**Escherichia coli** ribose binding protein based bioreporters revisited

Artur Reimer¹, Sharon Yagur-Kroll¹, Shimshon Belkin², Shantanu Roy¹ & Jan Roelof van der Meer¹

¹Department of Fundamental Microbiology, University of Lausanne, Bâtiment Biophore, Quartier UNIL-Sorge 1015 Lausanne, Switzerland, ²Department of Plant and Environmental Sciences, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel.

Bioreporter bacteria, i.e., strains engineered to respond to chemical exposure by production of reporter proteins, have attracted wide interest because of their potential to offer cheap and simple alternative analytics for specified compounds or conditions. Bioreporter construction has mostly exploited the natural variation of sensory proteins, but it has been proposed that computational design of new substrate binding properties could lead to completely novel detection specificities at very low affinities. Here we reconstruct a bioreporter system based on the native *Escherichia coli* ribose binding protein RbsB and one of its computationally designed variants, reported to be capable of binding 2,4,6-trinitrotoluene (TNT). Our results show in vivo reporter induction at 50 nM ribose, and a 125 nM affinity constant for in vitro ribose binding to RbsB. In contrast, the purified published TNT-binding variant did not bind TNT nor did TNT cause induction of the *E. coli* reporter system.

Construction of bioreporter bacteria typically starts with identifying a sensory protein that controls expression of a target gene promoter in dependence on one or more chemical inducers. As an example, the ArsR protein represses its cognate promoter P_{ars}, but when cells are exposed to arsenite (AsIII), this oxyanion will interact with ArsR causing it to lose affinity for the operator site close to P_{ars}, thus increasing the rate of transcription from P_{ars}. The expression of reporter genes, such as those for luciferase, autofluorescent proteins or beta-galactosidase, when coupled to Pars, will consequently increase in the presence of arsenite; it is this increase in reporter protein signal or activity that is quantified in the bioreporter assay. Despite the interest in and potential applicability of bioreporter assays, the weak part in their design is the availability of suitable sensory proteins for recognition of the target chemicals. Most of the "low-hanging fruits" in form of bacterial transcription regulators for e.g., heavy metals and metalloids, organic compounds or global stress responses, have been exploited. Although it has been shown to be possible to somewhat expand substrate recognition properties of known transcription regulators through mutagenesis and selection, this is a rather cumbersome approach. In this context, a landmark study over ten years ago suggested a completely different framework for the construction of bioreporter systems, based on computerized design of de novo substrate binding properties of periplasmic binding proteins (PBPs). Substrate binding to the redesigned PBP would lead to an interaction with a hybrid membrane receptor, thereby triggering reporter gene expression, as will be explained more in detail below (Fig. 1).

PBPs consist of a broad class of proteins that carry a conserved protein structure, the bilobal structural fold. PBPs scavenge molecules for the cell, which they can present to specific transporter channels, and/or link compound binding to chemotactic movement. As an example, the galactose- (GBP) and ribose-binding proteins (RBP) of *Escherichia coli* enable the cell to sense galactose and ribose, respectively. The sugars are bound by their respective PBP, and a fraction of sugar-bound GBP and RBP binds to the Trg chemoreceptor; the other fraction is presented to the transport channels MglAC (for GBP-galactose) or RbsAC (for RBP-ribose). The binding of the PBP to a chemoreceptor can be transformed into de novo gene expression by using a hybrid membrane chemoreceptor-histidine kinase. This was shown almost 20 years ago by the group of Hazelbauer, who linked theEnvZ histidine kinase of the *E. coli* osmoregulation system to the Trg receptor via the so-called HAMP domain (Fig. 1a). The HAMP domain is a conserved domain among histidine kinases, adenyl cyclases, methyl-accepting chemotaxis proteins and phosphatases, and plays a crucial role in signal transduction. The resulting hybrid receptor kinase (named TrzI) combines the 265 N-terminal amino acids of Trg with the 230 C-terminal amino acids of EnvZ. Galactose-GBP and/or ribose-RBP binding to TrzI will trigger histidine kinase activity of the cytoplasmic EnvZ-domain, leading to phosphorylation of the cognate DNA-binding response regulator OmpR. Phosphorylated OmpR (OmpR ~ P) binds the low affinity sites within the OmpC promoter (P_{ompC}) and increases transcription rate from this promoter (Fig. 1a).
fusing PompC with the gene coding for β-galactosidase (lacZ) in E. coli, demonstrating that trzI expression yielded enhanced β-galactosidase activity when exposed to increasing ribose concentrations24.

