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Research Article

Second-Generation *Escherichia coli* SuptoxR Strains for High-Level Recombinant Membrane Protein Production

Eleni Vasilopoulou, Artemis Giannakopoulou, Charalampos Kapsalis, Myrsini Michou, Aristeidis Michoglou-Sergiou, Fragiskos N. Kolisis, and Georgios Skretas*

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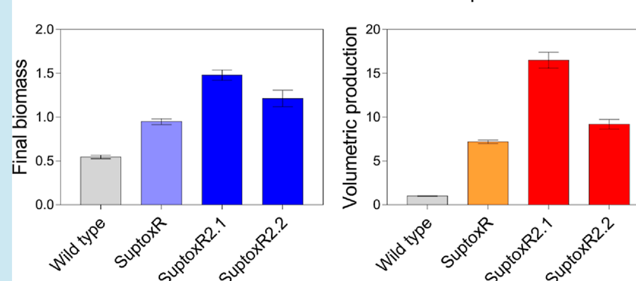

ABSTRACT: *Escherichia coli* is one of the most widely utilized hosts for recombinant protein production, including that of membrane proteins (MPs). We have recently engineered a specialized *E. coli* strain for enhanced recombinant MP production, termed SuptoxR. By appropriately co-expressing the effector gene *rraA*, SuptoxR can suppress the high toxicity, which is frequently observed during the MP-overexpression process, and, at the same time, enhance significantly the cellular accumulation of membrane-incorporated and properly folded recombinant MP. The combination of these two beneficial effects results in dramatically enhanced volumetric yields for various prokaryotic and eukaryotic MPs. Here, we engineered second-generation SuptoxR strains with further improved properties, so that they can achieve even higher levels of recombinant MP production. We searched for naturally occurring RraA variants with similar or improved MP toxicity-suppressing and production-promoting effects to that of the native *E. coli* RraA of the original SuptoxR strain. We found that the RraA proteins from *Proteus mirabilis* and *Providencia stuartii* can be even more potent enhancers of MP productivity than the *E. coli* RraA. By exploiting these two newly identified RraAs, we constructed two second-generation SuptoxR strains, termed SuptoxR2.1 and SuptoxR2.2, whose MP-production capabilities often surpass those of the original SuptoxR significantly. SuptoxR2.1 and SuptoxR2.2 are expected to become widely useful expression hosts for recombinant MP production in bacteria.

KEYWORDS: *Escherichia coli, SuptoxR, RraA, membrane protein, recombinant production, toxicity*

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INTRODUCTION

Membrane proteins (MPs) are the molecules responsible for the execution of numerous important functions in all living cells and organisms, such as nutrient import, maintenance of structural integrity, signaling and others.[1] Their great significance is well reflected by the fact that they are encoded by 20−30% of the genes in every living organism.[2] Furthermore, they constitute 30−50% of the targets of the marketed drugs and the ones currently in clinical development.[3]

Despite their enormous significance, our knowledge about the biochemical and structural properties of MPs is very much limited compared to that of soluble proteins. The difficulties in acquiring significant amounts of properly folded isolated protein, their frequent instability when extracted from their native membranes and solubilized in detergent micelles, and the increased complexity of the procedures required for their biochemical and biophysical characterization are some of the main factors, which have prevented faster development of this research area.[4,5]

Regarding the acquisition of isolated MPs at sufficient quantities for biochemical and biophysical analyses, this is typically done by recombinant overexpression in homologous or heterologous hosts, such as bacteria, yeasts, mammalian cells, insect cells, or transgenic animals.[6] Among these systems, and as it is the case also for soluble proteins, the bacterium *Escherichia coli* is among the most frequently utilized MP production hosts, primarily due to its speed, easiness, low cost, and capability for isotopic protein labeling.[2,7] Indeed, approximately 20% of all MP structures deposited in the Protein Data Bank have utilized MP material, which had been produced recombinantly in *E. coli*.[8] Despite these successes, however, recombinant MP production in *E. coli* and in other bacteria usually suffers from poor cellular accumulation and severe toxicity for the expression host.[2] MP-associated host toxicity, in particular, is a very frequently observed side effect of recombinant MP production and can lead to full growth

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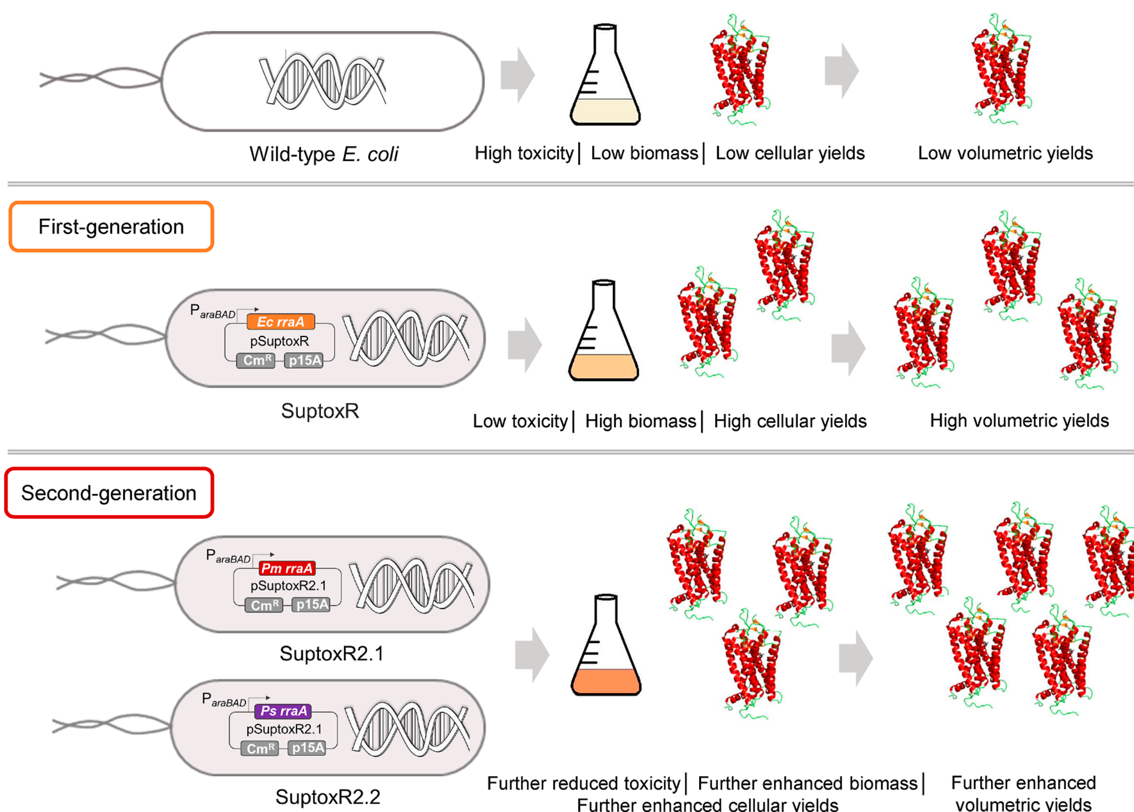

