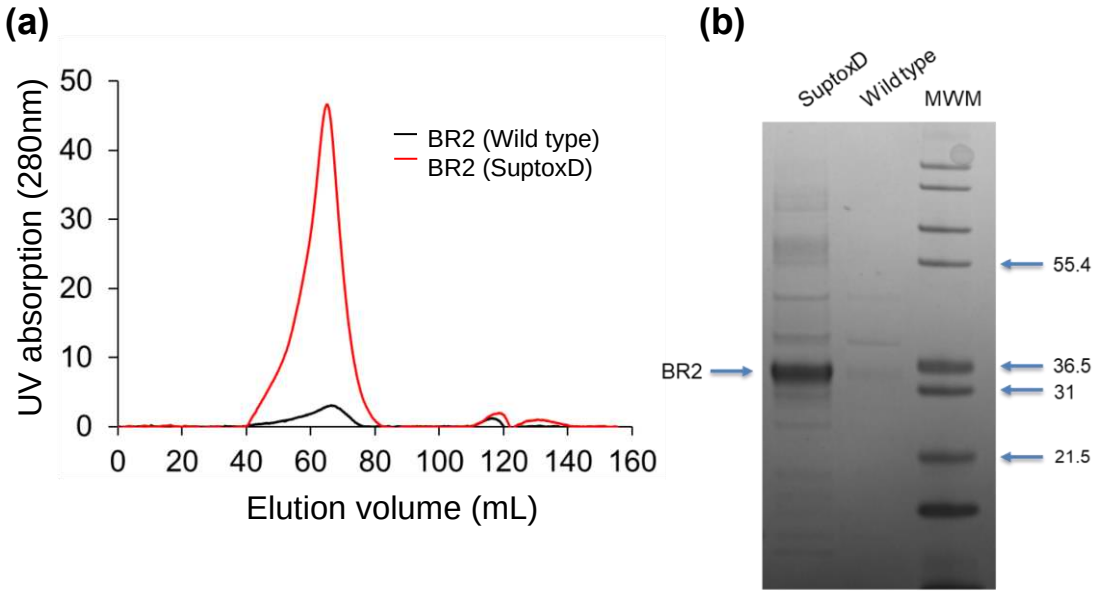


Figure 8. E. coli SuptoxD produce greatly enhanced amounts of isolated recombinant MP. (a). SEC profiles of purified BR2 from E. coli MC1061 (Wild type; black) and SuptoxD (red) **(b).** SDS-PAGE analysis of the BR2 SEC peaks (~67 mL on a Superdex200 16/60 GE column) obtained in (a). MWM: molecular weight marker. The numbers indicate the corresponding molecular masses in kDa.

The purity of the SEC peaks of the isolated BR2 was assessed by SDS-PAGE analysis. This revealed adequate sample purity for initial screening and characterization purposes (Figure 8b). The strong single band appearing after BR2 purification from SuptoxD exhibited the expected electrophoretic mobility corresponding to apparent molecular mass of ~35 kDa (detergent-extracted BR2 and other MPs run faster on SDS-PAGE than expected according to their molecular weight[31, 38]) and its identity was confirmed by western blot analysis and mass spectrometry following trypsin digestion (Supplementary Figure S1). It is important to note, that human BR2 is a particularly challenging MP to produce recombinantly, not only in microbial expression hosts[31] but also in human cell cultures[38].