A major conceptual advancement was made when it was proposed that by molecular dynamics modeling, on the basis of the resolved crystal structure of RBP, with and without ligand, it would be possible to predict the amino acid changes in RBP necessary for binding with new ligands27. This would create a possibly universal scaffold for engineering of new ligand-binding specificities, which could all be hosted in the same signaling “chassis” presented by the hybrid TrzI-OmpR system. To provide proof of principle, the binding pockets of glucose-binding protein (GBP), ribose-binding protein (RBP), arabinose-binding protein (ABP), glutamine-binding protein (QBP) and histidine-binding protein (HBP) were redesigned by computational simulation in order to bind toxic and non-natural molecules, such as serotonin, dinitrotoluene and TNT8. Simulation results suggested that nM binding affinities could be obtained, and experimental data were presented showing that expressing the mutant RBPs in an E. coli TrzI-OmpR background with ompCp-lacZ reporter led to nM detection specificity of TNT by measuring β-galactosidase activity8.

Motivated by the potential importance and implications of a universal scaffold for the engineering of bioreporter ligand specificity, we have set out to repeat the construction of one of the developed bioreporter strains with reported nM affinity for recognition of TNT8. A wide variety of methods is available for detection of TNT

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Figure 1 | Schematic outline of the ribose-binding protein based reporter signaling chain. (a) Ribose-binding protein (RBP) captures its ligand, leading to a conformational change. Ribose-RBP binds the TrzI hybrid transmembrane receptor, which causes a phosphorylation cascade leading to OmpR – P binding the ompC-promoter and consecutive gfpmut2 expression. (b) Relevant plasmid constructions. rbsB or tnt.R3 with original rbsB periplasmic export signal sequence and 3′-hexahistidine tag under transcriptional control of the weak constitutive PAA promoter29. Plasmid pSYK1 with gfpmut2 under the ompC promoter control and trzI under control of Ptac (note that pSYK1 carries the lacIq gene).
(see, for example, ref. 28), but bioreporter-based assays could be interesting for field application. Genes for both wild-type (rbsB) and mutant RBP (tntR3) were produced by DNA synthesis and cloned in an *E. coli* TrzI-OmpR background expressing the GFPmut2 protein from the ompC-promoter, to measure sensitivity of the reporter strains for ribose and TNT, respectively. We examined expression of wild-type and mutant proteins in the reporter strains, and have investigated ligand binding of the purified proteins by isothermal microcalorimetry (ITC). Whereas wild-type RBP produced an excellent and sensitive ribose sensor in *E. coli*, the published T Nbinding RBP variant did not show any significant binding to TNT, and the bioreporter cells harbouring it did not display any response to TNT or ribose.

**Results**

**In vitro characterization of RbsB and TNT.R3 substrate binding.** To test substrate binding by RbsB and TNT.R3 we purified both proteins from *E. coli* and determined the heat released by substrate addition to the purified protein fractions by isothermal microcalorimetry (ITC). Both proteins were overexpressed from the T7 promoter as C-terminal hexahistidine tagged variants in *E. coli* BL21 (DE3) pLysS using IPTG induction of the T7 RNA polymerase. Proteins were purified from culture-cleared lysates using Ni-NTA affinity chromatography. Figure 2 shows the different affinity binding steps and the purity of the final protein fraction (Elution II, at 250 mM imidazol). Concentrations of RbsB-His6 and TNT.R3-His6 after elution were between 0.2 and 0.8 mg/mL (Table 1).

Addition of 250 μM ribose to 34 μM RbsB-His6 solution produced clear evidence for ribose binding with successively decreased heat release upon accumulated ribose additions (Fig. 3a). Assuming a single binding site for ribose per RbsB, an affinity constant (Kd) of 125 nM was calculated from the fitted data sets. Titration of buffer into RbsB-His6 solution, or of ribose solution into buffer produced no heat release (Figure S1). In contrast, neither 1 mM TNT nor 1 mM ribose titration into 20 μM TNT.R3-His6 solution released heat that was different from the addition of buffer alone (Figure 3b, c). Titration of TNT alone into buffer did not produce any consistent heat release as well (Figure S1).

