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Escherichia coli strains with precise domain deletions in the ribonuclease RNase E can achieve greatly enhanced levels of membrane protein production

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Abstract

Escherichia coli is one of the most widely utilized hosts for production of recombinant membrane proteins (MPs). Bacterial MP production, however, is usually accompanied by severe toxicity and low-level volumetric accumulation. In previous work, we had discovered that co-expression of RraA, an inhibitor of the RNA-degrading activity of RNase E, can efficiently suppress the cytotoxicity associated with the MP overexpression process and, simultaneously, enhance significantly the cellular accumulation of membrane-incorporated recombinant MPs in bacteria. Based on this, we constructed the specialized MP-producing *E. coli* strain SuptoxR, which can achieve dramatically enhanced volumetric yields of well-folded recombinant MPs. In the present work, we have investigated whether domain deletions in the *E. coli* RNase E, which exhibit reduced ribonucleolytic activity, can result in suppressed MP-induced toxicity and enhanced recombinant MP production, in a manner resembling the conditions of *rraA* overexpression in *E. coli* SuptoxR. We have found that some strains encoding specific RNase E truncation variants can achieve significantly enhanced levels of recombinant MP production. Among these, we have found a single RNase E variant strain, which can efficiently suppress MP-induced toxicity and achieve greatly enhanced levels of recombinant MP production for proteins of both prokaryotic and eukaryotic origin. Based on its properties, and in analogy to the original SuptoxR strain, we have termed this strain SuptoxRNE22. *E. coli* SuptoxRNE22 can perform better than commercially available bacterial strains, which are frequently utilized for recombinant MP production. We anticipate that SuptoxRNE22 will become a widely utilized host for recombinant MP production in bacteria.

KEYWORDS

Escherichia coli, membrane protein, recombinant protein production, RNase E, RraA, SuptoxR

1 | INTRODUCTION

Membrane proteins (MPs) constitute a highly important protein group in all living cells and organisms. They are basic components of membrane bilayers, where they perform multiple essential functions, such as maintenance of structural integrity, cellular signaling, as well as nutrient and ion transport (von Heijne, 2007). Their great importance is reflected by the fact that, among all proteins encoded in the genome of prokaryotic and eukaryotic organisms, 20%–30% correspond to MPs (Wagner et al., 2006). Due to their critical functions, MPs represent more than half of all known drug targets (Yildirim et al., 2007). Furthermore, there is a constant focus on investigating their structure and function to gain in-depth knowledge, which can then be utilized for the development of new MP-targeting therapeutic molecules (Santos et al., 2017).

To acquire sufficient quantities of isolated MPs for performing the necessary biochemical and structural analyses, it is most common to produce them recombinantly in heterologous hosts, such as bacteria, yeast, insect cells, mammalian cells or transgenic animals. This occurs because, typically, natural abundance of MPs occurs at very low levels (Sarramegna et al., 2003). The bacterium *Escherichia coli* has been one of the most popular and successful host organisms for recombinant production of both soluble and membrane proteins (Makino et al., 2011). Among its many advantages, *E. coli* is preferred due to its facile and low-cost culture conditions, its rapid growth and the numerous available tools for its genetic manipulation (Mathieu et al., 2019). As a result, approximately 20% of all MP structures deposited at Protein Data Bank (PDB) have been derived from proteins, which have been produced recombinantly in *E. coli* (Dilworth et al., 2018). Despite the many successful examples, bacterial MP production has proven to be a particularly challenging process in many cases, which is often accompanied by severe toxicity for the expression host and low levels of cellular accumulation of the target recombinant MP in membrane-integrated form (Wagner et al., 2006). The combination of these two limiting factors, results in many failed attempts of recombinant MP production in bacteria, especially for the recombinant MPs of eukaryotic origin, which are also the most valuable ones for a pharmaceutical development perspective (Gubellini et al., 2011; Miroux & Walker, 1996).

In an effort to address these limitations, in our previous work, we searched within the *E. coli* genome for single genes, which could act as efficient suppressors of the toxicity caused by the MP overexpression process in this host. One of the genes we identified as a potent suppressor of MP-induced toxicity was *rraA* (Gialama, Kostelidou, et al., 2017). Remarkably, we also found that *rraA* additionally acts as an enhancer of the levels of cellular accumulation of properly membrane-integrated recombinant MPs (Gialama, Kostelidou, et al., 2017). Based on these results, we engineered a new *E. coli* strain, termed SuptoxR (Suppressor of toxicity—RraA), which upon co-expression of the *rraA* gene can become resistant to MP-induced cytotoxicity and reach significantly higher levels of final biomass, while, at the same time, it can accumulate significantly enhanced levels of membrane-incorporated recombinant MPs on a per cell basis. The combination of these two beneficial effects allowed *E. coli* SuptoxR to achieve dramatically enhanced volumetric MP yields for a variety of prokaryotic and eukaryotic recombinant MPs (Gialama, Kostelidou, et al., 2017; Michou et al., 2019).

The *rraA* gene encodes for the protein RraA, which acts as an inhibitor of mRNA-degrading activity of the *E. coli* ribonuclease E (RNase E) (Lee et al., 2003). We have found that the observed beneficial effects of RraA on recombinant MP production are mediated through the action of RNase E, likely due to inhibition of its ribonucleolytic activity upon *rraA* overexpression and stabilization of the mRNA and up-regulation of specific —yet unknown— cellular factors, with the ability to promote bacterial recombinant MP production (Gialama, Delivoria, et al., 2017).

In the present work, and based on these previous observations, we have investigated whether a variety of domain deletions in *rne*, the gene encoding for the *E. coli* RNase E, which exhibit reduced ribonucleolytic activity while being permissive for viability, can result in suppressed MP-induced toxicity and enhanced recombinant MP production in bacteria, in a manner resembling the conditions of *rraA* overexpression in *E. coli* SuptoxR. We have found that certain strains expressing specific RNase E truncation variants can achieve significantly enhanced levels of recombinant MP production. Among these, we have found a single RNase E deletion strain, which can efficiently suppress MP-induced toxicity and achieve greatly enhanced levels of recombinant MP production compared to wild-type *E. coli*, for proteins of both prokaryotic and eukaryotic origin. Based on its properties, and in analogy to the original SuptoxR strain, which served as the original motivation for this study, this strain is termed SuptoxRNE22 (Suppressor of toxicity—deletion 22 in RNase E). *E. coli* SuptoxRNE22 appears to be a versatile expression host, which is compatible with a variety of frequently utilized promoters and plasmid systems. Importantly, we have found that *E. coli* SuptoxRNE22 can perform better than commercially available bacterial strains, which are frequently utilized for recombinant MP production.

2 | RESULTS

2.1 | *E. coli* cells expressing truncated forms of RNase E can efficiently suppress MP-induced toxicity and enhance MP productivity

In our previous work, we used a genetic screen and we discovered that overexpression of the effector gene *rraA*, which encodes the protein RraA (Regulator of ribonuclease activity A), can efficiently suppress the cytotoxicity, which is frequently associated with the MP overexpression process and, simultaneously, enhance significantly the cellular accumulation of membrane-incorporated recombinant MPs in bacteria (Gialama, Kostelidou, et al., 2017). Based on this, we constructed the specialized MP-producing *E. coli* strain SuptoxR (Gialama, Kostelidou, et al., 2017) and its second-generation derivatives (Vasilopoulou et al., 2022), which can achieve dramatically enhanced volumetric yields of well-folded recombinant MPs of both prokaryotic and eukaryotic origin (Gialama, Kostelidou, et al., 2017; Michou et al., 2019; Vasilopoulou et al., 2022).