Figure 1. Characteristics of the first- and second-generation specialized *E. coli* strains for recombinant MP production SuptoxR, SuptoxR2.1, and SuptoxR2.2. The toxicity-suppressing and cellular production-promoting capabilities of SuptoxR, SuptoxR2.1, and SuptoxR2.2 are based on the overexpression of the *E. coli rraA* (*EcrraA*), the *Proteus mirabilis rraA* (*PmrraA*), and the *Providencia stuartii rraA* (*PsrraA*), respectively. *EcrraA*, *PmrraA*, and *PsrraA* are overexpressed from the vectors pSuptoxR, pSuptoxR2.1, and pSuptoxR2.2, respectively, under the control of the *araBAD* promoter and its inducer L(+)-arabinose.

arrest, very low levels of final biomass even under optimized conditions, and minute volumetric protein yields.[9−11]

In a previous work, we had searched for single *E. coli* genes, which can efficiently suppress the host toxicity associated with the MP overexpression process in bacteria. Following a genome-wide screen, one of the potent suppressors we identified was *rraA*, the gene encoding for the protein RraA, which functions as an inhibitor of the mRNA-degrading activity of the *E. coli* RNase E.[12] Based on this, we constructed the engineered *E. coli* strain SuptoxR, which upon overexpression of *rraA*, is capable of achieving significantly higher levels of final biomass and of producing dramatically increased yields for various recombinant MPs of both prokaryotic and eukaryotic origin.[12,13]

In the present work, we aimed at constructing second-generation SuptoxR strains, which can achieve even higher levels of recombinant MP production. For this, we mined the genomes of various *rraA*-encoding bacteria and plants to search for naturally occurring RraA variants with similar or improved MP toxicity-suppressing and production-promoting effects to that of the native *E. coli* RraA of the original SuptoxR strain. Through this process, we identified two RraA proteins from the bacteria *Proteus mirabilis* and *Providencia stuartii*, which can be even more potent enhancers of MP productivity than the *E. coli* RraA. By utilizing these two newly identified RraAs, we constructed two second-generation SuptoxR strains, termed SuptoxR2.1 and SuptoxR2.2, whose MP-production capabilities often surpass those of the original SuptoxR significantly. Based on these results, we anticipate that SuptoxR2.1 and SuptoxR2.2 will become widely useful expression hosts for recombinant MP production in bacteria.

■ RESULTS AND DISCUSSION

Various RraA Proteins from Bacteria and Plants can Efficiently Suppress MP-Induced Toxicity and Enhance MP Production when Co-Expressed in *E. coli*. The toxicity-suppressing and MP-production-promoting effects of SuptoxR are based on the overexpression of the *E. coli rraA* gene from the vector pSuptoxR under the control of the *araBAD* promoter and its inducer L(+)-arabinose[12−14] (Figure 1; Supporting Information Table S1). RraA (regulator of ribonuclease activity A) acts as an inhibitor of the mRNA-degrading activity of RNase E, and it has been found previously that *rraA* can affect the levels of more than 2000 different mRNAs when overexpressed in *E. coli*.[15] In a prior work, we demonstrated that the beneficial effects of RraA on recombinant MP production are mediated by the action of RNase E.[14]

With the ultimate goal of constructing second-generation SuptoxR strains with further improved properties, we searched for naturally occurring RraA variants with similar or improved MP toxicity-suppressing and production-promoting effects to that of the native *E. coli* RraA of the original SuptoxR strain. Apart from *E. coli*, homologous RraA sequences are encoded in the genomes of various archaea, proteobacteria, and plants.[15] Based on this, we first mined the genomes of *rraA*-encoding bacteria and plants and selected a broad panel of RraA homologues with high (>85%), intermediate (65−85%), and

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Table 1. Natural RraA Protein Variants Selected for Investigation in This Study

organism	length (aminoacids)	sequence Identity to SuptoxR RraA (%)	sequence similarity to SuptoxR RraA (%)	source
Escherichia coli	162	100	100	Genomic cDNA (Gialama et al.[12])
Salmonella enterica	162	96.9	98.8	Genomic cDNA (Pullinger et al.[36])
Klebsiella pneumoniae	162	93.8	99.4	Genomic cDNA (kindly provided by Dr. V. Miriagou)
Enterobacter gergoviae	162	91.4	98.8	Genomic cDNA (kindly provided by Dr. V. Miriagou)
Enterobacter cloacae	162	93.2	99.4	Genomic cDNA (kindly provided by Dr. V. Miriagou)
Serratia marcescens	162	89.5	95.7	Genomic cDNA (kindly provided by Dr. V. Miriagou)
Proteus mirabilis	173	77.5	87.3	Genomic cDNA (Jones et al.[37])
Providencia stuartii	174	77.0	85.1	Genomic cDNA (kindly provided by Dr. V. Miriagou)
Acinetobacter baumannii	143	45.1	62.3	Genomic cDNA (kindly provided by Dr. V. Miriagou)
Arabidopsis thaliana	167	40.5	61.3	Synthetic gene (Genscript Corp.)
Malus domestica	166	41.7	59.5	Genomic cDNA (This work)

Table 2. Membrane Proteins Used in This Study

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
CB1	*Homo sapiens*	Central cannabinoid receptor (GPCR)	7	N^{out}–C^{in}	52.9
CB2	*Homo sapiens*	Peripheral cannabinoid receptor (GPCR)	7	N^{out}–C^{in}	39.7
CLRN1	*Homo sapiens*	Clarin-1—Member of the tetraspanin family	4	N^{in}–C^{in}	25.7
MscL	*Mycobacterium tuberculosis*	Large conductance mechanosensitive channel	2	N^{in}–C^{in}	16.0
MdfA	*Escherichia coli*	Multidrug efflux transporter	12	N^{in}–C^{in}	44.3
HtdR	*Haloterrigena turkmenica*	Deltarhodopsin	7	N^{out}–C^{in}	27.1

lower (<65%) levels of sequence similarity to the *E. coli* RraA of SuptoxR (Table 1). Because there is very little information available about the function of RraA proteins and their structure–function relationships, our choice of RraA variants was based solely on sequence diversity criteria. Among the selected variants, the highest sequence variability was detected in the N- and C-terminal regions, where also significant differences in sequence lengths were observed (Supporting Information Figure S1). It is interesting to note that all the selected sequences, irrespective of their level of homology and of their bacterial or plant origin, share several absolutely conserved motifs throughout their length (Supporting Information Figure S1). Despite these striking similarities, there is no information currently available about how these highly conserved residues affect RraA functionality.