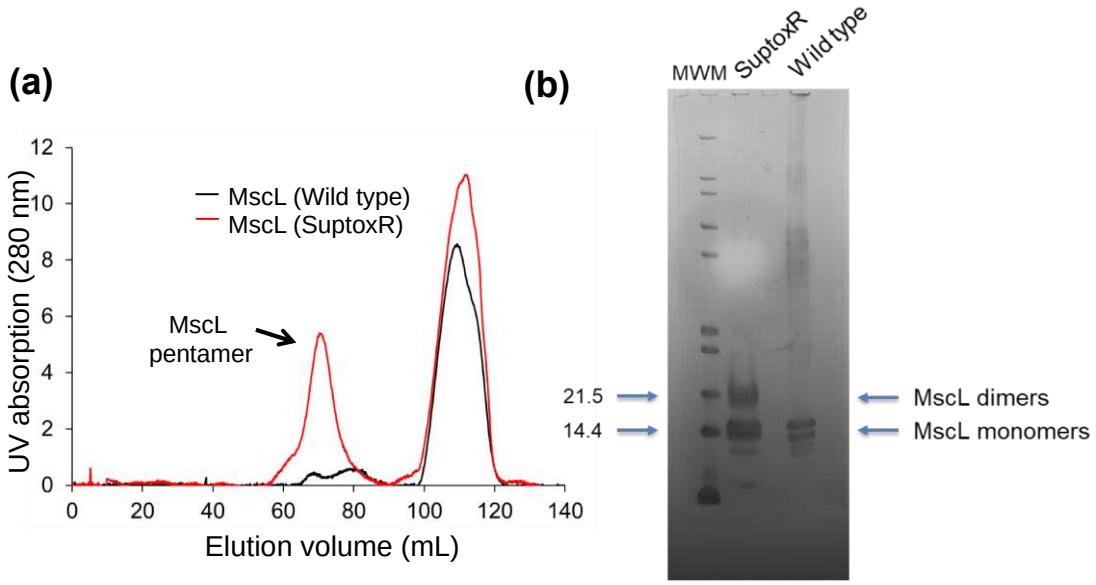


Figure 9. E. coli SuptoxR produce greatly enhanced amounts of isolated recombinant MP. (a). SEC profiles of purified MscL from E. coli MC1061 (Wild type; black) and SuptoxR (red), revealing a single homogeneous monodisperse peak of MscL pentamers. **(b).** SDS-PAGE analysis of the pentameric MscL(F88C) SEC peaks obtained from (a). The gel indicates the presence of highly pure protein containing only MscL(F88C) monomers and disulfide-linked dimers. F88C is located at the interface of two neighboring subunits and the appearance of reducing agent-resistant disulfide-linked dimers is in agreement with previous studies of single cysteine mutants of both E. coli MscL and the small conductance mechanosensitive ion channel (MscS)[39, 40]. MWM: molecular weight marker. The numbers indicate the corresponding molecular masses in kDa.