RraA acts as inhibitor of the mRNA-degrading activity of RNase E in *E. coli* and other bacteria (Lee et al., 2003). RNase E is a large, non-specific endonuclease, which is a major player in RNA processing and mRNA degradation in bacteria, which is essential for viability (Caruthers et al., 2006). The *E. coli* RNase E consists of 1061 amino acids and is divided in two distinct functional regions: a highly conserved N-terminal catalytic domain (NTD) responsible for its ribonucleolytic activity, and a C-terminal domain (CTD), which is intrinsically disordered and provides the scaffold for interaction with multiple proteins (Callaghan et al., 2004; Khemici et al., 2008) (Figure 1). For the degradation of mRNA molecules inside the cell, RNase E can act alone, or as part of a multifunctional enzyme complex, termed the RNA degradosome (Carpousis, 2007). This RNA-degrading molecular machine is created with the CTD as a core, onto which three additional enzymes are binding: a polynucleotide phosphorylase (PNPase), the ATP-dependent DEAD-box helicase RhlB, and the glycolytic enzyme enolase (Carpousis, 2007). RraA is normally interacting with the CTD of RNase E and functions as an inhibitor of its ribonucleolytic activity (Gorna et al., 2010).

In our previous work, we have shown that the MP production-promoting effects of RraA are mediated through the action of RNase E (Gialama, Delivoria, et al., 2017). Based on this, in the present work, we investigated whether specific genetic *rne* modifications encoding for truncation RNase E variants exhibiting reduced ribonucleolytic activity can result in suppressed MP-induced toxicity and enhanced recombinant MP production in bacteria, in a manner resembling the conditions of *rraA* overexpression in *E. coli* SuptoxR. As mentioned above, RNase E activity is essential for *E. coli* survival and, thus, *rne* null strains are not viable. However, since only the NTD is essential for viability, we used nine previously generated *E. coli* strains carrying different genetic deletions on their native *rne* gene (ENS134-X strains) (Figure 1, Table 1) (Leroy et al., 2002). These strains express either wild-type (WT) endonuclease (ENS134) or variants of RNase E with truncations (ENS134-10–ENS134-24) or complete deletion (ENS134-2) of the CTD (Lopez et al., 1999). As determined previously, these mutant RNase E proteins yield viable *E. coli* cells, which exhibit *bona fide* reduced levels of ribonucleolytic activity, in all cases but one (ENS134-10) (Lopez et al., 1999).

To study the effect of truncated RNase E proteins on MP-induced toxicity and MP productivity, we used the human bradykinin receptor 2 (BR2) as a model recombinant MP. BR2 is a member of the G protein-coupled receptor (GPCR) superfamily, whose overexpression in *E. coli* has been shown to be highly toxic (Gialama, Kostelidou, et al., 2017; Link et al., 2008). To enable easy monitoring of the relative accumulation levels of membrane-incorporated MP, BR2 was expressed as a fusion with the enhanced green fluorescent protein (GFP) at its C terminus. We selected to use BR2-GFP fusions for this purpose because it has been found that the fluorescence levels of *E. coli* cells expressing MP-GFP fusions, correlate well with the amount of MP, which is being produced and properly integrated in the bacterial inner membrane (Drew et al., 2001). Furthermore, MP-GFP fusions have been utilized extensively for these purposes by us (Gialama, Delivoria, et al., 2017; Gialama, Kostelidou, et al., 2017; Michou et al., 2020; Skretas et al., 2012; Skretas & Georgiou, 2009, 2010) and many others groups in previous studies (Angius et al., 2018; Daley et al., 2005; Drew et al., 2006; Kim et al., 2017; Schlegel et al., 2012; Wagner et al., 2008).

The potential toxicity-suppressing effects exerted by the different *rne* mutants were investigated by recording the final growth levels of *E. coli* cells expressing wild-type (WT) RNase E (ENS134 strain) or one of its nine different truncated forms (ENS134-X strains) upon BR2-GFP overexpression from the pASK75 vector and the addition of its inducer anhydrotetracycline (aTc) (Skerra, 1994). The levels of final bacterial biomass were recorded by measuring the optical density at 600 nm (OD_{600}) at the end of the overexpression process. We observed that the strains ENS134-2, ENS134-21 and ENS134-22 carrying the genetic deletions *rne131*, *rneΔ21* and *rneΔ22*, respectively, exhibited increased cell densities upon cell harvesting (Figure 2a). More specifically, ENS134-2, ENS134-21 and

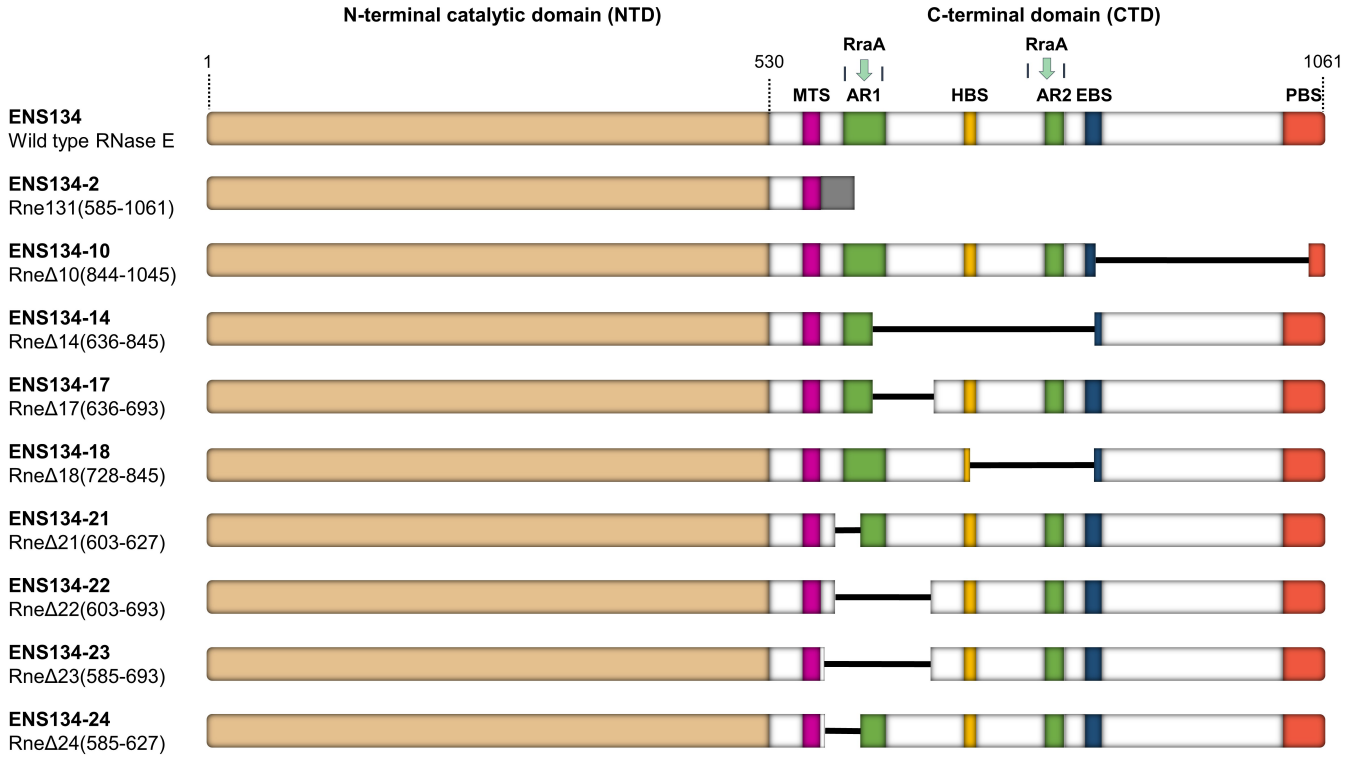

FIGURE 1 Schematic representation of the *E. coli* RNase E and the different truncated RNase E variants utilized in this study. RNase E is organized in two functional domains. The N-terminal domain (NTD; residues 1–529) encodes the catalytic activity of RNase E. The C-terminal domain (CTD; residues 530–1061) mediates all protein–protein interactions and forms the core of the RNA degradosome. The microdomains of the CTD are noted with different colors: white indicates intrinsically disordered regions, purple indicates the membrane targeting sequence (MTS; residues 565–582), green indicates the arginine-rich regions 1 and 2 (AR1 and AR2; residues 604–644 and 796–814, respectively), yellow indicates the helicase binding site (HBS; residues 719–731), blue indicates the enolase binding site (EBS; residues 834–850), and red indicates the PNPase-binding site (PBS; residues 1021–1031) (Bouvier & Carpousis, 2011). Known RraA interaction sites are indicated by light green arrows (Gorna et al., 2010).