We cloned the selected *rraA* genes into the original pSuptoxR vector in place of the *E. coli rraA* and tested the effect of their overexpression on MP-induced toxicity and MP productivity by using the human bradykinin receptor 2 (BR2), a member of the G-protein-coupled receptor superfamily (Table 2), as a model of a highly toxic recombinant MP as we have described previously.[12,16] To facilitate the rapid monitoring of the relative accumulation levels of membrane-incorporated MP, we used a C-terminal BR2 fusion with the enhanced green fluorescent protein (GFP). This choice was made because the fluorescence of *E. coli* cells expressing MP-GFP fusions has been found to correlate well with the amount of membrane-integrated recombinant MP[17] and has been utilized extensively for these purposes by our group[12,14,18−22] and many others.[23−28] It is known that *E. coli* cells producing MP-GFP fusions do not always accumulate exclusively intact MP-GFP fusion, but sometimes yield truncated forms corresponding to cleaved GFP or other products of proteolysis as well,[14,28] and thus, this assay is not typically used for absolute MP quantification. However, whole cell fluorescence of *E. coli* cells producing MP-GFP fusions is widely utilized as a facile metric for comparing relative MP productivities under different conditions and has been used extensively by various groups for determining optimal conditions and for identifying factors enhancing recombinant MP productivity.[12,14,18−22]

The potential toxicity-suppressing effects of the selected RraA proteins were evaluated by recording the final growth levels of *E. coli* MC1061 cells producing BR2-GFP, in the presence or absence of *rraA* overexpression, as measurements of the optical density at 600 nm (OD_{600}) of the bacterial cultures upon completion of the MP overexpression process. We observed that, apart from *Enterobacter cloacae* and *Malus domestica*, co-expression of all the other *rraA* genes resulted in 45−165% increases in final OD_{600} values, thus indicating significantly reduced levels of BR2-induced toxicity in these cultures (Figure 2a). Then, BR2 accumulation on an individual cell basis upon co-expression of the selected *rraA* genes was monitored by measuring BR2-GFP fluorescence using flow cytometry. We observed that most of the selected RraA variants were also capable of enhancing cellular BR2-GFP fluorescence, thus indicating increased relative cellular productivity of membrane-embedded BR2 (Figure 2b). The combination of the suppressed MP-induced toxicity and the enhanced BR2-GFP productivity on an individual cell basis (Figure 2a,b) resulted in greatly enhanced levels of relative volumetric BR2-GFP fluorescence, that is, the bulk BR2-GFP fluorescence of equal-volume bacterial cultures, for the majority of the co-expressed *rraA* genes (Figure 2c). Apart from the *E. cloacae, M. domestica,* and *Acinetobacter baumannii*

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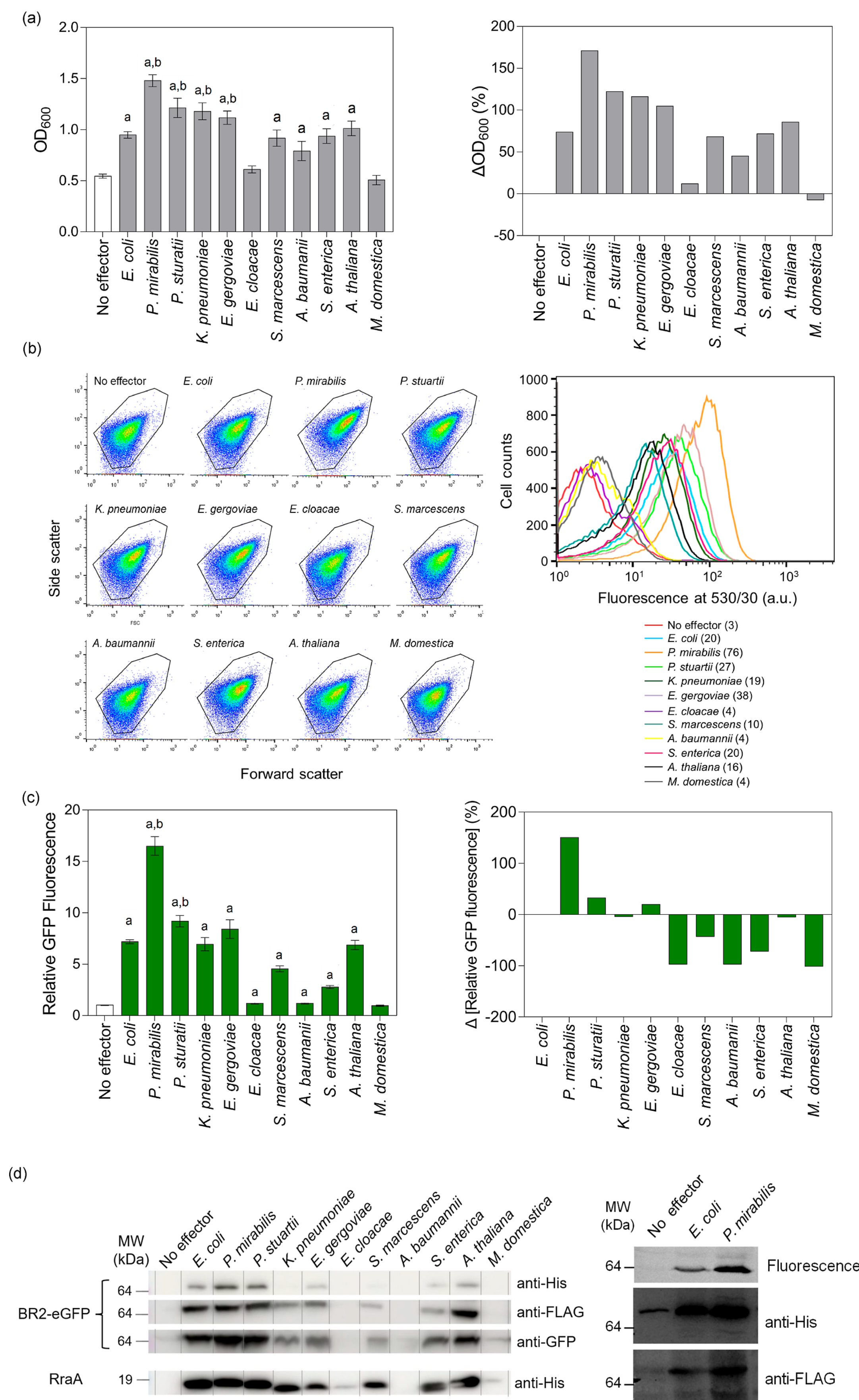

Figure 2. Co-expression of various RraA homologues from bacteria and plants results in suppressed BR2-induced toxicity and enhanced accumulation of membrane-embedded BR2 at levels similar to or higher than those of the *E. coli* RraA of SuptoxR. (a) (left) Effect of the