Because the maximum aqueous solubility of TNT, 140 mg/L at 20 °C, may have been limiting for saturation of TNT.R3 binding sites, we also tested the opposite titration in ITC (i.e., protein into substrate solution). In this manner, the TNT concentration in the measurement cell can be maintained below aqueous solubility. However, titration of 50 μM TNT.R3-His6 solution into 150 μM TNT also did not produce consistent heat release (Figure S1A, B). In contrast, titration of 100 μM RbsB-His6 solution into 4 μM ribose did produce heat release (Figure S1C), although in comparison to the binding curve in Figure 3a the injection peaks were not as clear. This may have been due to secondary effects caused by the dissolution of protein agglomerates during the injection. We thus concluded from the *in vitro* experiments that purified RbsB-His6 is indeed capable of binding ribose, but that TNT.R3-His6 neither binds TNT nor ribose, although the protein can be purified and is detectable on SDS-PAGE without any apparent degradation (Fig. 2b).

**RBP-based bioreporter assays.** To verify that *E. coli* expressing the TrzI-hybrid-OmpR signaling chain is indeed a good chassis for an RbsB-based bioreporter, we reconstructed a reporter strain using *gfpmut2* instead of the original lacZ under control of the ompC promoter (Fig. 1b, plasmid pSYK1), and measured induction of GFPmut2 over time in the presence of different sugars. This *E. coli* (strain 4175) lacks the chromosomal rbsB but constitutively expresses rbsB-his6 from a moderately constitutive promoter (PAA, plasmid pAR3). In addition, this strain expresses trzI from the lac promoter and carries the ompC-gfpmut2 fusion (both on plasmid pSYK1). Background fluorescence of *E. coli* BW25113 strain 4175 grown on MM with fumarate as sole carbon and energy source was very low, and the lowest measured ribose concentration that resulted in statistically significant GFPmut2 induction compared to the medium-only control within 2 h incubation time was 50 nM (Fig. 4a). Maximum induction reached 25-fold at a ribose concentration of 10 μM (Fig. 4c). To determine the reporter strain’s specificity we examined GFPmut2 production upon addition of a variety of other sugars, the majority of which (xylose, arabinose, sucrose, fructose, maltose, mannonse, lactose) did not elicit any GFPmut2 induction from the *E. coli* reporter strain at
concentrations below 1 mM. In contrast, significant GFPmut2 production was observed upon incubation with galactose and glucose between 300 nM and 10 μM (Fig. 4d, e). This is likely caused by interference from GBP, which can bind either galactose or glucose, and interacts in its closed (substrate-bound) configuration with the hybrid receptor TrzI (Fig. 4f). The reaction to glucose is lower than to galactose at the same concentration, possibly due to the more rapid interference from GBP, which can bind either galactose or glucose, between 300 nM and 10 μM (Fig. 4f).

Next, we examined the possible influence of a number of key proteins in the chemotaxis or osmolarity sensing pathways on reporter gene induction in the trzI-ompR ompC-gfpmut2 bioreporter strain. Figure 4b displays the characteristic GFPmut2 induction profiles of E. coli BW25113 carrying individual gene deletions as a function of ribose concentration, whereas Figure 4c shows the fold induction levels. In comparison to BW25113 lacking native rbsB, the isogenic strain with envZ interruption showed a loss of responsiveness to ribose (Fig. 4b). In contrast, interruption of rbsK increased the sensitivity of the reporter to ribose (Fig. 4b). A less drastic increase in sensitivity was observed when deleting trg or fliC. Deleting ompC or ompF had very little effect on the sensitivity of the reporter strain for ribose (Fig. 4b).

Interestingly, depending on the host, on ribose concentration and on the deleted gene, the heterogeneity of GFPmut2 production among cells in the population varied significantly (Fig. 5). In general, the per-cell variability in GFPmut2 expression decreased at higher inducer concentrations (Fig. 5). Compared to the host without functional chromosomal rbsB, deletion of fliC, ompC, or rbsK led to a more homogeneously reacting population at lower ribose concentrations (i.e., lower coefficient of variation, Fig. 5c, d, f). Conversely, deleting ompF or envZ resulted in higher cellular variability of GFPmut2 production, almost irrespective of ribose concentrations (Fig. 5a, e). Deleting the chromosomal trg chemoreceptor did not result in any difference of GFPmut2 heterogeneity compared to the ΔrbsB strain (Fig. 5b, g).

