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Article

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Tailoring *Escherichia coli* for the L-rhamnose P_{BAD} promoter-based production of membrane and secretory proteins

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Summary

Membrane and secretory protein production in *Escherichia coli* requires precisely controlled production rates to avoid the deleterious saturation of their biogenesis pathways. Based on this requirement, the *E. coli* L-rhamnose P_{BAD} promoter (*PrhaBAD*) is often used for membrane and secretory protein production since *PrhaBAD* is thought to regulate protein production rates in an L-rhamnose concentration dependent manner. By monitoring protein production in real-time in *E. coli* wild-type and an L-rhamnose catabolism deficient mutant, we demonstrate that the L-rhamnose concentration-dependent tunability of *PrhaBAD*-mediated protein production is actually due to L-rhamnose consumption rather than regulating production rates. Using this information, a RhaT-mediated L-rhamnose transport and L-rhamnose catabolism deficient double mutant was constructed. We show that this mutant enables to regulate *PrhaBAD*-based protein production rates in an L-rhamnose concentration dependent manner and that this is critical to optimize membrane and secretory protein production yields. The high precision of protein production rates provided by the *PrhaBAD* promoter in an L-rhamnose transport and catabolism deficient background could also benefit other applications in synthetic biology.

Keywords

E. coli, protein production, L-rhamnose promoter, L-rhamnose metabolism, membrane protein, secretory protein

59 **Introduction**

60 The Gram-negative bacterium *Escherichia coli* is widely used for the production of
61 membrane and secretory proteins^{1, 2}. It is preferred to produce membrane proteins in
62 the cytoplasmic membrane rather than in inclusion bodies in the cytoplasm, since
63 production in the membrane greatly facilitates their isolation for structural and
64 functional studies³. Disulfide bond-containing proteins, like antibody fragments and
65 many hormones, are preferably produced in the periplasm since in this compartment
66 the disulfide bond formation (Dsb) system is present⁴. In addition, it is easier to
67 isolate proteins from the periplasm than from whole cell lysates⁵. However, the
68 expression of genes encoding recombinant membrane and secretory proteins is often
69 toxic to *E. coli*, which makes their production challenging. The toxicity of membrane
70 and secretory protein production appears to be mainly caused by saturation of the
71 capacity of the Sec-translocon, which is a protein-conducting channel in the
72 cytoplasmic membrane assisting the biogenesis of both membrane and secretory
73 proteins^{6, 7}. To optimize membrane and secretory protein production yields in *E. coli*
74 it is critical to be able to precisely set protein production rates such that saturation of
75 the Sec-translocon capacity is avoided^{6, 7}. In contrast to many other commonly used
76 promoter systems, it is thought that the *E. coli* *PrhaBAD* promoter system, which is
77 derived from the *rhaBAD* operon (Figure 1a), allows for precise setting of protein
78 production rates⁸. Therefore, *PrhaBAD* has been used for the *E. coli*-based production
79 of membrane and secretory proteins⁸.

80 In addition to the aforementioned *rhaB*, *A* and *D* genes, the *rhaS*, *R* and *T*
81 genes are also involved in L-rhamnose utilization in *E. coli* (Figure 1a)⁹⁻¹⁴. The *rhaB*,
82 *A* and *D* genes, which are organized in an operon, encode for the L-rhamnulose
83 kinase, L-rhamnose isomerase, and the rhamnulose-1-phosphate aldolase,

respectively¹⁵⁻¹⁸. The *rhaR* and *S* genes are also organized in an operon and encode for the RhaR and S transcriptional activators, respectively (Figure 1a)⁹. The *rhaT* gene encodes for the L-rhamnose transporter RhaT¹⁹. Expression of *rhaT* is governed by *PrhaT*, expression of the *rhaSR* operon is governed by *PrhaSR* and expression of the *rhaBAD* operon is governed by *PrhaBAD* (Figure 1a)¹⁴. RhaR is always present independent of the presence of L-rhamnose²⁰. In the presence of L-rhamnose RhaR activates transcription of *rhaR* and *rhaS* resulting in the accumulation of RhaS^{9, 14, 21}. RhaS in turn acts as the L-rhamnose-dependent positive regulator of *PrhaBAD* and *PrhaT*^{9, 14, 22}. RhaS, when bound to L-rhamnose and when its levels are sufficiently high to saturate RhaS-activated promoters, can also negatively autoregulate *rhaSR* expression²³. All three *PrhaT*, *PrhaSR* and *PrhaBAD* are cyclic AMP receptor protein (CRP)-dependent promoters, *i.e.*, the expression of the genes encoding the proteins involved in L-rhamnose utilization is subject to catabolite repression^{14, 22, 23}.