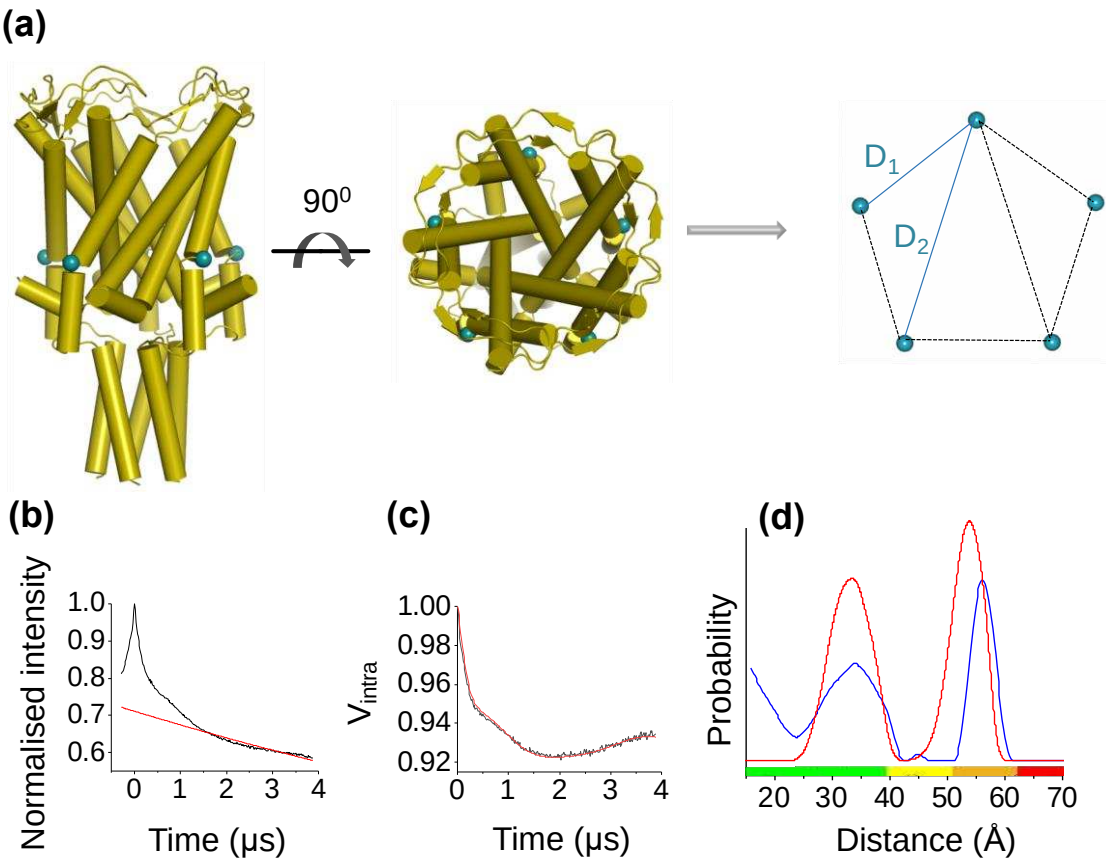


Figure 10. E. coli SuptoxR produce well-folded MP at the expected oligomerization and conformational state. (a). Side and top MscL channel pore view. F88C is highlighted (cyan spheres). Pentameric MscL is labelled with five spin labels resulting in pentagon symmetry and thus two expected distances in PELDOR/DEER. **(b).** Raw, uncorrected time domain spectrum (black) with the background function (red). **(c).** Background corrected time domain spectrum (black) with the fitting function (red). **(d).** PELDOR distance distribution of MscL(F88C) covalently modified with MTSSL (blue area, corresponding to mean $\pm 2\sigma$ confidence interval as calculated by the DeerAnalysis validation tool) compared to in silico-modelled distances (red) of the MscL crystal structure (PDB 2OAR), using MtsslWizard. The rainbow colour bar indicates the reliability of the distance range (calculated by DeerAnalysis), based on the experimental 4 μs time window used.

Similarly to the results acquired with SuptoxD, a >10-fold increased yield was observed for the purified mechanosensitive ion channel MscL in SuptoxR compared to wild-type E. coli (~5.40 mAU absorption for SuptoxD compared to ~0.47 for wild-type) (Figure 9a). The yields of purified MscL were calculated to be ~0.33 mg and 0.03

mg per L of shake flask culture for SuptoxR and wild-type E. coli, respectively. The recorded SEC peak for SuptoxR-purified MscL was monodisperse and highly homogeneous and eluted at ~69 mL from a Superdex200 16/60 column, thus indicating a highly monodisperse and well-folded multimeric channel protein. The purity of these SEC peaks was further assessed by SDS-PAGE (Figure 9b) and the identity of the MscL bands was confirmed by mass spectrometry (Supplementary Figure S2).

In order to assess whether the SuptoxR-produced MscL is well folded and so as to determine its oligomeric and conformational state in DDM solution, we combined pulsed electron electron double resonance (PELDOR) (also known as double electron electron resonance (DEER)) spectroscopy with site-directed spin labelling[41-43]. This method allows for measurement of interspin distances of engineered/introduced paramagnetic species (carrying unpaired electrons) within protein complexes between 1.5 to 7 nm and irrespective of their size. PELDOR has served as a powerful tool for studying conformation, oligomeric state and complex dynamics of various MP types, such as ion channels, transporters and GPCRs[39, 44-49], at molecular resolution and in complete agreement with X-ray crystallography studies[50]. To perform PELDOR analysis of MscL, we introduced the S-(2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methyl methanesulfonothioate (MTSSL) spin label by covalent attachment to the single-cysteine variant MscL (F88C), resulting in a MscL pentamer carrying five spins per macromolecule (Figure 10a). Due to the expected pentagon symmetry, two distinct distances, i.e. D_1 and D_2, are anticipated (Figure 10a), thus allowing the determination of the oligomerization and conformational state of the purified MscL in detergent solution. We obtained a PELDOR time trace with clear dipolar oscillations in the raw data and a 4-μs time window (Figure 10b), which allowed accurate distances to be determined, after background correction (Figures 10c and d). The resulting distance