**A non-functional TNT bioreporter.** In contrast to the wild-type rbsB-based reporter, replacing rbsB by tnt.R3 in an isogenic host background (E. coli BW25113 ΔrbsB, strain 4176, Table 2) led to complete loss of sensitivity to ribose (Fig. 4a). More importantly, the tnt.R3-based bioreporter was completely unresponsive to TNT over a broad concentration range, 0.06 to 4 μM (Fig. 4a). Since we did not observe any reporter signal from the reconstructed TNT.R3 bioreporter, but excellent sensitivity from the wild-type RbsB bioreporter for ribose, we also examined whether E. coli BW25113 expressed the TNT.R3 protein from the P_{AA-promoter} and periplasmic transport signal sequence at the same level as the RbsB protein (Fig. 1b). Expression of the RbsB-His6 and TNT.R3-His6 proteins in the BW25113 bioreporter strain is under control of the same low constitutive P_{AA-promoter} in the same plasmid background. Unfortunately, anti-His6-antibodies did not produce sufficient sensitivity and selectivity to detect both proteins in Western blots of cytoplasmic cell extracts from the BW25113 strains (not shown). In-gel staining of the His6-tag revealed fluorescent bands with an apparent size of around 30 kDa in the cytoplasmic protein fraction, which were not completely specific for the expressed RbsB-His6 and TNT.R3-His6 in extracts from E. coli BW25113 (Fig. 2c). Analysis of the periplasmic fraction indicated presence of a detectable RbsB-His6 but not TNT.R3-His6 (Fig. 2c). Gel-extracted and trypsin-digested protein fractions in the size range of 28–36 kDa were further analyzed by nano-liquid chromatography followed by direct peptide mass identification (Table 3). This analysis confirmed that both RbsB-His6 and TNT.R3-His6 are produced by E. coli BW25113 ΔrbsB from the P_{AA-promoter}. However, whereas we identified RbsB-His6 in the whole soluble and in the periplasmic

| Table 1 | Protein concentrations during purification of RbsB-His6 and TNT.R3-His6 from E. coli BL21 (DE3) |
|----------------|--------------------------------------------------|
| Sample         | Total protein concentration (mg/ml) |
| Column flow through | 43.9 | 27.1 |
| Washing step I   | 22.9 | 14.4 |
| Washing step II  | 2.4  | 1.2  |
| Elution I        | 5.0  | 0.7  |
| Elution II       | 0.8  | 0.2  |
| Elution III      | 0.12 | 0.01 |

Figure 3 | In vitro substrate binding using isothermal microcalorimetry. (a) Injections of 250 nM ribose into 34 μM purified RbsB-His6 solution. (b) Injections of 1 μM TNT into 50 μM purified TNT.R3-His6 solution. (c) Injections of 1 μM ribose into 50 μM purified TNT.R3-His6 solution. Graphs display immediate heat release in μcal/s (upper panels) and calculated heat release per mol of injectant (lower panels). Note the expected binding of ribose to RbsB (A), but the absence of any detectable binding of TNT or ribose by TNT.R3. For further controls see SI Figure 1.
protein extracts, TNT.R3-His6 was only detectable in the whole soluble but not in the periplasmic protein extract (Table 3). We conclude from this part that whereas both RbsB-His6 and soluble but not in the periplasmic protein extract (Table 3). We conclude this is also true for TNT.R3-His6 (Fig. 2c, Table 3). The absence of appears to reach the periplasmic space we did not find evidence that are produced in such

detection limit of TNT, contrary to the reported lowest detection limit of between 10^-2 and 10^-3 μM and a Kd of 2 nM, a value 65 times lower than the affinity we measured for the wild type RbsB protein towards ribose (125 nM). These findings, however, do not null the notion that a platform using biosensors based on periplasmic binding proteins could be a powerful tool. Indeed, E. coli expressing RbsB in combination with the TrzI-hybrid-OmpR ompCp-gfpmut2 signaling chain turned out to be an excellent reporter for ribose (method detection limit of ~50 nM), with a good selectivity (no reaction to multiple sugars and 10-fold lower detection threshold of ribose than glucose, galactose or ribose. Data points show means of GFPmut2 fluorescence from biological triplicate assays, each sampling 10,000 cells. Error bars indicate calculated standard deviations from the mean (when not visible, inside symbol size).