Here, we have characterized *PrhaBAD*-mediated protein production kinetics in *E. coli* wild-type and in an L-rhamnose catabolism deficient strain. This revealed that the tunability of protein production in *E. coli* is based on L-rhamnose consumption rather than the precise setting of protein production rates. By abolishing RhaT-mediated L-rhamnose transport in the catabolism deficient strain, *PrhaBAD*-mediated protein production rates could be set in an L-rhamnose concentration dependent manner. Importantly, we show that the ability to set protein production rates is critical to optimize membrane and secretory protein production yields.

Results and Discussion

The tunability of PrhaBAD-mediated protein production in E. coli depends on L-rhamnose consumption

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3 109 It is widely believed that the *E. coli PrhaBAD* promoter system allows for precise
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5 110 setting of protein production rates in *E. coli* by varying the concentration of L-
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7 111 rhamnose⁸. Therefore, we sought to monitor the effect of using different amounts of
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10 112 the inducer L-rhamnose on the production kinetics of super folder green fluorescent
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12 113 protein (SfGFP) in real-time in *E. coli* wild-type (*i.e.*, *E. coli* containing all genes
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14 114 involved in L-rhamnose utilization) (Figure 1a, b). Production of SfGFP is easy to
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16 115 monitor and in addition the protein is very stable and not toxic, making it a suitable
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18 116 target for monitoring protein production kinetics²⁴. The *PrhaBAD*-based expression
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20 117 vector pRha67Km^R was used throughout this study (see the ‘Methods’ section). This
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22 118 expression vector is a variant of the recently published high copy number pRha67
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24 119 vector⁸. Notably, in pRha67 also *rhaS* and *rhaR* with their promoter were included to
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26 120 ensure the presence of sufficient copies of RhaS/R (Figure 1a)⁸.

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29 121 To study *PrhaBAD*-mediated production of SfGFP in real-time, cells were
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31 122 cultured in a 96-well plate in a spectrofluorometer and GFP fluorescence was
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33 123 monitored every 5 min (Figure 1b)²⁵. Surprisingly, the initial SfGFP production rate
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35 124 was not affected by varying the L-rhamnose concentration used to induce protein
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37 125 production. However, SfGFP accumulation halted in an L-rhamnose concentration
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39 126 dependent manner; the lower the L-rhamnose concentration the earlier the SfGFP-
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41 127 based fluorescence accumulation halted and *vice versa*. Notably, standard LB
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43 128 medium, which consists of tryptone, yeast extract and sodium chloride, was used to
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45 129 culture cells. Since LB medium only contains neglectable amounts of sugars, *E. coli*
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47 130 uses catabolizable amino acids as carbon and energy source when cultured on this
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49 131 medium²⁶. Thus, the use of LB medium does not lead to sugar induced catabolite
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51 132 repression of the *PrhaBAD* promoter system. This was supported by the observation
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53 133 that adding glucose to the LB medium decreases the *PrhaBAD*-mediated production
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3 134 of SfGFP in a glucose concentration-dependent manner (data not shown). This also
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5 135 indicates that cAMP-CRP is not a limiting factor for the efficient *PrhaBAD*-mediated
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7 136 production of proteins in LB medium.
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9
10 137 Using flow cytometry, we monitored the accumulation of SfGFP in individual
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12 138 cells, harvested 1 and 4 hrs after the addition of L-rhamnose (Figure 1c). This showed
13
14 139 that all cultures were homogeneous and that after 1 hr SfGFP fluorescence per cell
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16 140 was the same for all different L-rhamnose concentrations used, whereas after 4 hrs
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18 141 there was a clear correlation between L-rhamnose concentration and SfGFP
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20 142 fluorescence per cell. Furthermore, SfGFP accumulation levels per cell in the cultures
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22 143 induced with the three lowest L-rhamnose concentrations were lower after 4 hrs
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24 144 compared to after 1 hr, and after 24 hrs SfGFP levels per cell had decreased even in
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26 145 the culture in which protein production was induced with the highest concentration of
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28 146 L-rhamnose. Thus, both the flow cytometry experiments and monitoring SfGFP
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30 147 accumulation in real-time suggest that the tunability of *PrhaBAD*-mediated protein
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32 148 production in *E. coli* wild-type is due to L-rhamnose consumption.
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36 149 To explore the impact of L-rhamnose consumption on SfGFP production in *E.*
37
38 150 *coli* directly, we constructed an *E. coli* strain in which the ability to catabolize L-
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40 151 rhamnose was abolished by introducing a frameshift mutation in the gene encoding L-
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42 152 rhamnulose kinase (RhaB) (Figure 2a)²⁷. This strain is hereafter referred to as *E. coli*
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44 153 *rhaB'*. SfGFP production rates in *E. coli rhaB'* were constant and hardly affected by
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46 154 the L-rhamnose concentration used (Figure 2b). Notably, the L-rhamnose
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48 155 concentration required to optimize SfGFP production in this strain was considerably
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50 156 lower than the one required in the wild-type strain. As shown by flow cytometry, the
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52 157 accumulation of fluorescence in individual *E. coli rhaB'* cells was also independent of
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the L-rhamnose concentration used to induce protein production and the fluorescence signal increased over time (Figure 2c).