distribution was in very good agreement with the in silico modelled distances of the closed state pentameric MscL (PDB 2OAR)[51], suggesting that the SuptoxR-produced MscL channel is correctly folded and adopts a symmetric pentameric arrangement in the closed conformation. The pentameric state of the SuptoxR-isolated MscL was further confirmed by the resulting distance peak ratio D_2/D_1 ~1.6 (for a perfectly symmetric pentamer this ratio is 1.6[41]). PELDOR/DEER, therefore, provides robust experimental evidence for the correct folding and structural integrity of the MscL channel protein produced in SuptoxR.

Overall, our results demonstrate that E. coli SuptoxD and SuptoxR are two specialized strains that achieve greatly enhanced recombinant MP production in bacteria. In our present and previous work[12, 15] we have found that this enhanced productivity occurs for membrane proteins (1) from all three domains of life (archaeal, eubacterial and eukaryotic); (2) with different functions (GPCR, mechanosensitive channel, light-driven proton pump proton pump and other); and (3) with different molecular and topological characteristics (size, number of transmembrane helices, oligomerization states etc.). Under the optimized production conditions determined here, the use of these strains allows the production of high-quality recombinant MPs at quantities sufficient for functional and structural studies. Recombinant MP production in these strains yields well-folded and homogeneous proteins, which maintain their structural integrity. Based on these results, we anticipate that SuptoxD and SuptoxR will become broadly utilized expression hosts for recombinant MP production in bacteria.

Methods

Plasmid construction. All enzymes for cloning of recombinant DNA were purchased from New England Biolabs. The plasmid pASKMscL-EGFP was generated by amplifying the sequence encoding the gene mscL by PCR from the vector pJ411TbMscL (DNA 2.0), with the primers MscLwtM.tuberculosisFOR (5' AAAAATCTAGAAGGAGGAAACGATGTTGAAAGGCTTTAAAG-3') and MscLwtM.tuberculosisREV (5'-AAAAAGGATCCCTCGAGCTGGCTTTCGGTAG-3'), digesting with XbaI and BamHI, and by cloning the resulting fragment into the similarly digested vector pASKBR2-EGFP in place of the BR2-encoding gene. The plasmid pASKHtdR was generated by amplification of the htdR gene from pHtdR200 [12] with the htdR-specific DNA primers HtdRFOR (5'-AAAAAATCTAGAAGGAGGAAACGATGTGTTACGCTGCTCTAGCACC-3') and HtdRREV (5'-AAAAAAAGCTTTTAGTGGTGATGGTGGTGATGGGTCGGGGCAGCCGTCGGCG-3') carrying XbaI and HindIII recognition sequences, digestion with XbaI and HindIII, and ligation into similarly digested pASK75. The plasmid pASKMscL(F88C) was generated by amplifying the gene encoding MscL(F88C) by PCR from the vector pJ411TbMscL(F88C) (DNA 2.0) with the primers MscLwtM.tuberculosisFOR (5'-AAAAATCTAGAAGGAGGAAACGATGTTGAAAGGCTTTAAAG-3') and MscLwtM.tuberculosisREVHISTAG (5'-AAAAAAAGCTTTTAGTGGTGATGGTGGTGATGCTCGAGCTGG-3'), digesting with XbaI and HindIII, and by cloning the resulting fragment into the similarly digested vector pASK75.