Figure 2. continued

overexpression of different *rraA* genes from bacteria and plants on the growth of *E. coli* MC1061 cells producing BR2-GFP. OD_{600}: optical density at 600 nm at the end of the BR2-GFP overexpression process; (right) percentage of increase in the OD values from Figure 2a (left) compared to wild-type *E. coli*. ΔOD (%): [(OD with *rraA* co-expression—OD without effector)/OD without effector] × 100. (b) Individual fluorescence of *E. coli* MC1061 cells producing BR2-GFP in the absence (no effector) and presence of *rraA* overexpression as measured by flow cytometry. (c) (left) Bulk fluorescence of *E. coli* MC1061 cells producing BR2-GFP in the absence (no effector) and presence of *rraA* overexpression corresponding to equal culture volumes; (right) percentage of increase in GFP fluorescence compared to SuptoxR. Δ[Relative GFP fluorescence] (%): [(GFP fluorescence with *rraA* co-expression from each organism—GFP fluorescence with *E. coli rraA* co-expression)/GFP fluorescence with *E. coli rraA* co-expression]x100. (d) SDS−PAGE analysis and visualization of the produced fusion by in-gel fluorescence and western blotting using an N-terminal anti-FLAG or C-terminal anti-GFP and anti-polyHis antibodies. In (a,c), the reported values correspond to the mean value from at least three independent experiments, each one performed in replica triplicates ± s.e.m. *a* denotes a statistically significant difference ($p < 0.05$) with the "no effector" sample, while *b* denotes a statistically significant difference with the "*E. coli*" sample.

rraA genes, whose co-expression resulted in only modest or negligible increases, all other overexpressed *rraA* genes yielded 3-to17-fold higher levels of volumetric BR2-GFP fluorescence (Figure 2c). Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE)/Western blot analysis of clarified cell lysates confirmed that this enhanced BR2-GFP fluorescence upon co-expression of the selected *rraA* genes occurs due to increased BR2 accumulation (Figure 2d).

We observed that the accumulation levels of the different RraA variants in the *E. coli* cytoplasm significantly affected the efficiency of *rraA* co-expression in promoting MP productivity. More specifically, the *rraA* genes from *E. cloacae*, *M. domestica,* and *A. baumannii*, which exhibited poor or no MP-production-promoting activity, yielded barely detectable or undetectable levels of RraA accumulation as revealed by western blotting. On the contrary, all other *rraA* genes, whose co-expression resulted in enhanced MP productivity, were also found to enable the accumulation of the corresponding RraA variants at significant levels (Figure 2d, left). Codon optimization of the co-expressed *rraA* genes and other expression modifications could enhance the observed MP-production-promoting effects for some of the selected RraAs, which accumulated at very low or undetectable levels. Such an effect was recorded in the case of the *Arabidopsis thaliana rraA*, which was the only codon-optimized variant tested here (Table 1). Thus, due to the observed variability in the accumulation levels of the tested RraA variants and in the absence of knowledge about how their co-expression affects the transcriptome profile of *E. coli* cells, we cannot compare directly the efficiency of the different RraA variants tested here in a quantitative manner. By monitoring the effects of *rraA* co-expression on the suppression of MP-induced toxicity and on the enhancement of MP productivity, however, we can identify RraA variants with MP toxicity-suppressing and production-promoting effects similar to that of the native *E. coli* RraA of the original SuptoxR strain, such as the ones from *P. mirabilis*, *P. stuartii*, *Klebsiella pneumoniae*, *Enterobacter gergoviae,* and *A. thaliana*, which is one of the main goals of the present study.

MP Production-Promoting Effects of the RraA Proteins from *P. mirabilis* and *P. stuartii* can Frequently Surpass Those of the *E. coli* RraA. Very interestingly, among the natural RraA variants, which were found to function as efficient enhancers of recombinant BR2 production, the potency of some of them was found to be on par with that of the native *E. coli* RraA of SuptoxR (EcRraA) or even higher (Figure 2a−c). Among them, the RraAs from *P. mirabilis* (PmRraA) and *P. stuartii* (PsRraA) were the most active and their potency surpassed that of the native EcRraA of SuptoxR with statistical significance both in terms of toxicity suppression (52 and 24% higher final OD_{600}, respectively) and productivity (280 and 35% higher specific productivity, and 110 and 18% higher volumetric productivity, respectively) (Figure 2a−c). To investigate the folding quality of the overproduced MP in the presence of the most potent effector, PmRraA, we analyzed BR2-GFP production in isolated total membranes by semi-denaturing SDS−PAGE and in-gel fluorescence. Previous studies have shown that in-gel fluorescence provides a quantitative measure of the amount of properly folded protein for bacterially overexpressed MP−GFP fusions.[29] Our analyses demonstrated that co-expression of PmRraA results not only in enhanced accumulation of total membrane-incorporated protein but also produces significantly increased levels of well-folded recombinant MP (Figure 2d, right). A fluorescence size-exclusion chromatography (FSEC)[30] analysis of BR2-GFP in the presence of PmRraA co-expression similarly revealed overexpressed recombinant MP, which is in reasonably well-folded form (Supporting Information Figure S2), especially when considering how difficult of a target BR2 is for recombinant production in bacteria.[16]

To examine whether the recorded effects for the different *rraA* genes tested are specific for BR2 or can be observed for other MPs as well, we evaluated the effect of their co-expression on the toxicity and volumetric fluorescence for six additional recombinant MPs of both prokaryotic and eukaryotic origin, which are characterized by different sizes, number of *trans*-membrane helices, topologies, and biochemical properties: the human central and peripheral cannabinoid receptors (CB1 and CB2), the human tetraspan-like MP clarin-1 (CLRN1), the *E. coli* multidrug transporter MdfA, the large mechano-sensitive ion channel (MscL) from *Mycobacterium tuberculosis,* and the deltarhodopsin from the archaeon *Haloterrigena turkmenica* (HtdR) (Table 2). Overexpression of CLRN1-GFP, CB1-GFP, and CB2-GFP, in the absence and presence of the selected *rraA* variants, resulted in effects similar to those observed with BR2, that is, the *rraA* genes from *P. mirabilis*, *P. stuartii*, *K. pneumoniae*, *E. gergoviae*, *Serratia marcescens*, *Salmonella enterica,* and *A. thaliana* were again the most efficient suppressors of MP-induced toxicity and enhancers of volumetric MP-GFP fluorescence, thus indicating increased productivity for all three proteins tested. Among them, PmRraA was again the most efficient and its capabilities surpassed those of the EcRraA of SuptoxR both in terms of toxicity suppression (10−60% higher final OD_{600}) and volumetric fluorescence (suggesting 30−60% higher volumetric productivity) (Figure 3a−c).