ENS134-22 achieved 47%–126% increases in final OD_{600}, thus indicating that the severe toxicity associated with BR2 overexpression is significantly reduced in these strains. It must be noted that, in the absence of BR2 overexpression, the utilized ENS134-X strains exhibited reasonably similar growth kinetics and most of them did not present significant growth delays (Figure S2). The only notable exception was ENS134-14, which was found to grow more slowly compared to the other strains.

To compare the levels of cellular accumulation of membrane-embedded recombinant MP, that is, the relative productivity of membrane-bound MP on a per cell basis, we measured the individual cell fluorescence in the cultures of each bacterial strain expressing BR2-GFP using flow cytometry. BR2-GFP individual cell fluorescence was found to be significantly increased in ENS134-2, ENS134-21 and ENS134-22 compared to WT, a fact suggesting considerably enhanced cellular MP accumulation in these strains (Figure 2b). Thus, individual ENS134-2, ENS134-21 and ENS134-22 cells appear capable of suppressing the cytotoxicity induced by BR2 overexpression and, at the same time, of accumulating enhanced amounts of the otherwise highly toxic and poorly expressed recombinant MP. This behavior is very similar to the properties of *E. coli* SuptoxR, where the inhibitor of RNase E activity, RraA, is being overexpressed (Gialama, Kostelidou, et al., 2017).

As in *E. coli* SuptoxR, as well as its second-generation derivatives, the combination of suppressed BR2-induced toxicity and enhanced cellular MP accumulation in ENS134-2, ENS134-21 and ENS134-22 led to significantly increased levels of relative BR2-GFP fluorescence corresponding to equal volumes of bacterial cultures (volumetric productivity; Figure 2c). More specifically, ENS134-2, ENS134-21 and ENS134-22 achieved increases in relative volumetric BR2-GFP fluorescence ranging from 40% to 430%, with the most pronounced effects being recorded for ENS134-22. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)/western blot analysis of isolated total membrane fractions of ENS134, ENS134-2, ENS134-21 and ENS134-22 strains producing BR2-GFP,

TABLE 1 *E. coli* strains used in this study.

Strain	Genotype	Source
ENS134	BL21(DE3), *lacZ*::Tn10, *malP*pA534::PT71acZ::RBSIamB-Arg5	Prof. A. Carpousis
ENS134-2	ENS134 *rne131*	Prof. A. Carpousis
ENS134-10	ENS134*rneΔ10*	Prof. A. Carpousis
ENS134-14	ENS134*rneΔ14*	Prof. A. Carpousis
ENS134-17	ENS134*rneΔ17*	Prof. A. Carpousis
ENS134-18	ENS134*rneΔ18*	Prof. A. Carpousis
ENS134-21	ENS134*rneΔ21*	Prof. A. Carpousis
ENS134-22	ENS134*rneΔ22*	Prof. A. Carpousis
ENS134-23	ENS134*rneΔ23*	Prof. A. Carpousis
ENS134-24	ENS134*rneΔ24*	Prof. A. Carpousis
C41 (DE3)	F⁻*ompT gal dcm lon hsdSB* (rB- mB-) *λ*(DE3 [*lacI* lacUV5-T7 *gene* 1 *ind1 sam7 nin5*]) with modifications described by (Kwon et al., 2015) and (Schlegel et al., 2015)	Lucigen
C43 (DE3)	F⁻*ompT gal dcm lon hsdSB* (rB- mB-) *λ*(DE3 [*lacI* lacUV5-T7 *gene* 1 *ind1 sam7 nin5*]) with modifications described by (Kwon et al., 2015)	Lucigen
Lemo21 (DE3)	F⁻*ompT gal dcm lon hsdSB* (rB- mB-) *λ*(DE3 [*lacI* lacUV5-T7 *gene* 1 *ind1 sam7 nin5*]) pLemo	New England Biolabs

revealed that the observed enhancements in fluorescence occur indeed due to increased levels of BR2-GFP accumulation in the cell membrane and that this increased accumulation is particularly striking in the case of ENS134-22 (Figure 2d).

To examine the folding quality of the produced BR2-GFP, we also analyzed the in-gel fluorescence of these SDS-PAGE-analyzed total membrane fractions under semi-denaturing conditions. This method has been to found to provide a reliable semi-quantitative measurement of the amount of recombinant MP produced in well-folded form (Geertsma et al., 2008) and has been used extensively for quality control of recombinantly produced MPs in bacteria in different studies (Cuesta Bernal et al., 2021; Mathieu et al., 2019; Yousefian et al., 2021). This analysis showed that ENS134-21 and ENS134-22, not only achieve increased productivity of total membrane-bound protein but can also accumulate significantly enhanced amounts of well folded recombinant MP (Figure 2d). These results indicate that specific genetic *rne* modifications leading to reduced RNase E activity can result in significantly suppressed MP-induced toxicity and dramatically enhanced recombinant MP production in bacteria, which occurs in a manner similar to what we have observed when RNase E activity is being inhibited upon overexpression of its inhibitor RraA in *E. coli* SuptoxR (Gialama, Kostelidou, et al., 2017; Michou et al., 2019; Vasilopoulou et al., 2022).

2.2 | *E. coli* cells carrying the RneΔ22 truncation can accumulate significantly enhanced levels of both prokaryotic and eukaryotic recombinant MPs

Among all tested *E. coli* strains expressing truncated variants of RNase E, ENS134-2, ENS134-21 and ENS134-22 increased the accumulation of bacterial biomass and membrane-incorporated BR2-GFP. Interestingly, ENS134-22 carrying the RneΔ22 truncation was the most effective of all strains both in terms of toxicity suppression (>2-fold increase in final OD) and productivity (>2.5-fold increase in cellular productivity, >5-fold increase in volumetric productivity), compared to ENS134 expressing WT RNase E (Figure 2).

To investigate whether the observed positive effects on MP production are general and can be applied to more MPs or if they are specific only to BR2, we overexpressed three additional recombinant MPs of eukaryotic and prokaryotic origin in the selected bacterial strains and examined again final biomass accumulation and volumetric MP productivity in the resulting cultures. More specifically, we tested the human tetraspan-like MP clarin 1 (CLRN1), the large mechano-sensitive ion channel (MscL) from *Mycobacterium tuberculosis* and the deltarhodopsin from the archaeon *Haloterrigena turkmenica* (HtdR). The selected MPs have variable sizes, topologies, number of transmembrane helixes and biochemical properties (Table 2).

Overexpression of CLRN1-GFP and MscL-GFP in strains expressing truncated RNase E, resulted in suppressed MP-induced toxicity compared to WT strain in most cases. In cultures expressing the eukaryotic