MP overexpression in liquid cultures. E. coli cells freshly transformed with the appropriate expression vector(s) were used for all protein production experiments. Single bacterial colonies were used to inoculate liquid LB cultures containing the appropriate combination of antibiotics (100 μg/mL ampicillin, 40 μg/mL chloramphenicol or 50 μg/mL kanamycin (Sigma)). These cultures were used with a 1:50 dilution to inoculate fresh LB cultures with 0.01% (MC1061 and SuptoxD) or 0.2% arabinose (SuptoxR), which were grown at 30 °C to an optical density at 600 nm (OD_{600}) of ~0.3−0.5 with shaking, unless specified otherwise. The temperature was then decreased to 25 °C and after a temperature equilibration period of 10−20 min, MP expression was induced by the addition of 0.2 μg/mL aTc (Sigma) overnight, unless specified otherwise. For rhodopsin overproduction, we followed the same procedure, but when the cell density reached OD_{600} ~0.3−0.5, protein production was induced by the addition of 0.2 μg/mL aTc in the presence of 10 μM all-trans-retinal (Cayman Chemical) overnight in dark.

Membrane isolation. Total membrane fractions were isolated from 1 L LB cultures in all cases, except for rhodopsin (250 mL). Cells were harvested by centrifugation (4,000 x g for 10 min) and resuspended in 10 mL of cold lysis buffer (300 mM NaCl, 50 mM NaH_2PO_4, 15% glycerol, 5 mM dithiothreitol, pH 7.5). The cells were lysed by brief sonication steps on ice and the resulting lysates were clarified by centrifugation at 10,000 x g for 15 min. The supernatant was then subjected to ultracentrifugation on a Beckman 70Ti rotor at 42,000 rpm (130,000 x g) for 1 h at 4 °C. The resulting pellet was finally re-suspended in 5 mL of cold lysis buffer and homogenized.

Western blot and in-gel fluorescence analyses. Proteins samples were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) in 12-15%

gels with or without prior boiling of the samples for 10 min for western blotting and without prior boiling for in-gel fluorescence analysis. In-gel fluorescence was analyzed on a UVP ChemiDoc-It^2 Imaging System equipped with a CCD camera and a GFP filter, after exposure for about 3 s. For western blotting, proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Merck) for 45 min at 12 V on a semidry blotter (Thermo Scientific). Membranes were blocked with 5% nonfat dried milk in Tris- buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature. After washing with TBST three times, membranes were incubated with the appropriate antibody dilution in TBST containing 0.5% nonfat dried milk at room temperature for 1 h. The utilized antibodies were a mouse monoclonal antipolyhistidine antibody (Sigma) at 1:3,000 dilution (conjugated with horseradish peroxidase), a mouse monoclonal anti-FLAG antibody (Sigma) at 1:1,000 dilution, with a horseradish peroxidase-conjugated goat anti-mouse as secondary antibody at 1:5,000 dilution. The proteins were visualized using a ChemiDoc-It^2 Imaging System (UVP).

Bulk fluorescence measurements. Cells corresponding to 0.5 mL of culture were harvested and resuspended in 100 μL PBS. The cell suspension was then transferred to a black 96-well plate and after fluorophore excitation at 488 nm, fluorescence was measured at 510 nm using a TECAN SAFIRE plate reader.

Fluorescence analysis by flow cytometry. ~10^7 cells were re-suspended in 1 mL PBS and after fluorophore excitation at 488 nm, the fluorescence of 50,000 cells was measured at 530/30 nm using a CyFlow ML flow cytometer (Partec) and analyzed statistically using FlowJo 7.6.2.

Rhodopsin extraction and quantification. Pellets from 250 mL of cell culture were re-suspended in 7.5 ml cold lysis buffer. Cells were lysed by brief sonication steps on

ice and rhodopsin was extracted from total cell lysates by the addition of 2.5% (w/v) DDM (Glycon Biochemicals) and rotation at 180 rpm for 24 h at 4 ºC in the dark. The mixture was subjected to ultra-centrifugation and, if a colorless pellet was acquired, the supernatant (detergent-extractable fraction) was collected analyzed by measuring absorbance at 550 nm as described previously[32, 52].