HtdR is a light-driven outward proton pump, which can bind the chromophore all-trans-retinal and acquire a characteristic

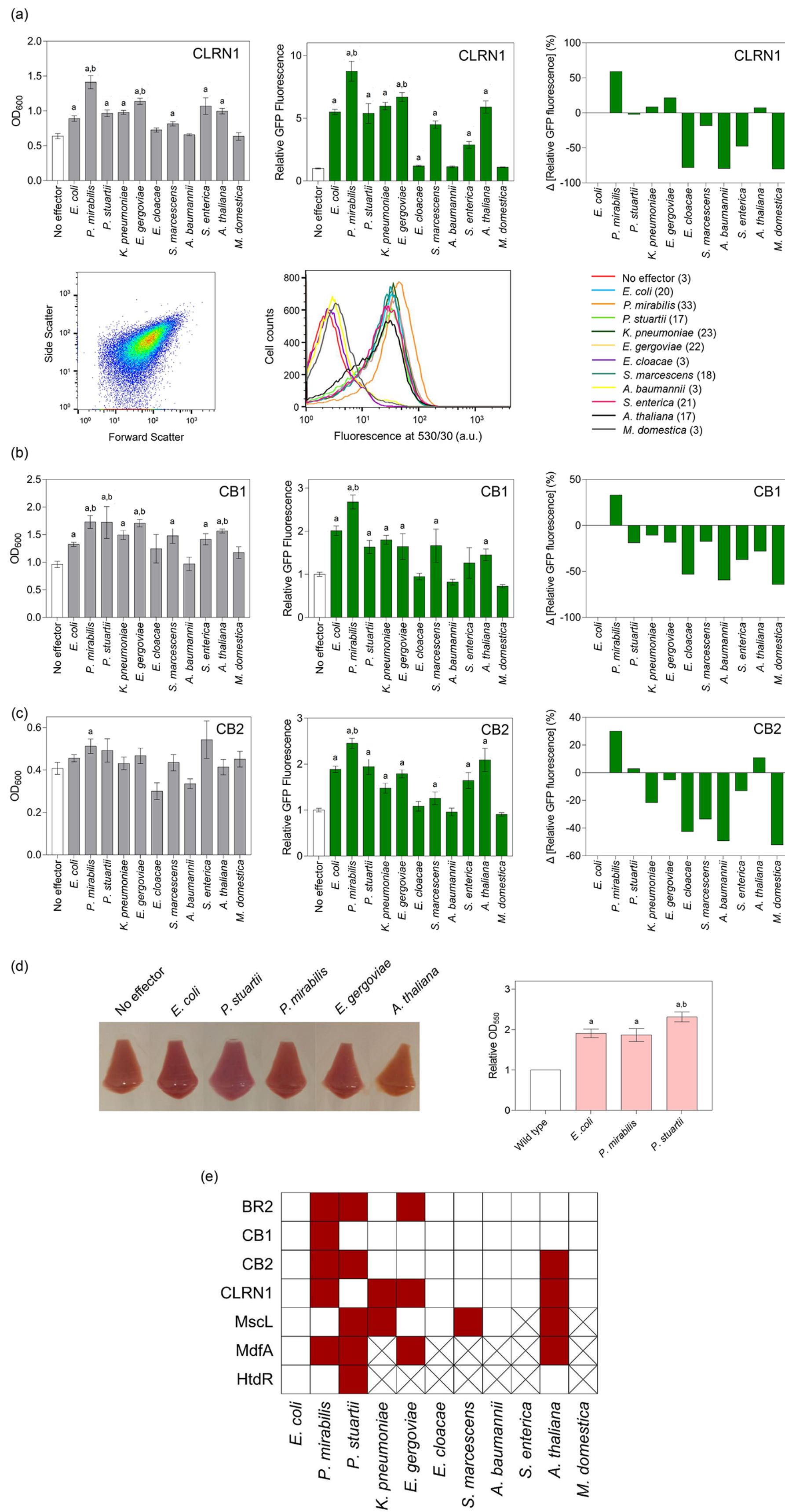

Figure 3. Co-expression of various RraA homologues from bacteria and plants results in suppressed MP-induced toxicity and enhanced accumulation of membrane-embedded protein at levels similar to or higher than those of the *E. coli* RraA of SuptoxR for a number of recombinant MPs with different characteristics. (a) (top left) Effect of the overexpression of different *rraA* genes on the growth of *E. coli* MC1061 cells producing CLRN1-GFP. OD_{600}: optical density at the end of the CLRN1-GFP overexpression process; (top middle) fluorescence of *E. coli*

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Figure 3. continued

MC1061 cells producing CLRN1-GFP in the absence (no effector) and presence of *rraA* overexpression corresponding to equal culture volumes; (top right) percentage of increase in the CLRN1-GFP fluorescence compared to SuptoxR. Δ[Relative GFP fluorescence] (%): [(GFP fluorescence with *rraA* co-expression from each organism—GFP fluorescence with *E. coli rraA* co-expression)/GFP fluorescence with *E. coli rraA* co-expression] × 100; (bottom) individual fluorescence of *E. coli* MC1061 cells producing CLRN1-GFP in the absence (no effector) and presence of *rraA* overexpression as measured by flow cytometry. (b) As in (a) for CB1-GFP. (c) As in (a) for CB2-GFP. (d) (left) Pellets of *E. coli* MC1061 cells producing HtdR in the presence of 10 μM all-*trans*-retinal in the absence (no effector) and presence of *rraA* overexpression derived from equal culture volumes. Purple coloring indicates the production of well-folded and functional HtdR. (right) Relative absorbance at 550 nm of DDM-extracted HtdR acquired from selected cell lysates from Figure 3c, left. (e) Diagram summarizing the effects of co-expression of the different *rraA* genes evaluated on the volumetric productivity of all the recombinant MPs tested in the present work, compared to the *E. coli rraA* of the original SuptoxR strain. A red square denotes an increase compared to the *E. coli rraA* of the original SuptoxR strain, while a white square denotes either no effect or decrease. A crossed square indicates that the particular test has not been performed. The reported values correspond to the mean value from at least three independent experiments each one performed in replica triplicates ± s.e.m. *a* denotes a statistically significant difference ($p < 0.05$) with the "no effector" sample, while *b* denotes a statistically significant difference with the "*E. coli*" sample.