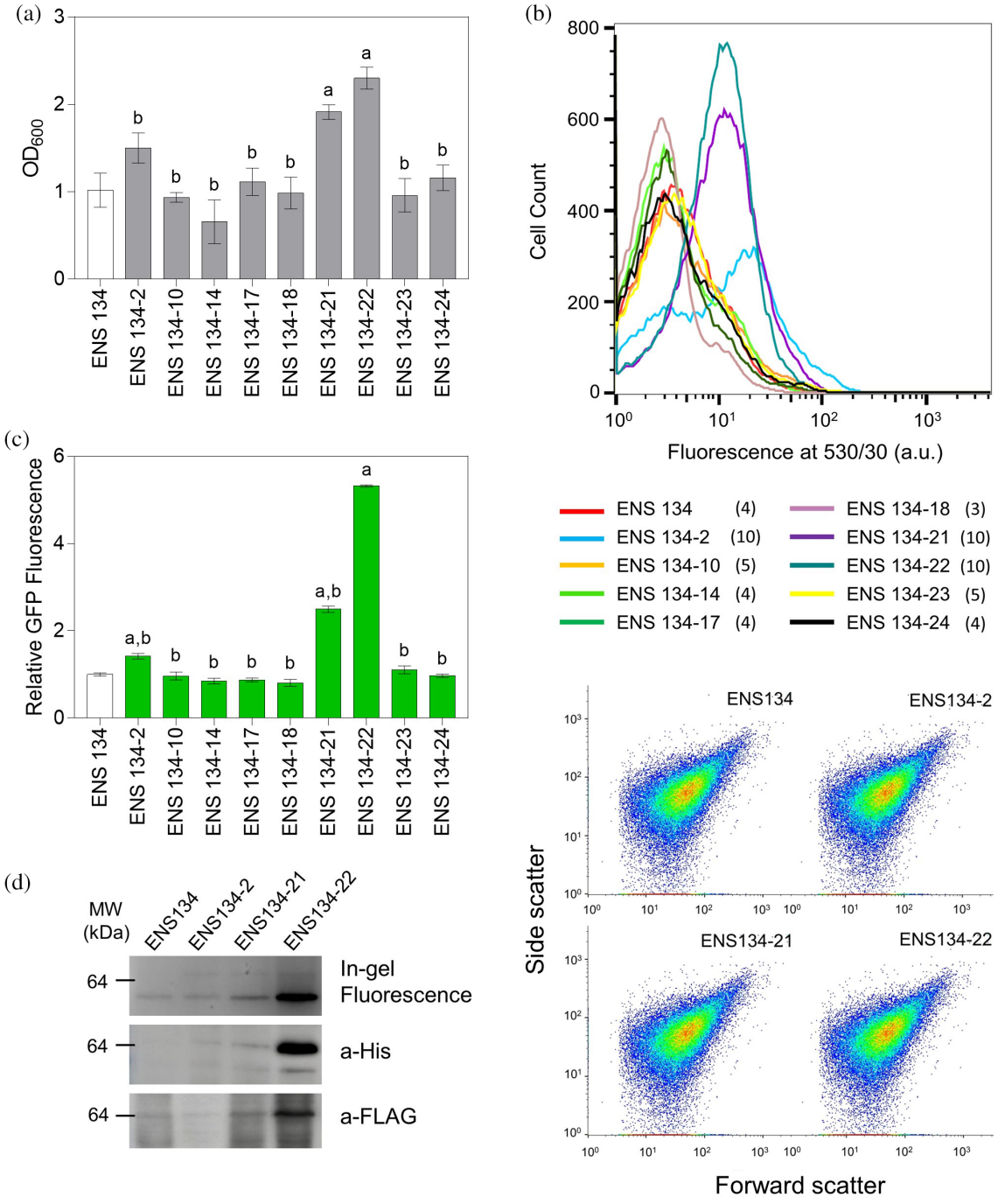

FIGURE 2 *E. coli* strains expressing truncation variants of RNase E results in suppressed MP-induced toxicity and enhanced MP accumulation. (a) Growth levels of WT *E. coli* and different ENS134-X cells expressing BR2-GFP. OD_{600}: optical density at 600 nm. (b) GFP fluorescence of individual ENS134-X cells expressing BR2-GFP as measured by flow cytometry. (c) Relative bulk fluorescence of equal culture volumes of different ENS134-X strains producing BR2-GFP compared to WT ENS134 strain. (d) Semi-denaturing SDS-PAGE analysis of isolated total membrane fractions of WT, ENS134-2, ENS134-21 and ENS134-22 cells expressing BR2-GFP, and visualization of the produced fusion by in-gel fluorescence (top), and western blotting using N-terminal anti-FLAG or a C-terminal anti-polyHis antibodies. In all panels BR2-GFP was expressed from pASKBR2-EGFP vector. In (a) and in (c) the reported values correspond to the mean value of at least three independent experiments performed in replica triplicates ± standard error of the mean (SEM). (a) denotes a statistically significant difference when compared with the ENS134 sample ($p < 0.05$), while (b) denotes a statistically significant difference when compared with the ENS134-22 sample ($p < 0.05$).

TABLE 2 MPs used in this study.

Membrane protein	Organism	Function	Number of TM helices	Topology	Mass (kDa)
BR2	*Homo sapiens*	Bradykinin receptor 2	7	N^{out}-C^{in}	44.5
CLRN1	*Homo sapiens*	Clarin-1—Member of the tetraspan family	4	N^{in}-C^{in}	25.7
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

CLRN1-GFP, OD_{600} values were 11%–103% higher than that of ENS134 in all cases but two (ENS134-10 and ENS134-14) (Figure 3a, left), while in the case of MscL-GFP overexpression, all strains carrying mutations in *rne* gene exhibited increased levels of final biomass (15%–280% increased OD_{600} values) (Figure 3b, left). Furthermore, measurements of relative bulk fluorescence of equal culture volumes, revealed that most of the truncated RNase E proteins enhanced the accumulation of recombinant CLRN1-GFP (20%–800% increased relative volumetric GFP fluorescence) (Figure 3a, right), while MscL-GFP accumulation was enhanced compared to the WT ENS134 strain in all cases (97%–425% increased relative volumetric GFP fluorescence) (Figure 3b, right). Among all tested strains, MP-GFP-overexpressing cultures of ENS134-22 exhibited the most pronounced increases in bulk fluorescence levels (425%–800% increased relative volumetric GFP fluorescence). SDS-PAGE under semi-denaturing conditions followed by in-gel fluorescence and western blotting analyses confirmed that these increases occur indeed due to enhanced production of well-folded recombinant MPs (Figure 3c). These results indicate that *E. coli* cells expressing the RneΔ22 deletion can yield greatly enhanced production for a variety of recombinant MPs.

It is very interesting to note that RNase E domain deletions, which result in suppressed MP-induced toxicity and enhanced recombinant MP productivity, appear to be highly specific. For example, ENS134-22 was found to be the most efficient MP-producing strain, while the closely resembling strain ENS134-23 differing only in 18 amino acids, demonstrated only marginal effects on MP productivity. Similarly, for the pair ENS134-21and ENS134-24, where *rne* deletions vary only in 18 amino acids their difference in performance is striking, as well as for ENS134-2 when compared to ENS134-10 and ENS134-14, in which case each one of the ENS134-10 and ENS134-14 contains practically 50% of the *rne* deletion in ENS134-2 (Figures 1, 2, and 3a–c).

The fourth example of a recombinant MP we tested, HtdR, is a light-driven outward proton pump. HtdR can bind the chromophore all-*trans* retinal when it is properly folded and, upon chromophore binding, acquires a characteristic purple color due to an absorption spectrum exhibiting a single maximum at ~550 nm (Kamo et al., 2006). Due to this feature, microbial rhodopsins can serve as convenient model proteins to monitor the levels of recombinant MP production in functional form (Michou et al., 2019; Nannenga & Baneyx, 2011). By monitoring the weight of wet pellets derived from cultures overexpressing HtdR in the selected ENS134-X strains, we observed that ENS134-2, ENS134-21 and ENS134-22 yielded increased final cellular biomass compared to WT *E. coli* (Figure 3d, left). Moreover, ENS134-2, ENS134-21 and ENS134-22 yielded increased volumetric production of well-folded and functional recombinant HtdR, as revealed by measurements of the relative amount of detergent-extracted and chromophore-bound MP after overexpression in each strain (Figure 3d, right).

Taken together, the results presented in Figures 2 and 3 demonstrate that the strains ENS134-2, ENS134-21 and ENS134-22 result in enhanced levels of recombinant MP production for all the four cases of the MPs tested here. Among these, ENS134-22 was found to be by far the most efficient in the cases of the two eukaryotic (human) recombinant MPs tested. Furthermore, ENS134-22 was the top-performing strain, together with ENS134-2, when MPs of prokaryotic origin were produced. Based on these results, we introduce *E. coli* ENS134-22 as a new expression host for high-level recombinant MP production in bacteria. Based on its properties, and in analogy to the original SuptoxR strain, which served as the original motivation for this study, we propose that this strain be named SuptoxRNE22 (Suppressor of toxicity—deletion 22 in RNase E). It must be noted that ENS134-2 is the commercially available strain BL21 Star(DE3) (Invitrogen).