**Discussion**

We revisit here the use of a PBP-based microbial biosensor, proposed over a decade ago as a general scaffold for computational design of new binding specificities. We show through independent de novo synthesis that a wild-type RbsB-based signaling cascade is fully functional in detecting low concentrations of ribose, but one of the most interesting computationally designed variants for detecting TNT is not. We conclude this from three different experimental lines of evidence. First, we demonstrated that the purified RbsB bound ribose, as expected, whereas purified TNT.R3 protein bound neither TNT nor ribose at detectable levels (Fig. 3). Second, constitutive expression of rbsb-his6 under a moderate promoter in an E. coli harboring the TrzI hybrid receptor and ompCp-gfpmut2 fusion led to a ribose-dependent production of GFPmut2 fluorescence already at 50 nM ribose (Fig. 4a, c). In contrast, assays in the same genetic background and plasmid constructions with a rbsB-his6 expression of TNT nor ribose at detectable levels (Fig. 2c, Table 3). We conclude this to ribose may be explained by the fact that deleting rbsK-mutant of E. coli rather than rbsB (Fig. 4C, D). The higher response of this strain to ribose may be explained by the fact that deleting rbsK.
interrupts ribose metabolism, leaving on average more ribose to activate the signaling chain through RbsB-Trz1-OmpR rather than going through the ribose transport system. Also, a trz1 host mutant background produced a more sensitive response, but with overall lower fold-induction (Fig. 4c), which may be due to less internal competition for ribose-bound RbsB by the natural Trg chemotaxis receptor. Deletion of envZ, ompF, and, surprisingly, flIC, resulted in a much poorer response to ribose (Fig. 4b, c). Disruption of native envZ may result, in spite of the presence of Trz1, in a lower overall amount of OmpR ~ P in the cell, causing less frequent binding to the weak-affinity OmpR-sites in the PompC promoter, and thus to a lower level of gfpmut2 transcription. These results show that the host chassis for a ribose-binding protein based reporter may be further improved and would prove useful, once the limitations in computational design are overcome.

Along with techniques such as directed evolution, computational protein design has been successful in the design of enzyme catalysts, new protein folds, or antigens, but its predictions still fall short especially in the protein-small molecule domain. Many methods adopted in the current context, including the technique of dead-end elimination along with a semi-empirical potential energy function, used in designing TNT.R3 and other PBP-variant receptors, do not take into consideration the flexibility of the protein backbone. This concerns specifically the substrate binding pocket flexibility, dynamics of the structure upon binding, and calculation of the protein stability or consistency of the 3D fold of the designs. Because of the magnitude of the combinatorial search problem to be tackled in protein design, the chemical accuracy of the calculations is significantly reduced. For a receptor like RBP, the energetic or entropic cost of the conformational re-organization or domain re-orientation during binding can be very important but difficult to calculate. This also holds for other relevant steps in the PBP signalling cascade, such as the binding of the ligand-bound PBP with the transmembrane receptor. So far, therefore, these steps have not been included in energy calculations.

Very recently, it has been demonstrated that in-silico design of small-molecule binding proteins can drive the binding affinity down to picomolar level. The computational method was modified so that the designs have binding pockets similar to the naturally occurring ones, in terms of the favourable hydrogen-bonding or van der Waals interactions with the ligand, and high shape complementarity to the ligand. These advances may be highly beneficial for the design of sensory proteins and extremely advantageous to the biosensing field, given that the set of known characterized transcription factors is small and in view of the difficulties involved in the identification of suitable transcription factors for the detection of new compounds of interest.

**Methods**

**Strains and growth conditions.** All E. coli strains used for this work are listed in Table 2. For cloning purposes, E. coli strains were cultured at 37°C on Luria Bertani (LB) medium, supplemented with appropriate antibiotics to select for plasmid maintenance. In case of ampicillin (Amp), a concentration of 100 μg/ml was used; for chloramphenicol (Cm), we used 30 μg/ml. Culturing conditions for protein overexpression and for reporter assays are specified below.