Taken together, these observations show that tunability of protein production in *E. coli* wild-type using the *PrhaBAD*-promoter system is due to L-rhamnose consumption rather than fine-tuning protein production rates.

Precisely setting PrhaBAD-based protein production rates in E. coli

Next, we hypothesized that if active L-rhamnose uptake is only mediated by the L-rhamnose transporter RhaT and L-rhamnose can enter the cell *via* diffusion, blocking RhaT-mediated transport of L-rhamnose may enable to precisely set SfGFP production rates in a concentration dependent manner. To test this hypothesis, we deleted, in *E. coli rhaB'*, the gene encoding the L-rhamnose transporter RhaT (Figure 3a). This double mutant is hereafter referred to as *E. coli rhaB'ΔrhaT*. Monitoring SfGFP production in *E. coli rhaB'ΔrhaT* at different L-rhamnose concentrations in real-time, showed that in this strain background, SfGFP production rates could indeed be precisely set by varying the concentration of L-rhamnose (Figure 3b). As shown by flow cytometry, both after 2 and 4 hrs after induction, SfGFP accumulation levels per cell correlate with the L-rhamnose concentrations used (Figure 3c). Notably, SfGFP accumulation levels in individual cells were monitored by flow cytometry first after 2 hrs rather than after 1 hr, since at low L-rhamnose concentrations SfGFP accumulation in *E. coli rhaB'ΔrhaT* could not yet be detected after only 1 hr. As expected, SfGFP accumulation levels in individual cells increased over time in *E. coli rhaB'ΔrhaT* (Figure 3c).

Taken together, by inactivating the ability of *E. coli* to both consume L-rhamnose and actively transport it into the cell *via* RhaT, *PrhaBAD*-mediated SfGFP

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3 183 production rates can be precisely and stably set in an L-rhamnose concentration
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5 184 dependent manner.
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10 186 ***Optimizing PrhaBAD-based production yields of the membrane protein GltP using***
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12 187 ***E. coli rhaB'ΔrhaT***
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14 188 Being able to precisely set production rates of membrane proteins is critical to avoid
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16 189 saturating the capacity of the Sec-translocon, which is key to improving production
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18 190 yields of these proteins^{6, 7}. Therefore, we explored if the *PrhaBAD* promoter system in
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20 191 combination with the *E. coli rhaB'ΔrhaT* strain could be used to improve the
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22 192 production yields of a model membrane protein, the *E. coli* glutamate proton
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24 193 symporter GltP²⁵.
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27 194 To facilitate monitoring GltP production in the cytoplasmic membrane, the
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29 195 protein was C-terminally fused to GFP²⁸. The GFP moiety folds properly and
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31 196 becomes fluorescent when the membrane protein-GFP fusion is inserted in the
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33 197 cytoplasmic membrane and not when it aggregates in the cytoplasm. GltP-GFP was
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35 198 produced in the presence of increasing amounts of L-rhamnose in *E. coli rhaB'ΔrhaT*
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37 199 (Figure 4a). The *E. coli* wild-type and *rhaB'* strains were used as reference points
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39 200 (Figure 4a). In *E. coli rhaB'ΔrhaT*, an L-rhamnose concentration of 50 μM led to the
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41 201 highest production of GltP-GFP, as assessed by monitoring fluorescence (relative
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43 202 fluorescence unit, RFU) per milliliter of culture after 24 hrs. Higher L-rhamnose
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45 203 concentrations led to lower yields of GltP-GFP in this strain. At the optimal L-
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47 204 rhamnose concentration growth was hardly affected as assessed by measuring the
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49 205 OD₆₀₀ of cultures, whereas higher L-rhamnose concentrations negatively affected
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51 206 growth (Figure S2a). In *E. coli* wild-type, GltP-GFP production yields increased with
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53 207 increasing amounts of L-rhamnose but did not match the ones obtained with *E. coli*
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rhaB'ΔrhaT (Figure 4a). In *E. coli rhaB'*, GltP-GFP production levels were, as expected, L-rhamnose concentration independent and were significantly lower than the highest level obtained in *E. coli rhaB'ΔrhaT* (Figure 4a).