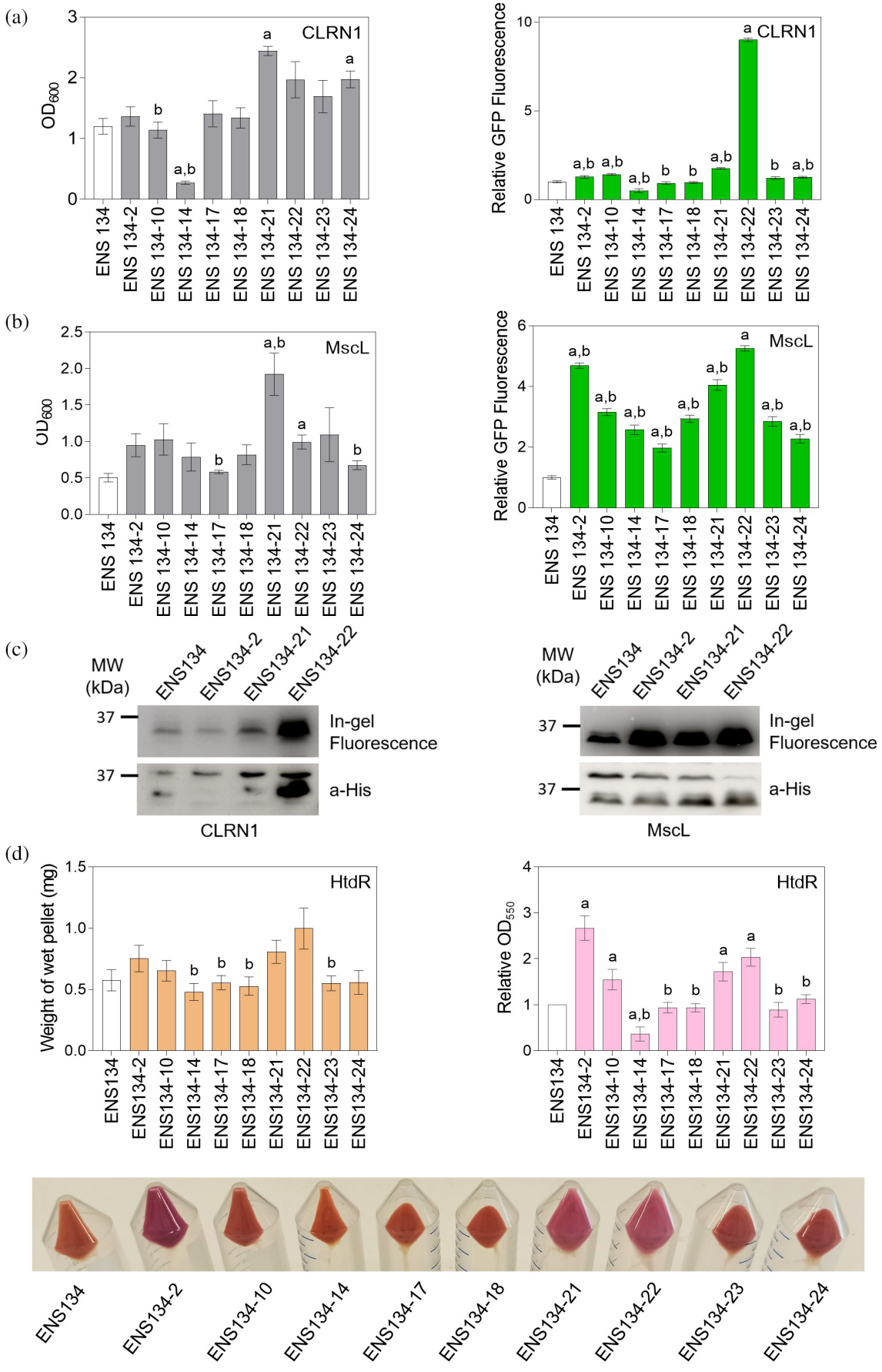

FIGURE 3 Legend on next page.

2.3 | Co-expression of RraA in *E. coli* SuptoxRNE22 does not further enhance recombinant MP production

We have shown here that specific *rne* genetic modifications leading to reduced RNase E activity can result in significantly suppressed MP-induced toxicity and dramatically enhanced recombinant MP production in bacteria in a manner reminiscent of what we have observed previously when RNase E activity is being inhibited upon overexpression of its inhibitor RraA in *E. coli* SuptoxR. Thus, we wondered whether the properties of SuptoxRNE22 could be further improved by further modulation/reduction of RNase E activity via the overexpression of *rraA*. In our previous studies, we had found that *rraA* overexpression resulted in a marginal, non-statistically significant increase in BR2 accumulation in *E. coli* carrying the *rneΔ22* deletion (Gialama, Delivoria, et al., 2017). To test if this is also the case for other recombinant MPs, we monitored the levels of cell growth, as well as the cellular and volumetric accumulation of CLRN1 and MscL in SuptoxRNE22 in the absence and presence of *rraA* overexpression. This analysis showed that the MP production-promoting effects in SuptoxRNE22 cannot be further enhanced upon RraA co-expression for anyone of the tested MPs (Figure S1).

2.4 | *E. coli* SuptoxRNE22 can be used as a broadly applicable expression host for enhanced recombinant MP production

An advantageous expression host for recombinant MP production should be broadly applicable and compatible with various expression vectors and promoters frequently utilized for recombinant production. The most extensively utilized set of expression vectors for recombinant protein production in bacteria are the well-established pET vector series, whose high target gene expression and protein production capabilities rely on the use of the strong T7 promoter (Dubendorff & Studier, 1991; Hattab et al., 2015). When these vectors are introduced in strains carrying a chromosomally encoded copy of the T7 RNA polymerase gene under the control of the lactose-inducible promoter *lacUV*5 (DE3 strains), high-level production can be achieved by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG).

Since SuptoxRNE22 is a DE3 strain, we tested whether this strain can also achieve high-level recombinant MP production with a pET-based expression system in addition to the Tet promoter-based expression system (pASK75 vector), which was utilized in all of our previous analyses described above. Indeed, SuptoxRNE22 cells transformed with a pET-28a(+)-BR2-GFP vector and expressing BR2-GFP by the addition of 0.1–0.4 mM IPTG achieved a 1.6-fold enhancement in volumetric BR2-GFP accumulation compared to the corresponding WT strain. Interestingly, SuptoxRNE22 cells producing BR2-GFP from the pET-based system appeared to accumulate significantly more recombinant MP compared to the same cells using the expression system based on the Tet promoter (approximately 2.7-fold enhancement) (Figure 4). Taken together, *E. coli* SuptoxRNE22 is an expression host with the ability to achieve greatly enhanced levels of recombinant MP production, which is compatible with different frequently utilized promoters and plasmid systems and, thus, can be utilized as a broadly applicable production host.

2.5 | *E. coli* SuptoxRNE22 can perform better than commercially available bacterial strains, which are frequently utilized for recombinant MP production

Finally, we compared the MP-producing capabilities of SuptoxRNE22 with those of the commercially available

FIGURE 3 *E. coli* cells carrying the RneΔ22 truncation can accumulate significantly enhanced levels of both prokaryotic and eukaryotic recombinant MPs. (a) Growth levels (left) and relative bulk GFP fluorescence corresponding to equal culture volumes (right) of WT *E. coli* and ENS134-X cells producing CLRN1-GFP from pASKCLRN1-EGFP vector. OD_{600}: optical density at 600 nm. (b) as in (a) for MscL-GFP expressed from pASKMscL-EGFP plasmid (c) (left) SDS-PAGE analysis of clarified lysates of WT, ENS134-2, ENS134-21 and ENS134-22 cells expressing CLRN1-GFP and visualization of the produced fusion by in-gel fluorescence and western blot analysis using a C-terminal anti-polyHis antibody. (right) as in (left) for isolated total membrane fractions of the same strains expressing MscL-GFP. (d) (top left) Weight of wet pellets derived from equal culture volume of WT and ENS134-X cells expressing HtdR. (top right) Relative absorbance at 550 nm of DDM-extracted HtdR from total cell lysates of equal culture volumes of WT and ENS134-X cells. (bottom) Purple-colored pellets derived from equal culture volumes of WT and ENS134-X strains expressing HtdR in the presence of 10 μM all-*trans*-retinal. The reported values correspond to the mean value of at least three independent experiments performed in replica triplicates ± SEM. (a) denotes a statistically significant difference with ENS134 sample ($p < 0.05$), while (b) denotes a statistically significant difference with ENS134-22 sample ($p < 0.05$).