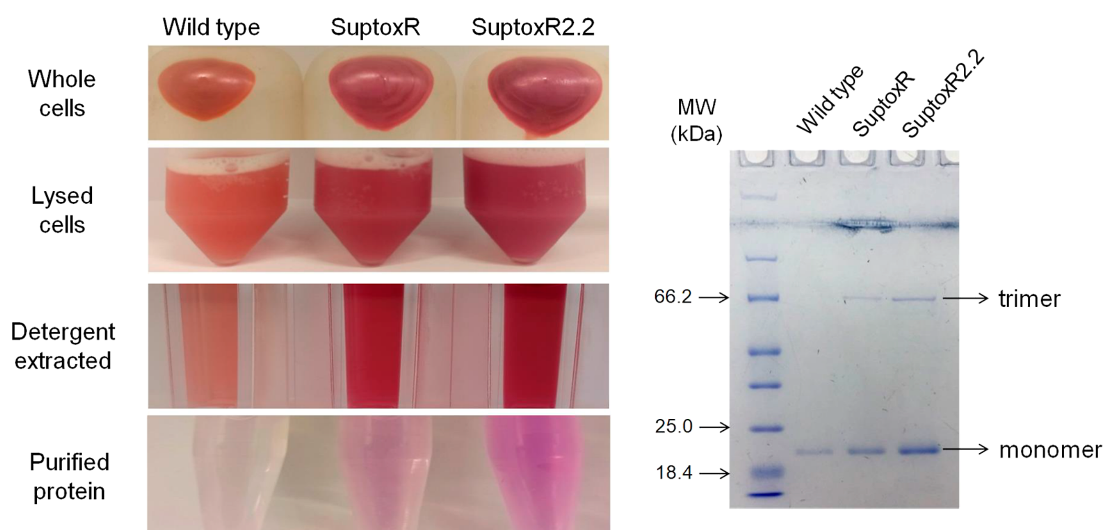

Figure 4. Second-generation *E. coli* SuptoxR strains produce significantly enhanced amounts of isolated functional recombinant MP. (a) Photographs of purple-colored bacterial cell pellets, lysates, detergent extracts, and purified protein derived from equal culture volumes of *i* MC1061 (wild type), SuptoxR, and SuptoxR2.2 cells producing HtdR in the presence of 10 μM all-*trans*-retinal. (b) SDS−PAGE analysis of the isolated HtdR from (a) and stained with Coomassie brilliant blue. MW: molecular weight marker. The numbers indicate the corresponding molecular masses in kDa.

purple color with an absorption maximum at ∼550 nm when properly folded.[31] Microbial rhodopsins, such as HtdR, have proven to be invaluable tools for optogenetic regulation applications.[32] Furthermore, monitoring the levels of their characteristic purple coloring is a very convenient reporter of functional MP production.[13,21,22,33] When HtdR was overexpressed along with each one of the five RraA variants, which were found as the most efficient production enhancers in the previous three cases (*E. coli*, *P. mirabilis*, *P. stuartii*, *E. gergoviae*, and *A. thaliana*), we observed that, apart from the *A. thaliana* RraA, all other variants were efficient in enhancing the levels of well-folded, functional HtdR compared to wild-type *E. coli* (Figure 3d, left). Among them, PsRraA was the most potent one and yielded levels of functional HtdR, which surpassed those of the EcRraA of SuptoxR (Figure 3d).

Finally, we evaluated the effect of the overexpression of the five best-performing *rraA* genes mentioned above on MdfA-GFP and MscL-GFP cytotoxicity and production. We observed, once again, that all five effectors were capable of enhancing recombinant MP volumetric fluorescence compared to wild-type *E. coli* (Supporting Information Figure S3). For MdfA, the *E. gergoviae* and the *P. stuartii* RraAs were found to be the most efficient effectors, while for MscL, the *A. thaliana* and *P. stuartii* RraAs yielded the highest volumetric productivity enhancements (Supporting Information Figure S3). In our previous studies, we have shown that SuptoxR-produced MscL is correctly folded and adopts a symmetric pentameric arrangement in the closed conformation that is in structural agreement with the solved crystal structure of this channel (PDB 2OAR).[13]

Construction of the Second-Generation Strains SuptoxR2.1 and SuptoxR2.2. Among the naturally occurring RraA proteins found to act as suppressors of MP-induced toxicity and enhancers of recombinant MP production and after taking into account the expression tests for all seven recombinant MPs we studied, PmRraA and PsRraA were the effectors that proved to have improved performance compared to the EcRraA of SuptoxR in most cases (Figure 3e). Based on this, we constructed two second-generation SuptoxR strains, termed SuptoxR2.1 and SuptoxR2.2, which overexpress the PmRraA and the PsRraA under the control of the *araBAD* promoter, respectively (Figure 1). Which SuptoxR2.X strain was optimal, depended on the particular MP target being produced: for all human MPs tested here, PmRraA was the most potent effector, while for the prokaryotic MPs, PsRraA typically yielded the largest or one of the largest increases in

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total and/or functional MP production. PmRraA and PsRraA are not necessarily the best MP-production-promoting effectors for all MP targets for recombinant production, and thus, it is likely that for other MPs, RraA proteins other than PmRraA and PsRraA from our collection or from other organisms not investigated here, may be optimal.

To evaluate the yields of purified recombinant MP, which can be achieved with our second-generation SuptoxR strains, we produced polyhistidine-tagged versions of HtdR in SuptoxR2.2. SuptoxR2.2 accumulated significantly enhanced levels of final bacterial biomass (Figure 4a), as well as total and detergent-extractable functional HtdR compared to both wild-type *E. coli* and the original SuptoxR (Figure 4a). HtdR overexpressed in wild-type *E. coli*, SuptoxR, and SuptoxR2.2 under identical conditions was solubilized using *n*-dodecyl *β*-D-maltoside (DDM) from total membrane fractions and was isolated using a two-step purification process, which included immobilized metal affinity chromatography (IMAC) and SEC. The yield of isolated HtdR from SuptoxR2.2 was increased by >400% compared to wild-type *E. coli* and by >100% compared to the original SuptoxR and calculated to result in ∼5 mg of pure protein per L of shake flask culture. SDS−PAGE analysis of the SEC peaks corresponding to isolated HtdR revealed primarily a band exhibiting electrophoretic mobility with an apparent molecular mass of ∼19 kDa, which corresponds to the monomeric protein (Figure 4b and Supporting Information Figure S4; detergent-extracted MPs frequently run faster on SDS−PAGE than expected according to their molecular weight[16]), while protein dimers and trimers were also visible by SDS−PAGE and western blotting, albeit to significantly lower levels. At the same time, the isolated protein exhibited the characteristic purple color of well-folded and functional HtdR (Figure 4a). When combined, these analyses demonstrate that our second-generation SuptoxR strains can provide isolated recombinant MP of high purity and quality, which are sufficient for biochemical and structural characterization purposes.

RraA was discovered in 2003 as a protein factor with the ability to inhibit the mRNA-degrading activity of RNase E, and it was found that *rraA* overexpression can affect the levels of more than 2000 different mRNAs in *E. coli*.[15] A number of these mRNAs were found to encode proteins, which are associated with cell envelope biosynthesis and anaerobic metabolism.[15] Interestingly, compromised cell envelope integrity and impaired formation of respiratory chain complexes in the cytoplasmic membrane have been found previously to be prominent consequences of MP overexpression.[9] In our prior work, we have demonstrated that the beneficial effects of RraA on recombinant MP production are mediated by the action of RNase E.[14] Based on this, we have hypothesized that RraA suppresses MP-induced toxicity and promotes MP accumulation by an indirect manner that involves stabilization of the mRNA and the subsequent increase of the cellular abundance of certain MP-productivity-promoting factors, possibly involved in cell envelope biosynthesis and/or (an)aerobic metabolism. We have favored the notion that these are multiple factors that function synergistically rather than one individual effector since, in the latter case, the single effector is expected to have been identified in our initial screen for suppressor of MP-induced toxicity, where RraA was initially selected.[12] Since it is currently unknown exactly how RraA interacts with RNase E, it is very difficult to predict how different sequence variations in RraA affect RNase E inhibition and, consequently, how the transcriptomic profile of *E. coli* changes upon overexpression of different RraA variants. Thus, we can only speculate that the different RraA variants investigated here, when overexpressed and produced at substantial levels, can interact with the *E. coli* RNase E in a slightly different way in each case, thus resulting in a slightly altered transcriptomic profile, where the hypothesized MP-promoting factors mentioned above are found in different relative abundances. The fact that different RraA variants appear to be optimal for different MPs may be suggesting that a fine balance between the optimal levels of the RraA-associated MP-promoting factors and the maximal levels of production for different recombinant MPs exist.