We have previously shown that saturation of the Sec-translocon capacity due to membrane and secretory protein production results in the aggregation of the target protein as well as the aggregation of a variety of other proteins in the cytoplasm^{6, 7, 29, 30}. The aggregation of proteins in the cytoplasm is accompanied by the induction of the expression of genes encoding proteins involved reversing protein aggregation, such as inclusion body protein B (IbpB)^{6, 7, 29-32}. Therefore, to assess if the Sec-translocon capacity was saturated upon inducing the production of GltP-GFP with increasing amounts of L-rhamnose or not, levels of IbpB were monitored in whole-cell lysates isolated from all cultures using immuno-blotting (Figure 4b)²⁹. In *E. coli* wild-type, IbpB accumulation levels increased with increasing L-rhamnose concentrations and in *E. coli rhaB'*, IbpB accumulation levels were high and independent of the concentration of L-rhamnose. In *E. coli rhaB'ΔrhaT*, IbpB could only be detected at L-rhamnose concentrations higher than the one optimal for GltP-GFP production in this strain. The formation of protein aggregates in the cytoplasm was corroborated by an increased granularity (side scatter) as monitored by flow cytometry (Figure S2b). The flow cytometry also showed that only under suboptimal conditions cell size was compromised (Figure S2b). The absence of any detectable IbpB and increased granularity when using the optimal L-rhamnose concentration to induce production of GltP-GFP in *E. coli rhaB'ΔrhaT* indicates that the Sec-translocon capacity is not saturated.

Previously, using flow cytometry we have shown that the GFP-moiety of a membrane protein GFP-fusion enables to monitor the production of a membrane

protein in the cytoplasmic membrane in individual cells³⁰. The saturation of the Sec-translocon capacity due to too high expression intensities of a gene encoding target membrane protein leads to the accumulation of cells that do not produce the membrane protein anymore, neither in the cytoplasmic membrane nor in inclusion bodies³⁰. Thus, the non-fluorescent cells that accumulate in cultures with amounts of inducer that result in target gene expression intensities leading to saturation of the Sec-translocon capacity have completely stopped producing the target membrane protein³⁰. Therefore, to monitor the effect of varying amounts of L-rhamnose on the stability of GltP-GFP producing cultures, we monitored GltP-GFP produced in the cytoplasmic membrane in individual cells using flow cytometry (Figure 4c). In *E. coli* wild-type-based cultures GltP-GFP production per cell increased with increasing amounts of L-rhamnose, however only to low levels. In *E. coli rhaB'*-based cultures GltP-GFP production per cell was L-rhamnose concentration independent and a significant fraction of the cells did not produce GltP-GFP. In *E. coli rhaB'ΔrhaT*-based cultures cells contained GltP-GFP at increasing levels and populations were homogenous up to the optimal L-rhamnose concentration for GltP-GFP production. Higher L-rhamnose concentrations led to a significant fraction of non-producing cells. The GltP-GFP level per cell was thus highest at the optimal L-rhamnose concentration in *E. coli rhaB'ΔrhaT*.

Finally, the activity of GltP-GFP isolated from the three strains cultured at their respective optimal L-rhamnose concentration was monitored (Figure 4d). The protein produced by *E. coli rhaB'ΔrhaT* cells was slightly, but significantly, more active than the proteins produced in the other strains. This indicates that optimizing the production rate leads to more properly folded GltP in the cytoplasmic membrane.

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3 257 Taken together, in *E. coli rhaB'ΔrhaT* GltP-GFP production could be
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5 258 optimized by harmonizing the GltP-GFP production rates with the membrane protein
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7 259 biogenesis machinery. Also for another membrane protein GFP fusion, YidC-GFP,
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10 260 we have made similar observations (Figure S3).

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14 262 ***Optimizing PrhaBAD-based production of a single-chain variable fragment using***

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17 263 ***E. coli rhaB'ΔrhaT***

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19 264 Production of secretory proteins can just like membrane proteins be hampered by
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21 265 saturation of the Sec-translocon capacity⁶. Therefore, we explored the potential of *E.*
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23 266 *coli rhaB'ΔrhaT* for optimizing production yields of a model secretory protein, the
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25 267 single-chain variable fragment (scFv) BL1 equipped with a DsbA-derived signal
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27 268 sequence⁶.

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30 269 The scFv BL1 was produced in *E. coli rhaB'ΔrhaT* at different L-rhamnose
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32 270 concentrations, and it was detected by means of immuno-blotting using an antibody
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34 271 recognizing the C-terminal His-tag of BL1 (Figure 5a). *E. coli* wild-type and *E. coli*
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36 272 *rhaB'* were used as reference points. In *E. coli rhaB'ΔrhaT* an L-rhamnose
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39 273 concentration of 100 μM led to the highest amount of properly processed BL1. At
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41 274 higher L-rhamnose concentrations production yields became only lower and
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43 275 increasing precursor accumulation in the cytoplasm was observed. In *E. coli* wild-
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45 276 type, any significant accumulation of processed BL1 was accompanied by severe
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47 277 precursor accumulation in the cytoplasm. Thus, it was not possible to simultaneously
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49 278 optimize production levels (of the mature BL1) and maximize quality (*i.e.*, reduce the
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51 279 amounts of precursor BL1) in this strain. In *E. coli rhaB'*, both the precursor and
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53 280 processed form of the protein could be detected to the same levels at all L-rhamnose
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55 281 concentrations. Notably, both in *E. coli* wild-type and in *E. coli rhaB'* production
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3 282 yields were significantly lower than the optimal yield obtained in *E. coli rhaB'ΔrhaT*
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5 283 (Figure S4). We also monitored the accumulation levels of inclusion body protein B
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7 284 (IbpB) in whole-cell lysates using immuno-blotting (Figure 5a). This showed that
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10 285 stress levels correlated with the initial protein production rate, in a similar manner to
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12 286 when producing GltP-GFP. The optimal condition for the production of BL1 in *E. coli*
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14 287 *rhaB'ΔrhaT* was accompanied with only mild stress. It is of note that the BL1
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16 288 produced in *E. coli rhaB'ΔrhaT* cells, induced with 100 μM L-rhamnose, was active
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19 289 and also that growth of these cells appeared to be unaffected by the production of BL1
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21 290 (Figure 5b and c).