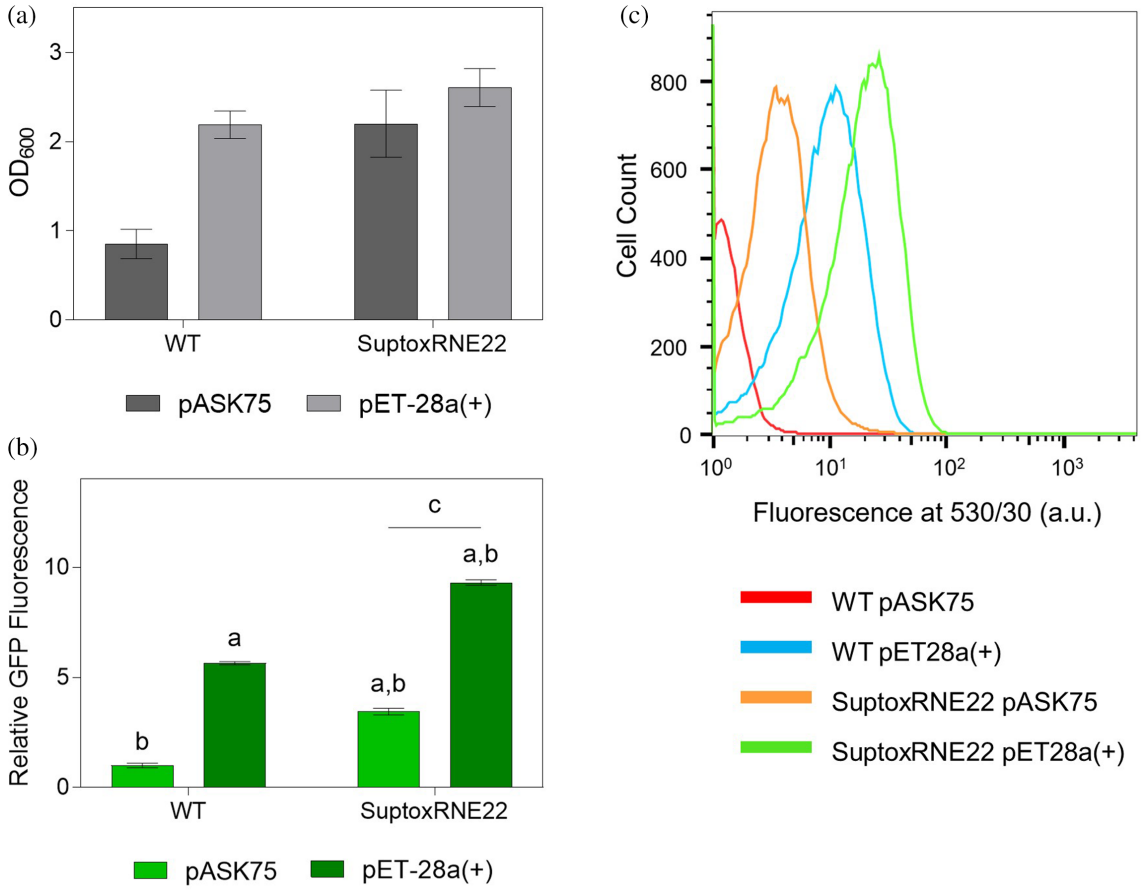

FIGURE 4 *E. coli* SuptoxRNE22 can be used as a broadly applicable expression host for enhanced recombinant MP production. (a) Growth levels of WT *E. coli* and SuptoxRNE22 cells expressing BR2-GFP from pASKBR2-GFP and pET-28a(+)-BR2-GFP plasmid vectors. (b) Relative bulk GFP fluorescence of equal culture volumes of WT *E. coli* and SuptoxRNE22 cells expressing BR2-GFP from pASKBR2-GFP and pET-28a(+)-BR2-GFP vectors. (c) Individual cell fluorescence of the same cultures as in (b) measured by flow cytometry. OD_{600}: optical density at 600 nm. The reported values correspond to the mean value of at least three independent experiments performed in replica triplicates ± SEM (a) denotes statistically significant difference with pASK75- WT sample ($p < 0.05$), (b) denotes statistically significant difference with pET-28a(+)-WT sample ($p < 0.05$), while C indicates statistically significant difference of the samples that are have the same bar above them ($p < 0.05$).

E. coli strains C41(DE3) and C43(DE3) (Lucigen) (Miroux & Walker, 1996), as well as Lemo21(DE3) (New England Biolabs) (Wagner et al., 2008), which are frequently utilized for the production of recombinant MPs and other toxic proteins. SuptoxRNE22, C41(DE3), C43 (DE3) and Lemo21(DE3) were all transformed with the same pET-28a(+)-BR2-GFP vector. For Lemo21(DE3), we first determined the optimal concentration of L-rhamnose for maximizing volumetric BR2-GFP production (Figure S3), which were consistent with our previous results (Gialama, Kostelidou, et al., 2017). Final levels of bacterial biomass and volumetric BR2-GFP accumulation were compared by measuring OD_{600} and cell fluorescence of equal culture volumes, respectively. Consistently with previous reports (Wagner et al., 2008), as well as our own observations (Gialama, Kostelidou, et al., 2017), Lemo21 (DE3) cells accumulated increased amounts of recombinant MP compared to C41(DE3) and C43(DE3). The comparison of SuptoxRNE22 with C41(DE3), C43 (DE3) and Lemo21(DE3) showed that SuptoxRNE22 is capable of accumulating significantly increased levels of recombinant MP compared to all three frequently utilized commercial strains and, thus, holds great potential for wide use as host for bacterial recombinant MP production (Figure 5).

3 | DISCUSSION

In our previous work, we had identified RraA, a protein acting as an inhibitor of the mRNA-degrading activity of RNase E in *E. coli* and other bacteria, as a potent suppressor of the toxicity caused by the MP overexpression process in bacteria and as an efficient enhancer of the

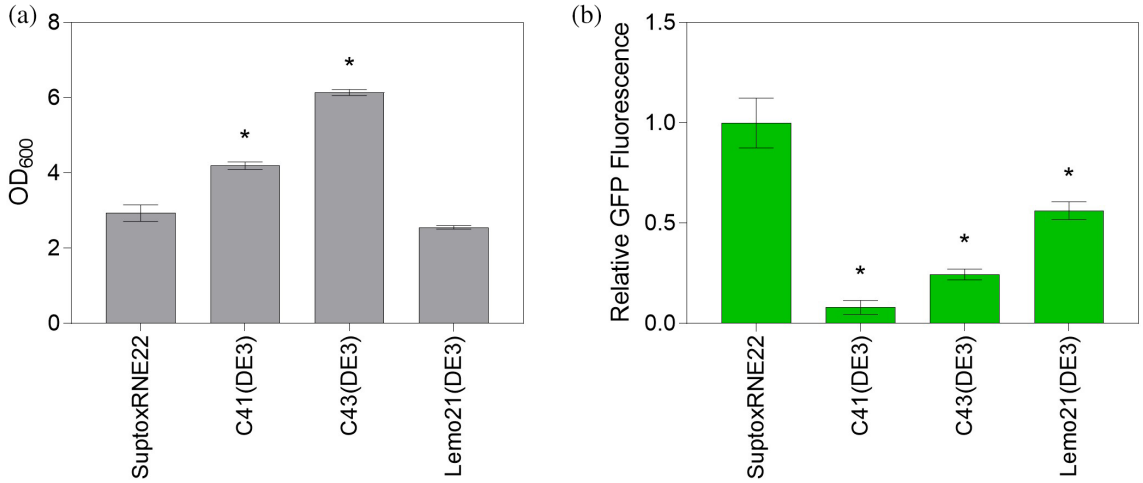

FIGURE 5 *E. coli* SuptoxRNE22 can perform better than commercially available bacterial strains, which are frequently utilized for recombinant MP production. (a) Growth of SuptoxRNE22, C41 (DE3), C43 (DE3) and Lemo21 (DE3) *E. coli* cells expressing BR2-GFP from pET-28a(+)-BR2-GFP plasmid vector upon harvesting. (b) Relative BR2-GFP fluorescence of equal culture volumes the same *E. coli* strains as in (a). OD_{600}: optical density at 600 nm. The reported values correspond to the mean value of at least three independent experiments performed in replica triplicates ± SEM. *denotes a statistically significant difference with SuptoxRNE22 sample ($p < 0.05$).