In conclusion, our results demonstrate that various RraA proteins from bacteria and plants can efficiently suppress MP-induced toxicity and enhance MP production when co-expressed in *E. coli*. More importantly, certain natural variants of the EcRraA, such as the ones from *P. mirabilis* and *P. stuartii*, can frequently outperform the MP-enhancing capabilities of the EcRraA of our original SuptoxR strain. By using these enhanced-performance variants from *P. mirabilis* and *P. stuartii*, we have constructed two second-generation *E. coli* SuptoxR strains, termed SuptoxR2.1 and SuptoxR2.2, respectively, and we have found that, either SuptoxR2.1 or SuptoxR2.2 or both, can often achieve even further increased accumulation of well-folded recombinant MPs compared to the original SuptoxR strain. Finally, we have demonstrated that the use of the new SuptoxR2.X strains enables the production of recombinant MPs of quality and quantity suitable for functional and structural studies. Thus, we anticipate that SuptoxR2.X will become broadly utilized expression hosts for recombinant MP production in bacteria.

■ MATERIALS AND METHODS

Identification and Selection of Natural RraA Variants. The search for RraA proteins from proteobacteria and plants was performed using NCBI BLASTp,[34] following a search for protein sequences similar to the *E. coli* RraA. The selection of specific organisms as sources of RraA proteins was based on the different levels of sequence similarity/identity.

Expression Vector Construction. All enzymes for cloning of recombinant DNA were purchased from New England Biolabs. The construction of the expression vectors pASKBR2-EGFP, pASKMdfA-EGFP, and pSuptoxR (pBAD33RraA) has been previously described.[12] pASKCLRN1-EGFP was constructed by replacing the *BDKRB*2 gene (encoding for BR2) in the pASKBR2-EGFP vector with the *CLRN*1 gene (Addgene NM_174878.2) using the restriction enzymes *Xba*I and *BamH*I. For the construction of the vectors pBAD33RraA$_{P.\ mirabilis}$ (pSuptoxR2.1), pBAD33RraA$_{P.\ stuartii}$ (pSuptoxR2.2), pBAD33RraA$_{K.\ pneumoniae}$, pBAD33RraA$_{E.\ gergoviae}$, pBAD33RraA$_{E.\ cloacae}$, pBAD33RraA$_{S.\ marcescencens}$, pBAD33RraA$_{A.\ baumanii}$, pBAD33RraA$_{S.\ enterica}$, and pBAD33RraA$_{M.\ dometica}$, the corresponding *rraA* genes were amplified using sequence-specific DNA primers and the genomic DNA of each organism as the template. pBAD33RraA$_{A.\ thaliana}$ was constructed using a codon-optimized synthetic gene (Genscript Corp). Each forward primer contained an *Xba*I site, an optimized Shine-Dalgarno sequence (AGGAGGAAACG), and a start codon, while each reverse primer contained a 6xHis tag, a stop codon, and a *Hind*III site. The resulting PCR products were cloned into the pBAD33 vector,[35] which was digested with the same

restriction enzymes. The correct sequences for all constructs were verified by DNA sequencing.

MP Overexpression. *E. coli* MC1061 cells freshly transformed with the appropriate vectors were used for all protein production experiments. Liquid Luria−Bertani (LB) cultures containing the appropriate combination of antibiotics (100 μg/mL ampicillin, 40 μg/mL chloramphenicol) were inoculated from single bacterial colonies and incubated overnight at 37 °C. These cultures were used with a 1:50 dilution to inoculate fresh LB cultures containing 0.2% arabinose and were grown at 30 °C to an optical density at 600 nm (OD_{600}) of 0.3−0.5 with shaking. 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 of anhydrotetracycline (aTc; Sigma) overnight.

Membrane Isolation. Total membrane fractions were isolated from 1 L LB cultures. Cells were harvested by centrifugation (4000 g for 10 min) and resuspended in 10 mL of cold lysis buffer (300 mM NaCl, 50 mM NaH_2PO_4, 15% glycerol, 2 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 g for 15 min. The supernatant was then subjected to ultracentifugation on a Beckman 70Ti rotor at 42,000 rpm (130,000 g) for 1 h at 4 °C. The resulting pellet was finally resuspended in 5 mL of cold lysis buffer and homogenized.

Western Blot and In-Gel Fluorescence Analysis. Proteins were analyzed by SDS−PAGE in 12% gels under fully denaturing conditions (boiling of the samples for 10 min prior to loading) or semi-denaturing conditions (no boiling of the samples prior to loading). In-gel fluorescence was analyzed on a UVP ChemiDoc-It2 imaging system equipped with a cooled CCD camera and a GFP filter, after exposure for about 3 s, following SDS−PAGE analysis under semi-denaturing conditions. For Western blot analysis, proteins were transferred to polyvinylidene fluoride membranes (Merck) for 45 min at 12 V on a semi-dry blotter (Thermo Fisher Scientific). Membranes were blocked with 5% (w/v) non-fat dried milk in Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST) overnight at 4 °C. After three washes with TBST, they were incubated with the appropriate antibody dilution in TBST containing 0.5% (w/v) non-fat dried milk at room temperature for 1 h. Specifically, the utilized antibodies were a mouse monoclonal anti-polyhistidine antibody (Sigma) at 1:3000 dilution (conjugated with horseradish peroxidase), a mouse monoclonal anti-GFP antibody (Takara) at 1:20,000 dilution, or a mouse monoclonal anti-FLAG antibody (Sigma) at 1:2500 dilution, with a horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody at 1:4000 dilution. The proteins were visualized using a ChemiDoc-It2 Imaging System (UVP), after triple membrane washing with TBST.

Bulk Fluorescence Measurements. Cells corresponding to 0.5 mL of culture were harvested and resuspended in 100 μL of 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 or a Cytation 5 (BioTek) plate reader.

Fluorescence Analysis by Flow Cytometry. ~10^7 cells were resuspended in 1 mL of 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). Data were analyzed with FlowJo 7.6.2.