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23 291 Taken together, also the production of a model secretory protein could be
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25 292 optimized in a strain background lacking the ability to both catabolize L-rhamnose
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27 293 and take it up *via* the RhaT transporter.
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31 295 ***An E. coli rhaB'ΔrhaT*-based membrane and secretory protein production screen**

32
33 296 The production yields of both the membrane proteins GltP-GFP and YidC-GFP
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35 297 (Figure S3), and the scFv BL1 could be significantly improved in *E. coli rhaB'ΔrhaT*.
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37 298 Therefore, we screened the production of five more membrane proteins and one more
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39 299 secretory protein in *E. coli rhaB'ΔrhaT* using *E. coli* wild-type as a reference. All
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41 300 membrane proteins were C-terminally fused to GFP as described before. As
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43 301 additional secretory protein we used SfGFP fused to the same DsbA-derived signal
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45 302 sequence used for the production of BL1⁶. Thus, for all targets we could conveniently
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47 303 use GFP fluorescence to monitor their production (Figure 6). For all targets tested
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49 304 yields were significantly improved in *E. coli rhaB'ΔrhaT*. In *E. coli rhaB'ΔrhaT*
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51 305 cells, none of the tested targets gave rise to any detectable IbpB accumulation,
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53 306 indicating that these cells did not experience any stress and that target gene expression
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intensity was balanced with Sec-translocon capacity (Figure 6). Notably, the L-rhamnose concentrations used for optimal protein production in *E. coli rhaB'ΔrhaT* spanned a 20-fold range (from 25 to 500 μM), but in all cases were considerably lower as compared to the wild-type strain.

Summary

To optimize membrane and secretory protein production yields in *E. coli* it is critical to harmonize the production rates of these proteins with the Sec-translocon capacity^{6, 7, 30}, *PrhaBAD*-based expression vectors have previously been used to produce membrane and secretory proteins in *E. coli*⁸, since it is thought that this promoter allows to precisely regulate protein production rates in a concentration dependent manner.

Here, using SfGFP as a target protein for monitoring protein production in real-time we have now shown that *PrhaBAD*-based protein production kinetics are highly dependent on the strain background used. In *E. coli* wild-type, initial protein production rates were independent of the amount of L-rhamnose added to the culture medium. Rather, protein production stopped in a L-rhamnose concentration dependent manner, most likely as a result of L-rhamnose consumption. Therefore, it had appeared, very deceptively, as if protein production rates can be regulated in an L-rhamnose concentration dependent manner in *E. coli* wild-type. Showing that protein production rates in an *E. coli* variant unable to catabolize L-rhamnose are constant and not affected by the L-rhamnose concentration confirmed that the apparent tunability of protein production rates in *E. coli* wild-type is due to L-rhamnose consumption. However, in an *E. coli* variant that neither can catabolize L-rhamnose nor take it up *via* the transporter RhaT, protein production rates could be precisely and

stably set by varying the concentration of L-rhamnose. Notably, in this study all strains were derived from *E. coli* B. However, also in *E. coli* K wild-type the tuneability of *PrhaBAD*-based protein production is based on L-rhamnose consumption and by introducing the *rhaB'* and *ArhaT* mutations protein production rates could also be precisely and stably set (Figure S6). We monitored the *PrhaBAD*-based production of both membrane and secretory proteins in *E. coli* wild-type and in the L-rhamnose catabolism and RhaT-mediated L-rhamnose uptake deficient single and double mutants. The ability to precisely and stably set protein production rates in the *E. coli rhaB'ArhaT* strain led to superior *PrhaBAD*-based production yields of all membrane and secretory proteins tested. The optimal conditions did not cause any notable protein-production related stress, which indicates that protein production rates were indeed harmonized with the Sec-translocon capacity in the *E. coli rhaB'ArhaT* strain. In *E. coli* wild-type any negative side effects such as hampered growth or protein aggregation in the cytoplasm could not be reduced without reducing the target protein yield. Furthermore, the L-rhamnose concentrations required were significantly lower in *E. coli rhaB'ArhaT* than the ones required in the *E. coli* wild-type strain, which leads to lowered protein production costs.