capacity of the bacterial cell machinery for recombinant MP production, in membrane-integrated and well-folded form (Gialama, Kostelidou, et al., 2017). Based on this, we had constructed the specialized MP-producing *E. coli* strain SuptoxR (Gialama, Kostelidou, et al., 2017) and its second-generation derivatives (Vasilopoulou et al., 2022), which can achieve dramatically enhanced volumetric yields of well-folded recombinant MPs of both prokaryotic and eukaryotic origin (Gialama, Kostelidou, et al., 2017; Michou et al., 2019; Vasilopoulou et al., 2022). In the present study, we have investigated a potentially alternative approach to RraA overexpression in SuptoxR for promoting recombinant MP production: direct modulation RNase E activity via mutagenesis. In this direction, we have investigated whether a variety of *rne* domain deletions in the gene encoding for the *E. coli* RNase E (*rne*), which exhibit reduced ribonucleolytic activity while being permissive for bacterial viability, can result in suppressed MP-induced toxicity and enhanced recombinant MP production in bacteria, in a manner resembling the conditions of *rraA* overexpression in *E. coli* SuptoxR. We have found that *E. coli* strains carrying the genetic C-terminal RNase E deletions *rne131*, *rneΔ21* and *rneΔ22* can suppress MP-induced toxicity and achieve increased accumulation of both final bacterial biomass and production of membrane-incorporated recombinant MPs. Among these, we have found a single strain, carrying the deletion *rneΔ22*, which can efficiently suppress MP-induced toxicity and achieve greatly enhanced levels of recombinant MP production for proteins of both prokaryotic and eukaryotic origin. Based on these observations, and in analogy to our original SuptoxR strain, we have named this strain SuptoxRNE22 (Suppressor of toxicity—deletion 22 in RNase E). *E. coli* SuptoxRNE22 is compatible with a variety of frequently utilized promoters and plasmid systems. Importantly, we have found that SuptoxRNE22 can perform better than commercially available bacterial strains, which are frequently utilized for recombinant MP production. Thus, we expect that SuptoxRNE22 will become a broadly applicable expression host for recombinant MP production.

In our previous studies, we have observed that the beneficial effects of RraA on MP overexpression in *E. coli* SuptoxR occur through a mechanism specifically involving RNase E, but not other paralogous ribonucleolytic proteins, such as RNase G (Gialama, Delivoria, et al., 2017). Furthermore, as part of our original study, where the development of *E. coli* SuptoxR was described, *rraA* overexpression was found not to affect the mRNA levels of the target recombinant MP (Gialama, Kostelidou, et al., 2017). This excluded a potential cytotoxicity-suppressing and MP production-increasing mechanism involving a reduced, more balanced expression of the target recombinant MP at the mRNA level, which prevents the overwhelming of the proteosynthetic and proteostatic capacity of the cell, as it is the case with various commercial strains, which are frequently utilized for recombinant production of toxic recombinant MPs, such as C41(DE3), C43(DE3) and Lemo21(DE3) (Kwon et al., 2015; Miroux & Walker, 1996; Schlegel et al., 2015; Wagner et al., 2008). As mentioned already, *rraA* overexpression can affect the levels of more than 2000 different mRNAs in *E. coli* (Lee et al., 2003). Interestingly, a number of

these affected mRNAs have been found to encode proteins, which are associated with cell envelope biosynthesis and anaerobic metabolism (Lee et al., 2003). Compromised cell envelope integrity and formation of respiratory chain complexes in the cytoplasmic membrane have been shown previously to be prominent consequences of MP overexpression (Wagner et al., 2007). Based on these observations, we had proposed previously that RraA suppresses MP-induced toxicity and promotes MP accumulation not by directly interacting with the target MP (or its coding mRNA), but rather in an indirect manner, which involves stabilization of the mRNA and increase of the cellular abundance of other productivity-promoting factors, possibly involved in cell envelope biosynthesis and/or anaerobic metabolism (Gialama, Delivoria, et al., 2017).

RNase E is the essential major endonuclease for mRNA turnover in *E. coli*, whose function affects many different transcripts, while it also participates in the processing of precursors of some structural RNAs, such as ribosomal and transfer RNAs (Carpousis et al., 2009). Since particular deletions in the *rne* gene, which result in reduced ribonucleolytic RNase E activity, resemble the MP productivity-promoting effects of *rraA* overexpression observed originally in *E. coli* SuptoxR as postulated at the beginning of our study, the toxicity-suppressing and MP accumulation-enhancing properties of *E. coli* SuptoxRNE22 must be occurring with the same mechanism as in the case for *E. coli* SuptoxR, when RraA is being overexpressed. A very noteworthy observation of the present work is that RNase E domain deletions, which result in suppressed MP-induced toxicity and enhanced recombinant MP productivity, are highly specific: we have found that closely resembling strains differing only in few amino acids in their RNase E sequence can exhibit striking differences in their MP production-promoting performance. Of course, one cannot rule out the possibility that other mutations spontaneously acquired since the initial construction of the herein utilized strains do not contribute to the phenotypes and properties reported in this study.

One of the RNase E-deficient strains with MP production-promoting properties presented here is the one encoding the RNase E variant Rne131, which carries a complete deletion of the CTD (mutation *rne131*), is the commercial *E. coli* strain BL21 Star(DE3) (ThermoFisher Scientific). BL21 Star(DE3) can achieve increased yields of recombinant protein production, presumably due to increased mRNA stability of the target recombinant protein, and its use is recommended by the manufacturer for "applications that require high-level expression of non-toxic recombinant proteins" (Lopez et al., 1999). In this study, our experiments involved the overexpression of genes encoding for both eukaryotic (BR2 and CLRN1) and prokaryotic MPs (MscL and HtdR). *E. coli* encoding the RNase E variant Rne131 (BL21Star (DE3)) performed very well compared to wild-type *E. coli* for the prokaryotic MPs but only poorly if better at all for the eukaryotic/human MPs tested here. On the contrary, SuptoxRNE22 achieved greatly enhanced volumetric accumulation for all the tested recombinant MPs.

The latter observation is—at first glance—somewhat surprising, given that the RNase E variants Rne131 and RneΔ22, produced by BL21Star (DE3) and SuptoxRNE22, respectively, have been found to exhibit similarly reduced levels of RNase E activity, despite the fact that Rne131 lacks the entire CTD (Leroy et al., 2002). These findings lead to the assumption that, while reduction of RNase E activity and stabilization of mRNA in the cell acts beneficially for MP production, preservation of specific parts of the CTD, which mediate additional regulatory interactions, may promote this process further. For example, the RNase E variant RneΔ22 in SuptoxRNE22 maintains in its sequence the areas of the protein, which mediate the interactions with all other known components of the *E. coli* RNA degradosome (RhlB, enolase and PNPase; Figure 1), whereas the RNase E variant Rne131 in BL21Star (DE3) lacks this entire region and, thus, the capacity to interact with these factors that affect global mRNA abundance in *E. coli* through their interaction with RNase E. Because RNase E and the RNA degradosome affect the levels of a great number of mRNAs in *E. coli*, it is impossible to predict how the transcriptomic profile of the host changes upon expression of different RNase E variants and MP overexpression. Based on the observations and the rationale described above, we hypothesize that different CTD deletions in each RNase E variant affect different groups of mRNAs and/or to a different extent, and this specificity in mRNA stabilization and increase in cellular abundance of certain factors might be the key in MP-production-promoting effect that RNase E deficiency exhibits. We are currently performing RNAseq and proteomic analyses to test these hypotheses.