MscL and BR2 purification. MscL(F88C) was expressed in E. coli MC161 (wild type) and SuptoxR, spin labelled and purified as previously described[41, 48]. The extent of cysteine modification (i.e. spin labelling efficiency) was assessed as previously described [53]. In brief, protein pellets were resuspended in phosphate-buffered saline (PBS), lysed with a cell disrupter at 30,000 psi and centrifuged at 4,000 x g for 20 min. The supernatant was then ultra-centrifuged at 100,000 x g for 1 h. The resulting membrane pellet was mechanically resuspended in solubilisation buffer (50 mM sodium phosphate of pH 7.5, 300 mM NaCl, 10% v/v glycerol, 50 mM imidazole, 1.5% w/v DDM (Glycon, GmbH) and incubated at 4 ºC for 1 h. The sample was then centrifuged at 4,000 x g for 10 min and the supernatant was passed through a column containing 0.5 mL Ni^{2+}-nitrilotriacetic acid (Ni^{2+}-NTA) beads (Sigma). Subsequently, the column was washed with 10 mL wash buffer (50 mM sodium phosphate pH 7.5, 300 mM NaCl, 10% v/v glycerol, 50 mM imidazole, 0.05% w/v DDM) and 5 mL wash buffer supplemented with 3 mM tris(2-carboxyethyl)phosphine (TCEP). Then, MTSSL (Glycon) dissolved in wash buffer at 10x excess of the expected protein concentration was added to the column and left to react for 2 h at 4 ºC. The protein was then eluted with 5 mL of elution buffer (50 mM sodium phosphate of pH 7.5, 300 mM NaCl, 10% v/v glycerol, 300 mM imidazole, 0.05% w/v DDM) before being subjected to SEC using a Superdex 200 column (GE Healthcare) equilibrated with SEC buffer (50 mM

sodium phosphate of pH 7.5, 300 mM NaCl, 0.05% w/v DDM). Finally, the protein was concentrated to ~800 μM monomer concentration for PELDOR measurements.

The purification protocol used for BR2 from E. coli MC161 (wild type) and SuptoxD was similar, with the exception that different buffers were used: solubilisation buffer (10 mM HEPES of pH 7.2, 400 mM NaCl, 10% v/v glycerol, 30 mM imidazole, 0.5% w/v fos-14 (Anatrace) wash buffer (10 mM HEPES of pH 7.2, 400 mM NaCl, 10% v/v glycerol, 30 mM imidazole, 0.05% w/v DDM); elution buffer (10 mM HEPES of pH 7.2, 400 mM NaCl, 10% v/v glycerol, 300 mM imidazole, 0.05% w/v DDM); and SEC buffer (10 mM HEPES of pH 7.2, 400 mM NaCl, 0.05% w/v DDM).

PELDOR measurements, data analysis and in silico spin labelling and distance modelling. Purified MscL(F88C) was mixed at a 1:1 ratio with deuterated ethylene glycol and 70 μL of the mixture were loaded in a 3 mm quartz tube and flash frozen in liquid N_2. PELDOR measurements were performed with a Bruker ELEXSYS E580 pulsed Q band (34 GHz) spectrometer with a TE012 cavity at 50 ^{o}K. The offset between the detection (ν_A) and pump (ν_B) frequencies was 80 MHz and the pulse sequence used was $(\pi/2)_A$ - τ_1 – π_A - (τ_1+t) - π_B - (τ_2–t) - π_A - τ_2 – echo[54]. ν_A pulses were 16 ns ($\pi/2$) and 32 ns (π) and separated by τ_1= 380 ns, while the ν_B pulse was 12 ns long. The shot repetition time was set to 3 ms. The data acquired were analysed with the DeerAnalysis 2016 Matlab plugin[55]. Time domain spectra were fitted with an exponential decay function, background-corrected and analysed by Tikhonov regularization[56]. The validation tool was used as previously described[48], with the background starting point varying between 5% and 80% of the length of the trace in 16 steps and 50% random noise added in 50 trials for each step, resulting in 800 total trials. Finally, datasets more than 15% above the best (lowest) root-mean-square deviation (RMSD) were discarded. In silico spin labelling and distance measurements were done using the