Recently, it was shown that the L-rhamnose analogue L-mannose, which is not catabolised by *E. coli*, can also be used for the *PrhaBAD*-mediated controlled production of proteins³³. It would be very interesting to compare the use of L-mannose as an inducer and the L-rhamnose-based setup described in this study both in terms of protein production yields and cost-effectiveness.

Taken together, to improve *PrhaBAD*-based production of membrane and secretory proteins in *E. coli* when using L-rhamnose as an inducer, a strain background deficient in both L-rhamnose catabolism and RhaT-mediated L-rhamnose

uptake is critical. The high precision of protein production rates provided by the *PrhaBAD* promoter in a L-rhamnose transport and catabolism deficient background could also benefit other applications in synthetic biology.

Methods

Strains, plasmids and culture conditions

E. coli BL21(DE3) was used as wild-type strain and as a starting point to construct the *E. coli rhaB'* and *E. coli rhaB'ΔrhaT* mutants³⁴. In *E. coli rhaB'* a frame-shift mutation was introduced in the gene encoding RhaB essentially as described by Wilms *et al.*²⁷. *E. coli rhaB'ΔrhaT* contains the aforementioned frame-shift mutation in the gene encoding RhaB and in addition the gene encoding RhaT was deleted using recombineering as described by Datsenko and Wanner³⁵. All genes encoding the target proteins used in this study were expressed from the pRha67-derived vector pRha67Km^{R8}. Cloning was done using standard procedures³⁶. All membrane protein targets were produced as C-terminal GFP-His₈ fusions as described before²⁸. Secretory proteins were equipped with a previously described DsbA-derived signal sequence⁶. The scFv BL1 was C-terminally fused to a His-tag⁶. Strains and pRha67Km^R (and derivatives thereof) will be made available under an MTA. The costs involved in shipping materials are for the requesting party. Cells were grown aerobically at 30°C and 200 rpm, in 5 ml Lysogeny broth (LB) medium (Difco) supplemented with 50 µg/ml kanamycin in a 24-well plate format. At an OD₆₀₀ of ~0.5 target gene expression was induced by adding varying amounts of L-rhamnose. For *E. coli* wild-type the following L-rhamnose concentrations were used: 0, 100, 500, 1000, 2500, 5000, 8000 µM, if not indicated otherwise. For *E. coli rhaB'* the following L-rhamnose concentrations were used: 0, 5, 25, 50, 100, 250, 500 µM. For

E. coli rhaB'ΔrhaT the following L-rhamnose concentrations were used: 0, 25, 50, 100, 250, 500, 5000 μ M, if not indicated otherwise. Notably, these L-rhamnose concentrations were selected based upon initial screening of SfGFP production (results not shown). Growth was monitored by measuring the OD₆₀₀ with a UV-1601 spectrophotometer (Shimadzu). For online GFP fluorescence measurements 200 μ l of the induced (or not induced) cultures were transferred at an OD₆₀₀ of $\sim$ 0.5 to a 96 well plate and fluorescence was automatically detected every 5 minutes and the 96 well plate was shaken every 30 seconds²⁵.

Whole cell fluorescence measurements and flow cytometry

Production of membrane protein GFP fusions and secretory SfGFP were monitored using whole-cell fluorescence as described before²⁸. Standard deviations are based on a minimum of three biologically independent experiments. GFP fluorescence, cell size (forward scatter) and granularity (side scatter) were analyzed on a single cell level by flow cytometry using a FACSCalibur instrument (BD Biosciences) as described before²⁹. FM4-64 membrane staining was used to discriminate between cells and background signal. The FlowJo software (Treestar) was used for raw data analysis/processing.

SDS-PAGE, in-gel fluorescence and immuno-blotting

Whole cell lysates (0.05 OD₆₀₀ units) were analyzed by standard SDS-PAGE using 12% polyacrylamide gels followed by immuno-blotting as described before³⁰. His-tagged target membrane proteins were detected using an HRP-conjugated α -His antibody (ThermoFisher) recognizing the C-terminal His-tag. IbpB levels were monitored using antisera from our sera collection, followed by incubation with a secondary HRP-conjugated goat- α -rabbit antibody (Bio-Rad). Proteins were

visualized using the ECL-system (GE Healthcare) according to the instructions of the manufacturer and a Fuji LAS-1000 charge coupled device (CCD) camera and exposure times were kept the same for all conditions used.

BL1 activity measurement

The proper folding of BL1 produced in *E. coli rhaB'ΔrhaT* cells induced with 100 μM L-rhamnose was assayed by the recognition of its substrate, *E. coli* β-galactosidase, using a dot-blot assay and whole cell lysate as described before⁶. As a control the whole cell lysate was treated with the reductant β-mercaptoethanol as describe before⁶.