It is very interesting to note that genetic modifications leading to impaired RNase E activity have arisen as hits also from unbiased genetic screens aiming at enhanced recombinant MP production in *E. coli* (Heyde & Norholm, 2021). In this study, Nørholm and co-workers identified a transposable element insertion in *rne* causing a CTD truncation in RNase E, which can result in significantly enhanced bacterial recombinant MP production. This truncation variant resembles closely the RNase E variant Rne131 of BL21Star (DE3) as it lacks the largest part of the CTD, but it is also slightly different as it contains amino acid residues 1–702 versus residues 1–584 of Rne131 of BL21Star (DE3). The authors found that the

E. coli strain carrying this 702-amino acid variant, which they termed Evo21(DE3), can achieve enhanced levels of recombinant MP production, which can surpass the yields of BL21Star (DE3) for various recombinant MPs (Heyde & Norholm, 2021). Additionally, the authors reported the serendipitous discovery of the missense mutation D346N in RNase E, which also results in decreased ribonucleolytic activity of the enzyme, and whose overexpression from the vector pLysS-Max also leads to increased MP productivity and to levels even higher than those achieved with the 702-amino acid RNase E variant of Evo21(DE3) (Heyde & Norholm, 2021).

Taken together, the results reported previously by us (Gialama, Delivoria, et al., 2017) and others (Heyde & Norholm, 2021), as well as our new observations presented in this work, point to the conclusion that recombinant MP production in bacteria can be enhanced greatly by global modulation of RNA abundance in the host, which can be achieved via reduction of RNase E activity in highly specific ways. Proper modulation of RNase E activity can be achieved either directly, for example, with domain deletions or point mutations as in strains BL21Star, Evo21 and SuptoxRNE22 (Heyde & Norholm, 2021; Leroy et al., 2002), or indirectly, for example, by co-expression of factors interfering with RNase E activity like the inhibitor RraA as in SuptoxR and its second-generation derivatives (Gialama, Kostelidou, et al., 2017; Vasilopoulou et al., 2022). We have proposed previously (Gialama, Delivoria, et al., 2017) and still favor the notion that productive modulation of RNase E activity results in an altered transcriptome profile of the expression host, in which the cellular abundance of certain factors that promote recombinant MP production is increased and, thus, the expression host exhibits enhanced resistance to the stress associated with recombinant MP production together with an improved capacity for proper biosynthesis of these difficult-to-express targets. We anticipate that SuptoxRNE22 and other *E. coli* strains with similar mechanisms of action will become widely utilized hosts for recombinant MP production in bacteria for various purposes.

4 | MATERIALS AND METHODS

4.1 | Bacterial strains

E. coli ENS134 and all of its *rne* mutants were previously described by Leroy et al., 2002 (Leroy et al., 2002). C41 (DE3) and C43(DE3) were purchased from Lucigen, while Lemo21(DE3) were acquired from New England Biolabs.

4.2 | Plasmid construction

The expression vectors pSuptoxR, pASKBR2-EGFP and pET-28a(+)-BR2-GFP were constructed previously by Gialama et al. (Gialama, Kostelidou, et al., 2017). pASKMscL-EGFP and pASKHtdR were constructed by Michou et al. (Michou et al., 2019), while pASKCLRN1-EGFP by Vasilopoulou et al. (Vasilopoulou et al., 2022) and pASKBR2-GFP by Link et al. (Link et al., 2008). In all plasmids, GFP refers to GFPmut2, whereas EGFP refers to enhanced green fluorescent protein.

4.3 | MP overexpression

E. coli cells were freshly transformed with the appropriate vector(s) for each protein-production experiment. Single colonies were picked to inoculate liquid LB cultures containing the appropriate combination of antibiotics (100 μg/mL ampicillin, 40 μg/mL chloramphenicol, 50 μg/mL kanamycin) and cultured overnight at 37°C. These cultures were used to inoculate fresh liquid LB with a 1:50 dilution. In case of *rraA* co-expression, LB contained also 0.2% arabinose. Cultures were grown at 30°C with shaking to an optical density at 600 nm (OD_{600}) of 0.3–0.5, where the temperature was decreased to 25°C, and after an equilibration period of 5 min, MP production was induced by the addition of 0.2 μg/mL of anhydrotetracycline (aTc) for pASK75-based expression vectors and by the addition of 0.1–0.4 mM IPTG for pET28a(+)-based expression vectors, for 16 h. In case of rhodopsin overexpression, the induction of protein production was accompanied by the addition of 1 μM all-*trans* retinal (Cayman Chemical) and the overexpression process was carried out in the dark.

4.4 | Fluorescence measurements

For bulk fluorescence measurements, cell pellets from 0.5 mL cultures were resuspended in 100 μL of phosphate buffered saline (PBS) pH 7.4 and the cell suspension was transferred to a black 96-well plate. The fluorescence was measured at 510 nm after excitation at 488 nm using a Cytation 5 (BioTek) plate reader. To measure single-cell fluorescence, a CyFlow ML flow cytometer (Partec) was used. 1 mL PBS was added to $\sim 10^7$ cells and, after fluorophore excitation at 488 nm, the fluorescence of 50,000 cells was measured at 530/30 nm. The data that were obtained as a result were analyzed using FlowJo 7.6.2.

4.5 | Membrane isolation

Cell pellets from 1 L bacterial cultures were collected by centrifugation at 4000 *g* for 10 min and resuspended at 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 using a cell disruptor (Constant Systems) and the resulting total lysates were clarified by centrifugation at 10,000 *g* for 20 min. The supernatant was then subjected to ultracentrifugation using a Beckman 70Ti rotor at 42,000 rpm (100,000 *g*) for 1 h at 4°C. The supernatant was discarded, and the resulted membrane pellet was resuspended and homogenized in 5 mL of cold lysis buffer.

4.6 | Rhodopsin extraction and quantification

Pellets from 150 mL culture were resuspended in 9 mL of cold PBS pH 7.4 and lysed by four brief sonication steps on ice. Deltarhodopsin from the organism *Haloterrigena turkmenica* (HtdR) was extracted from the total cell lysate by the addition of 0.1% (w/v) *n*-dodecyl *β*-D-maltoside (DDM) (Glycon Biochemicals) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and rotation at 180 rpm for 24 h at 4°C in the dark. The samples were then ultracentrifuged at 42,000 rpm (100,000 g) at 4°C for 1 h using a Beckman 70Ti rotor, and if a colorless pellet was obtained, the supernatant containing the extracted HtdR was collected and further analyzed by measuring the absorbance at 550 nm.

4.7 | Western blot and in-gel fluorescence analyses

Proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 12% gels under semi-denaturing conditions (without boiling the samples prior to loading). In-gel fluorescence was detected by excitation at 460 nm after exposure for approximately 3 s on an Amersham ImageQuant 800 imaging system (Cytiva). For western blot analysis, proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Merck) under semi-dry conditions for 30 min at 25 V using a Trans-Blot Turbo transfer system (BioRad Laboratories) and blocked with 5% (w/v) non-fat dried milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) at 4°C overnight. After triple 10 min washing steps with TBST, membranes were incubated with the appropriate antibody dilution in TBST containing 0.5% (w/v) non-fat dried milk at room temperature for 1 h. The utilized antibodies were a mouse monoclonal anti-polyhistidine antibody conjugated with horseradish peroxidase (Sigma) at 1:3000 dilution and a mouse monoclonal anti-FLAG antibody (Sigma) at 1:2500 dilution paired with a horseradish peroxidase-conjugated goat anti-mouse IgG (BioRad Laboratories) as secondary antibody at 1:4000 dilution. The visualization of the proteins was performed after three 10-min washes with TBST using an Amersham ImageQuant 800 imaging system.

4.8 | Statistical analysis

Statistical analyses were performed using the Prism 7.00 (GraphPad Software Inc., La Jolla, CA) and Microsoft Office 365 Excel (Microsoft Corporation, Redmond, WA) software packages. Graphs were prepared using Prism 7.00. For flow cytometry data, FlowJo 7.6.2. was used. All reported measurements correspond to at least three independent experiments, each of them performed in replica triplicates. Error bars denote ±standard error of mean (SEM), and differences between mean values of samples consider to be statistically significant when *p*-value ≤ 0.05.

AUTHOR CONTRIBUTIONS

Eleni Vasilopoulou: Funding acquisition; writing – original draft; investigation; visualization; validation; software; data curation. **Tania Chroumpi:** Investigation; validation; data curation. **Georgios Skretas:** Conceptualization; funding acquisition; writing – review and editing; writing – original draft; supervision; project administration; methodology; visualization; resources.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interests.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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