Membrane Extraction, Purification and Quantification of *H. turkmenica* Deltarhodopsin. The procedure followed to extract HtdR from bacterial cultures has been described previously.[13] Briefly, HtdR was overexpressed in *E. coli* MC1061 cells (wild type), SuptoxR and SuproxR2.2 cells. Pellets derived from each culture were resuspended in 7.5 mL of cold lysis buffer (300 mM NaCl, 50 mM NaH_2PO_4, 15% glycerol, 2 mM dithiothreitol, pH 7.5) and lysed by brief sonication steps on ice. Total cell lysates were incubated with 2.5% (w/v) DDM (Glycon Biochemicals) and 0.1 mM phenylmethylsulfonylfluoride (PMSF) by rotation at 180 rpm for 24 h at 4 °C in the dark to extract HtdR. The samples were then ultracentrifuged using a Beckman 70-Ti rotor at 42,000 rpm (130,000 g) at 4 °C for 1 h. If after the centrifugation the pellet was colorless and all the purple-colored HtdR were successfully extracted, the supernatant was collected and analyzed by measuring the absorbance at 550 nm.

HtdR purification was performed by IMAC using an ÄKTA start system (GE Healthcare) with a 5 mL HisTrap column (GE Healthcare) equilibrated with 25 mL of equilibration buffer (300 mM NaCl, 50 mM NaH_2PO_4, 0.1% (w/v) DDM, pH 8). The DDM-extracted fraction was loaded onto the column, washed initially with 75 mL of equilibration buffer and then with 7.5 mL of the same buffer containing 100 mM imidazole. The protein was eluted from the column with 10 mL of elution buffer (200 mM imidazole, 300 mM NaCl, 50 mM NaH_2PO_4, 0.1% (w/v) DDM, pH 8). The eluate was concentrated to 600 μL using an Amicon Ultra-15 Centrifugal Filter Unit with a cutoff at 10 kDa (Merck Millipore). The IMAC eluate was purified further by SEC using a Superdex 200 column on an ÄKTA pure system (GE Healthcare), equilibrated with PBS (pH 7.4) by the addition of 0.1% (w/v) DDM. Quantification of purified protein was performed by measuring the absorbance at 280 nm using a Cytation 5 plate reader (BioTek) and by estimating the final concentration based on HtdR's extinction coefficient [$\varepsilon_{280} = 2.15(\text{mg/mL})^{-1}\cdot\text{cm}^{-1}$].

Fluorescence SEC. BR2-GFP was expressed in 1 L cultures as described above. The resulting pellet was resuspended in 7.5 mL of PBS and lysed by brief sonication steps on ice. Total cell lysate was centrifuged at 10,000 g for 30 min, the supernatant was collected and then ultracentrifuged using a 70-Ti rotor at 42,000 rpm (130,000 g) at 4 °C for 1 h. The membrane pellet was collected and solubilized in 8.5 mL of solubilization buffer (10 mM HEPES pH 7.4, 400 mM NaCl, 10% (v/v) glycerol, 1.5% (w/v) DDM, 0.12 mM PMSF) under rotation at 180 rpm at 4 °C for 1 h. The solubilized membrane pellet was then analyzed by SEC using a Superdex 200 column on an ÄKTA pure system (GE Healthcare) paired with a RF-20A fluorescent detector (Shimadzu). The column equilibration buffer contained 10 mM HEPES (pH 7.4), 400 mM NaCl, and 0.05% (w/v) DDM. The resulting fractions corresponding to fluorescent peaks were analyzed further by native PAGE/in-gel fluorescence and PAGE/western blotting.

Statistical Analysis. Graphs were prepared using Prism 7.00 (GraphPad Software Inc., La Jolla, CA). Statistical analyses were performed using Prism and Microsoft Office 2007 Excel (Microsoft Corporation, Redmond, WA) software packages. Mean values were compared using unpaired t tests. Data in all assays correspond to the mean values of at least three independent experiments, each one performed in three replicates. Error bars denote ±standard error of mean.

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Differences in the values among different samples were considered as statistically significant when $p \leq 0.05$.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.1c00598.

Sequence comparison of the tested RraA proteins with the *E. coli* RraA of SuptoxR; FSEC profile of BR2-GFP expressed in SuptoxR2.1; co-expression of various RraA homologues enhances bacterial recombinant production of the *E. coli* MdfA and the *M. tuberculosis* MscL; and second-generation *E. coli* SuptoxR strains increase the production of isolated and folded recombinant MPs (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Georgios Skretas – *Institute of Chemical Biology, National Hellenic Research Foundation, Athens 11635, Greece;* orcid.org/0000-0003-1320-9092; Phone: +302107273736; Email: gskretas@eie.gr

Authors

Eleni Vasilopoulou – *Institute of Chemical Biology, National Hellenic Research Foundation, Athens 11635, Greece; Department of Biochemistry and Biotechnology, University of Thessaly, Viopolis, Larisa 41500, Greece;* orcid.org/0000-0003-1071-5942

Artemis Giannakopoulou – *Institute of Chemical Biology, National Hellenic Research Foundation, Athens 11635, Greece*

Charalampos Kapsalis – *Institute of Chemical Biology, National Hellenic Research Foundation, Athens 11635, Greece*

Myrsini Michou – *Institute of Chemical Biology, National Hellenic Research Foundation, Athens 11635, Greece; Department of Biochemistry and Biotechnology, University of Thessaly, Viopolis, Larisa 41500, Greece*

Aristeidis Michoglou-Sergiou – *Institute of Chemical Biology, National Hellenic Research Foundation, Athens 11635, Greece*

Fragiskos N. Kolisis – *Laboratory of Biotechnology, School of Chemical Engineering, National Technical University of Athens, Athens 15772, Greece*

Complete contact information is available at: https://pubs.acs.org/10.1021/acssynbio.1c00598

Author Contributions

G.S. conceived, designed the research, and coordinated the project. E.V., A.G., C.K., M.M., and A.M.S. carried out the research. E.V., F.N.K., and G.S. secured funding and analyzed the data. G.S. and E.V. wrote the paper. All authors read and approved the final manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

MP, membrane protein; aTc, anhydrotetracycline; GPCR, G-protein-coupled receptor; BR2, bradykinin receptor 2; CB1, central cannabinoid receptor; CB2, peripheral cannabinoid receptor; CLRN1, tetraspan-like membrane protein clarin-1; MscL, large conductance mechanosensitive ion channel; MdfA, multidrug efflux transporter; HtdR, *Haloterrigena turkmenica* deltarhodopsin; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; IMAC, immobilized metal affinity chromatography; SEC, size-exclusion chromatography; DDM, *n*-dodecyl *β*-D-maltoside; PMSF, phenylmethylsulfonyl fluoride; LB, Luria–Bertani; PBS, phosphate-buffered saline; OD, optical density; TBST, Tris-buffered saline + 0.1% Tween-20; EcRraA, *E. coli* RraA; PmRraA, *P. mirabilis* RraA; PsRraA, *P. stuartii* RraA

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