Isolation of GltP-GFP and GltP activity assay

1 L cultures of cells producing the GltP-GFP-fusion were used as starting material for the isolation of membranes. Membrane isolations, IMAC-based purification of the GltP-GFP fusion and the GltP activity assay were performed as described previously²⁵.

Author Contributions

A.H. designed and conducted experiments and wrote the paper, E.R. designed and conducted experiments, Z.Z designed and conducted experiments, A.K. designed and conducted experiments, D.V. designed and conducted experiments, D.J.S. designed experiments and wrote the paper, J.W.d.G. designed experiments and wrote the paper.

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Conflict of interest

David Vikström is employed by Xbrane Biopharma AB.

Supporting Information

Figures S1 – 6, and Table S1.

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Figure legends

Figure 1. *PrhaBAD*-mediated production of SfGFP in *E. coli*. (a) On the left. Schematic representation of the genes encoding the proteins involved in L-rhamnose utilization in *E. coli*. The *rhaB*, *A* and *D* genes encode the L-rhamnulose kinase, L-rhamnose isomerase, and the rhamnulose-1-phosphate aldolase, respectively. The *rhaR* and *S* genes encode the RhaR and S transcriptional activators. In the presence of rhamnose, RhaR, which is always present independent of the presence of L-rhamnose, activates transcription of *rhaR* and *rhaS* resulting in the accumulation of RhaS. RhaS in turn acts as the L-rhamnose-dependent positive regulator of the *rhaBAD* promoter. The *rhaT* gene encodes the RhaT L-rhamnose transporter. L-lactaldehyde can be metabolized further by an assimilatory aerobic pathway, to pyruvate *via* L-lactate, or an aerobic pathway, to L-1,2-propanediol^{37,38}. The *PrhaBAD*-based expression vector pRha67Km^R used in this study is a variant of the recently published pRha67 vector⁸. In pRha67Km^R the ampicillin resistance marker of pRha67 is replaced with a kanamycin resistance marker. The parts of the *rhaBAD* and *RhaSR* operons that were used to make pRha67 are indicated by the dotted arrows in grey. On the right. Schematic representation of the L-rhamnose uptake and degradation in *E. coli*. (b) *PrhaBAD*-mediated production of SfGFP in *E. coli* wild-type induced with different amounts of L-rhamnose (L-rhamnose concentrations used range from 100 - 8000 μ M, 0 μ M L-rhamnose was used as a control) was monitored on-line by measuring SfGFP fluorescence every 5 min in cells cultured in a 96-well plate in a spectrofluorometer. The vertical lines at 60 and 240 min indicate the timepoints cells were harvested for the flow cytometry experiments presented in panel (c) of this figure. (c) The production of SfGFP per cell was determined using flow cytometry one hour (left panel) and four hours (right panel) after the addition of L-rhamnose. The dotted trace in the right panel represents cells induced with 8000 μ M L-rhamnose that were harvested 24 hrs after induction.

Figure 2. SfGFP production in *E. coli rhaB'*. (a) On the left. To make an *E. coli* strain unable to catabolize L-rhamnose a frameshift mutation in *rhaB* was introduced. On the right. Schematic representation of how the frameshift mutation in *rhaB* affects L-rhamnose degradation. (b) *PrhaBAD*-mediated production of SfGFP in *E. coli rhaB'* induced with different amounts of L-rhamnose (L-rhamnose concentrations used range from 5 - 500 μ M, 0 μ M L-rhamnose was used as a control) was monitored on-line by measuring GFP fluorescence every 5 min in cells cultured in a 96-well plate in a spectrofluorometer. The vertical lines at 60 and 240 min indicate the timepoints cells were harvested for the flow cytometry experiments presented in panel (c) of this figure. The frameshift mutation in *rhaB* did not affect growth in the presence of L-rhamnose (Figure S1). (c) The production of SfGFP per cell was determined using flow cytometry one hour (left panel) and four hours (right panel) after the addition of L-rhamnose. The dotted trace in the right panel represents cells induced with 5 μ M L-rhamnose that were harvested 24 hrs after induction.

Figure 3. SfGFP production in *E. coli rhaB'ΔrhaT*. (a) On the left. To make an *E. coli* strain unable to catabolize L-rhamnose and take up L-rhamnose *via* the RhaT L-rhamnose transporter, the *rhaT* gene in *E. coli rhaB'* was deleted. On the right. Schematic representation of how the deletion of *rhaT* and frameshift mutation in *rhaB* affect the uptake and degradation of L-rhamnose, respectively. (b) *PrhaBAD*-mediated production of SfGFP in *E. coli rhaB'ΔrhaT* induced with different amounts of L-rhamnose (L-rhamnose concentrations used range from 25 - 5000 μM, 0 μM L-rhamnose was used as a control) was monitored on-line by measuring GFP fluorescence every 5 min in cells cultured in a 96-well plate in a spectrofluorometer. The vertical lines at 120 and 240 min indicate the timepoints cells were harvested for the flow cytometry experiments presented in panel (c) of this figure. The inability to catabolize L-rhamnose and take up L-rhamnose *via* the RhaT L-rhamnose transporter did not affect growth in the presence of L-rhamnose (Figure S1). (c) The production of SfGFP per cell was determined using flow cytometry one hour (left panel) and four hours (right panel) after the addition of L-rhamnose. The dotted trace in the right panel represents cells induced with 500 μM L-rhamnose that were harvested 24 hrs after induction.