MTSSLWizard[57] PyMOL plugin, with F88 mutated to C. The "thorough search" option was used for the MTSSL rotamers and the Van der Waals restraints were set to tight.

Statistical analyses. Graphs were prepared using SigmaPlot (Systat Software Inc. Ver. 10. Systat Software, Point Richmond, CA, USA). Data in all assays correspond to the mean values of one to three independent experiments, each one performed in a least three replicates as mentioned in the corresponding figure legends.

Supporting information

The Supporting Information is available free of charge on the ACS Publications website. Figure S1, BR2 purification from E. coli SuptoxD cultures; Figure S2, MscL purification from E. coli SuptoxR cultures.

Abbreviations

MP: membrane protein; DjlA: DnaJ-like protein A; RraA: regulator of ribonuclease activity A; aTc: anhydrotetracycline; GPCR: G-protein-coupled receptor; BR2: bradykinin receptor 2; NTR1: neurotensin receptor 1; MscL: large conductance mechanosensitive ion channel; MscS: small conductance mechanosensitive ion channel; HtdR, archaeal deltarhodopsin from Haloterrigena turkmenica; GFP: green fluorescent protein; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; WT: Wild type; IMAC: immobilized metal affinity chromatography; SEC: size-exclusion chromatography; Fos14: fos-choline-14; DDM: n-dodecyl β-D-maltoside; PELDOR: pulsed electron electron double resonance; DEER: double electron electron resonance; MTSSL: S-(2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methyl methanesulfonothioate.

Author Contributions

GS conceived and coordinated the project. GS and CP designed the research. MM and CK carried out the research. MM, CK, CP and GS analyzed the data. GS wrote the paper with contributions from MM, CK and CP. All authors read and approved the final manuscript.

Competing financial interests

GS is an inventor on a patent application for SuptoxD and SuptoxR (PCT/EP2017/025168).

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Table 1. Plasmids used in this study.

Plasmid	Protein expressed	Marker	Origin of replication	Source
pASKBR2-EGFP	FLAG-BR2-TEV-EGFP-His_6	Amp^R	ColE1	Gialama et al.[12]
pASKNTR1(D03)-EGFP	FLAG-NTR1(D03)-TEV-EGFP-His_6	Amp^R	ColE1	Gialama et al.[12]
pASKMscL-EGFP	MscL-EGFP-His_6	Amp^R	ColE1	This work
pASKBR2	BR2-His_6	Amp^R	ColE1	Link et al.
pASKMscL(F88C)	MscL-His_6	Amp^R	ColE1	This work
pASKHtdR	HtdR-His_6	Amp^R	ColE1	This work
pSuptoxD	DjlA-His_8	Cm^R	ACYC	Gialama et al.[12]
pSuptoxR	RraA-His_8	Cm^R	ACYC	Gialama et al.[12]

Table 2. Membrane proteins studied in this work.

Membrane protein	Organism	Function	Number of TM helices	Topology	Mass (kDa)
BR2	Homo sapiens	Bradykinin receptor 2 (GPCR)	7	N^{out}-C^{in}	44.5
NTR1(D03)	Rattus norvegicus	Neurotensin receptor 1 variant D03[6] (GPCR)	7	N^{out}-C^{in}	44.6
MscL	Mycobacterium tuberculosis	Large conductance mechanosensitive channel	2	N^{in}-C^{in}	16.0
HtdR	Haloterrigena turkmenica	Deltarhodopsin	7	N^{out}-C^{in}	27.1