**Plasmid constructions.** The mutant tnt.R3 gene was produced by DNA synthesis (DNA2.0, CA, USA) on the basis of the mutant sequence provided in Looger et al. The gene sequence further encoded a C-terminal hexa-histidine (His6) tag, restriction sites for NdeI (N-terminal), Xhol and BamHI (C-terminal), and for NcoI at the end of the signal sequence (Figure S2). The wild type rbsB gene was amplified from pAI12 (a kind gift of Hazelbauer’s lab, Pullman, Washington) using primers with restriction sites for NdeI and XhoI. The PCR product was digested and placed into pET22b (+) in order to attach the vector-located hexa-histidine tag to the C-terminus of the rbsB gene. The resulting rbsB-His6 gene was again amplified with primers containing Ndel and SalI restriction sites. The PCR product was digested and inserted into pET22b (+) to give the expression vector pET22b(+)-rbsB. The PCR product was digested and inserted into pET22b (+) to give the expression vector pET22b(+)-rbsB. The PCR product was digested and inserted into pET22b (+) to give the expression vector pET22b(+)-rbsB. The PCR product was digested and inserted into pET22b (+) to give the expression vector pET22b(+)-rbsB.
control of the BamHI site of plasmid pRB020, which carries a BamHI restriction site on either end. This fragment was ligated into the unique and tnt.R3-His6 transformation in (Table 2).

For overexpression the synthesized intR3-His6 fragment was digested with Ncol and BamHI and placed into pET3d resulting in plasmid pRB020. This removes the signal sequence for transport in the periplasmic space. RbsB-His6 was amplified from plasmid pAI12, now without the signal sequence using primers containing Ndel and Xhol restriction sites. The Ndel-Xhol fragment was purified and placed into pET22b (+) digested with the same enzymes, directly in front of the hexahistidine tag. After transformation this resulted in plasmid pAR1. All final constructs were verified by sequencing. Plasmids mL LB medium containing Amp and Cm were inoculated with a single colony from a 8.0. Half of the suspension (for the preparation of the total soluble protein fraction or for overexpression were transformed into resulted in plasmid pAR1. All final constructs were verified by sequencing. Plasmids

Table 2 | List of strains used in this study with their relevant characteristics

| Strain No | Host | Plasmid(s) | Relevant characteristics | Reference |
|----------|------|------------|--------------------------|-----------|
| 97       | *E. coli* BL21 (DE3) | pLYS5 | Host strain for overexpression from the T7 promoter | 40 |
| 3325     | *E. coli* BL21 (DE3) pLYS5 | pAR2 | Host 97, cytoplasmic overexpression of His6-tagged TNT.R3 | This study |
| 3725     | *E. coli* BL21 (DE3) pLYS5 | pAR1 | Host 97, cytoplasmic overexpression of His6-tagged RbsB | This study |
| 4076     | *Esherichia coli* BW25113 ΔrbsB | rbsB | expression of His6-tagged RbsB with signal sequence from PAA | This study |
| 4175     | *E. coli* BW25113 ΔrbsB | pAR3 pSYK1 | expression of TrzI, gfpmut2 fusion to ompC promoter | This study |
| 4176     | *E. coli* BW25113 ΔrbsB | pAR4, pSYK1 | as 4175, but expressing the TNT.R3 mutant protein | This study |
| 4497     | *E. coli* BW25113 ΔrbsB | pSTV28, pSYK1 | as 4175, but with empty vector. | This study |
| 4500     | *E. coli* BW25113 ΔompF | ompF | | |
| 4501     | *E. coli* BW25113 Δtrg | trg | | |
| 4502     | *E. coli* BW25113 ΔrlC | rlC | | |
| 4503     | *E. coli* BW25113 ΔompC | ompC | | |
| 4504     | *E. coli* BW25113 ΔenvZ | envZ | | |
| 4505     | *E. coli* BW25113 ΔrbsK | rbsK | | |
| 4515     | *E. coli* BW25113 ΔompF | pAR3, pSYK1 | as 4175 in host 4500 | This study |
| 4516     | *E. coli* BW25113 Δtrg | pAR3, pSYK1 | as 4175 in host 4501 | This study |
| 4517     | *E. coli* BW25113 ΔrlC | pAR3, pSYK1 | as 4175 in host 4502 | This study |
| 4518     | *E. coli* BW25113 ΔompC | pAR3, pSYK1 | as 4175 in host 4503 | This study |
| 4519     | *E. coli* BW25113 ΔenvZ | pAR3, pSYK1 | as 4175 in host 4504 | This study |
| 4520     | *E. coli* BW25113 ΔrbsK | pAR3, pSYK1 | as 4175 in host 4505 | This study |