Figure 4. Production of GltP-GFP in *E. coli* wild-type, *E. coli rhaB'* and *E. coli rhaB'ΔrhaT*. (a) The production of GltP-GFP in *E. coli* wild-type, *E. coli rhaB'* and *E. coli rhaB'ΔrhaT* induced with different amounts of L-rhamnose was assessed by monitoring fluorescence (relative fluorescence unit, RFU) per milliliter of culture 24 hrs after the addition of L-rhamnose²⁸. The L-rhamnose concentrations used for each strain are shown below the bar graph. The concentrations of L-rhamnose optimal for GltP-GFP production in each strain are highlighted in red. Data shown are based on three independent biological replicates. (b) Protein folding/aggregation stress in the cytoplasm of cells from cultures in (a) was monitored by determining the levels of IbpB in whole-cell lysates using immuno-blotting. Equal amounts of cells were loaded per lane. The lanes representing the samples induced with the optimal concentration of L-rhamnose are marked with an asterisk in red. (c) The production of GltP-GFP per cell in the cultures used in (a) and (b) was determined using flow cytometry. (d) GltP-GFP was purified from membranes isolated from *E. coli* wild-type, *E. coli rhaB'* and *E. coli rhaB'ΔrhaT* producing the protein. Subsequently, GltP-GFP was incorporated in liposomes and glutamate uptake was determined.

Figure 5. Production of the scFv BL1 in *E. coli* wild-type, *E. coli rhaB'* and *E. coli rhaB'ΔrhaT*. (a) The scFv BL1 N-terminally fused to a recently described modified DsbA signal sequence was produced in *E. coli* wild-type, *E. coli rhaB'* and *E. coli rhaB'ΔrhaT*-based cultures induced with different amounts of L-rhamnose. Notably, BL1 contains a C-terminal His-tag, facilitating its detection by means of immuno-blotting⁶. For immuno-blotting equal amounts of cells were loaded per lane and an HRP-conjugated α-His antibody was used for detection. * = precursor form of the protein (DsbA-BL1; cytoplasmically localized) and ** = processed form of the protein (BL1; periplasmically localized). Protein folding/aggregation stress in the cytoplasm of cells from the different cultures was monitored by determining the levels of IbpB in whole-cell lysates using immuno-blotting. Optimal conditions for the production of BL1 in *E. coli* wild-type and *E. coli rhaB'ΔrhaT* are marked with a red box. (b) Growth of the cultures producing BL1 at different L-rhamnose concentrations was monitored by measuring their OD₆₀₀ values. Data shown are based on three independent biological replicates. (c) The proper folding of BL1 produced in *E. coli rhaB'ΔrhaT* cells induced with 100 μM L-rhamnose was assayed by the recognition of its substrate, *E. coli* β-galactosidase, using a dot-blot assay and whole cell lysate as described before⁶. Bottom panel. As a control the whole lysate was treated with the reductant β-mercaptoethanol as describe before.

Figure 6. Screening the production of membrane and secretory proteins in *E. coli rhaB'ΔrhaT*. The production of a set of membrane protein GFP-fusions (Table S1) and secretory SfGFP, *i.e.*, SfGFP N-terminally fused to the same signal sequence used for the scFv BL1, was assessed in *E. coli rhaB'ΔrhaT* cells cultured in the presence of different amounts of L-rhamnose. Membrane protein-GFP and secreted SfGFP production was monitored by measuring GFP fluorescence per ml of 24 hours after the addition of L-rhamnose (relative fluorescence unit, RFU) and the optimal L-rhamnose concentration for every target was determined (data not shown). Here, only production yields for the optimal L-rhamnose concentrations are shown. *E. coli* wild-type was used as reference point for all targets in the screen and induced with 8000 μM L-rhamnose (apart from SfGFP where the *E. coli* wild-type-based culture was induced with 5000 μM L-rhamnose). On top of each bar the corresponding L-rhamnose concentration (μM) is given. Data presented are based on three independent biological replicates. Also protein folding/aggregation stress in the cytoplasm of cells from cultures was monitored by determining the levels of IbpB in whole-cell lysates using immuno-blotting as seen in the lower panel. See Figure S3 and Figure S5 for a more detailed characterisation of *PrhaBAD*-based production of YidC-GFP and DsbA-SfGFP, respectively.

a **b** **c**

a **b** **c**

a **b** **c**

a **b** **c** **d**

a **b** **c**