Table 3 | Occurrence of RbsB-His6 and TNT.R3-His6 in *E. coli* BW25113 ΔrbsB (pSYK1) protein fractions

| Identified protein(s) | Periplasmic protein fraction | Whole soluble protein fraction |
|-----------------------|-----------------------------|-------------------------------|
|                       | Strain 4175 | Strain 4176 | Strain 4497 | Strain 4175 | Strain 4176 | Strain 4497 |
| RbsB-His6             | 18 [8]a | 5 [5] | 4 [4] | 14 [5] | 1 [1] | 1 [1] |
| TNT.R3-His6           | ND | ND | ND | ND | 2 [2] | ND |
| RbsB-His6 + TNT.R3-His6 | 18 [5] | 6 [6] | ND | 10 [5] | 5 [3] | ND |
| Total RbsB-His6 + TNT.R3-His6 | 36 [13] | 11 [11] | 4 [4] | 24 [10] | 8 [6] | 1 [1] |
| Total proteins        | 116 [387]b | 86 [277]b | 104 [349]b | 259 [1273]b | 234 [971]b | 254 [1061]b |

1) 28–36 kDa fragments on SDSPAGE gel from extracts as shown in Fig. 2C, originating from *E. coli* BW25113 ΔrbsB (pSYK1) expressing rbsB-his6 (from plasmid pAR3, strain 4175), *E. coli* BW25113 ΔrbsB (pSYK1) expressing intR3-his6 (from plasmid pAR4, strain 4176), or *E. coli* BW25113 ΔrbsB (pSYK1) carrying empty pSTV (strain 4497, Ctrl).

2) Normalized number of peptide mass fragments identifying the protein(s) as total number of specific peptide fragments by the averaged total peptide count in the sample; between brackets, number of unique peptide mass fragments identifying the protein(s).

3) ND, not detected.

4) Peptides covering regions common to both RbsB-His6 and TNT.R3-His6.

5) All peptides identifying both RbsB-His6 and TNT.R3-His6.

6) Total number of identified proteins within the 27–32 kDa purified gel region; within brackets: total number of identified peptide mass fragments.
Expression and export of RbsB-His and TNT.R3-His from the P$_{rpa}$ promoter was separately analyzed using direct peptide mass identification. E. coli BW25113 cultures were grown and periplasmic or whole soluble protein fractions were prepared as described above. Proteins were separated by SDS-PAGE and proteins in a size region of 28-36 kDa were excised. Proteins were subsequently digested with trypsin and peptides were separated on an Ultimate 3000 Nano LC System (Dionex), followed by detection with the Thermo Scientific LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Mass spectra were analyzed using Scaffold Viewer (http://www.proteomescf.com/), using protein and peptide identification thresholds of 99.9% and 99.99%, respectively. The minimum number of peptides for identification was 2.

**RbsB-His, and TNT.R3-His, overexpression and purification.** In order to analyze in vitro substrate binding, we first overexpressed and purified RbsB-His and TNT.R3-His. We used the C-terminal added hexahistidine tag in combination with Ni-NTA affinity chromatography (Qiagen, Germany). Proteins were overexpressed in E. coli BL21(DE3)pLysS carrying the appropriate plasmid (pR11 for RbsB-His or pPARP for TNT.R3-His). Cultures were launched at 37°C overnight at 37°C. Cells were harvested by centrifugation at 3,200 g for 40 min. Falcon tubes. Cell pellets were stored at −80°C until protein isolation.

For purification of the His$_6$-tagged proteins, one cell pellet was resuspended in 4 mL of buffer A (see above). The suspension was transferred into 1.5 mL screw-capped plastic tubes containing 0.1 g glass beads (see above), and homogenized in a bead beater (FastPrep 2400, MP Biomedicals, Santa Ana, CA) at a speed of 4.0 with intermittent cooling on ice. After centrifugation for 30 min, at 16,000 x g and 4°C the supernatant was transferred to a clean tube, and mixed with 750 µL of Ni-NTA resin (Qiagen, Germany) for one hour at 4°C using a multi axle rotator (A257, Denley Instruments LTD, United Kingdom). Subsequently, the protein-Ni-NTA suspension was poured onto a 1 mL polypropylene column (Qiagen, Germany) (2.5 cm x 1.5 cm). The resin was washed with buffer A and then 80 mM imidazole, the proteins were eluted in fractions of 600 µL using buffer A with 250 mM imidazole. The suspension was transferred into 1.5 mL screw-cap tubes and then aliquots of 150 µL were auto-sampled, and values of $m^/$s and the cell density was measured using a multi axle rotator (A257, Denley Instruments LTD, United Kingdom). The next morning, 2 mL of this suspension was auto-sampled, and values of $m^/$s and the cell density was measured using a multi axle rotator (A257, Denley Instruments LTD, United Kingdom). A volume of 280 µL of the suspension was transferred to 3 tubes at 20°C and a speed of 4.0 with intermittent cooling on ice. After centrifugation for 30 min, at 16,000 x g and 4°C the supernatant was transferred to a clean tube, and mixed with 750 µL of Ni-NTA resin (Qiagen, Germany) for one hour at 4°C using a multi axle rotator (A257, Denley Instruments LTD, United Kingdom). Subsequently, the protein-Ni-NTA suspension was poured onto a 1 mL polypropylene column (Qiagen, Germany) (2.5 cm x 1.5 cm). The resin was washed with buffer A and then 80 mM imidazole, the proteins were eluted in fractions of 600 µL using buffer A with 250 mM imidazole. Flow-through from the column was collected and analysed by SDS-PAGE and Coomassie blue staining (see above). Protein concentrations were determined using the Bradford assay and by NanoDrop (Thermo Scientific, USA), using the “Protein A280” mode with calculated theoretical molar extinction coefficient and molecular weight as parameters. Purified protein was stored on ice and used in 5 h for the substrate-binding assay, without further dialysis.

**Analysis of substrate binding using isothermal microcalorimetry (ITC).** Quantified amounts of purified protein, typically 280 µL of between 0.6 and 1 µg/µL, were pipetted into the measurement cell of an isothermal titration calorimetry instrument (MicroCal iTC200, GE Healthcare Life Sciences, USA). A volume of 280 µL of the buffer (buffer A with 250 mM imidazole) was used as a reference. An appropriate concentration of the test ligand (between 0.25 and 1 mM in buffer A containing 250 mM imidazole) was filled into the injection syringe. In other experiments we tested injecting purified protein solution (of between 1.4 and 2.8 µg/µL) into substrate solution in the measurement cell. The substrates tested were ribose and TNA and were taken at 20°C, at 25 µL/s, with a stirring velocity of 1,000 rpm and a “low feedback” mode. Raw data were recorded in µcal/s over time and integrated to kcal/mol over molar ratio. Wherever possible regression curves were calculated based on a one-binding site model.

**RBP-based bioreporter assays using the Trrl-OmpR hybrid signaling chain.** In order to measure the capacity of RbsB or TNT.R3 to induce the Trrl-hybrid-OmpR ompC-gfpmut2 signaling chain in the presence of appropriate inducer, we used E. coli BW25113Arbs cotransformed with pSTV-based plasmids (pAR3, to express ribb, or pPAR4 for trnt.R3) and plasmid pSY1 (to provide the hybrid signaling chain, see Table 1) on a single plasmid. On a single plasmid, these strains produce the same transcriptional intensity of which was measured using flow cytometry. The bioreporter assay was optimized for minimal background GFPmut2 expression and medium fluorescence. Hereto, 5 mL of minimal medium with Amp and Cm (SI Table 1) with 20 mM fumarate as sole carbon and energy source were inoculated with a single colony from a freshly grown LB plate containing the same antibiotics. Cultures were incubated further for 16 h at 20°C, after which the cells were harvested by centrifugation at 3,200 g in four 50 mL Falcon tubes. Cell pellets were stored at −80°C until protein isolation.

**Cell bioreporters for the detection of TNT in environmental samples.** P. aeruginosa PAO1 strains expressing RbsB-His and TNT.R3-His overexpression and purification. Cells were grown and periplasmic or whole soluble protein fractions were prepared as described above. Proteins were separated by SDS-PAGE and proteins in a size region of 28-36 kDa were excised. Proteins were subsequently digested with trypsin and peptides were separated on an Ultimate 3000 Nano LC System (Dionex), followed by detection with the Thermo Scientific LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Mass spectra were analyzed using Scaffold Viewer (http://www.proteomescf.com/), using protein and peptide identification thresholds of 99.9% and 99.99%, respectively. The minimum number of peptides for identification was 2.

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**Experimental Materials and Methods.** Details about the procedures used in this study can be found in the Supporting Information.

**Supplementary Information.** Supplementary Information is available for this article.

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**Author contributions**

A.R. and S.Y.K. performed experiments. A.R. and J.R.M. prepared Figures 1–5. S.B. and S.Y.K. contributed strains and experimental advice. A.R., S.R. and J.R.M. wrote the main manuscript. All authors reviewed the final manuscript.

**Additional information**

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Artur Reimer, Sharon Yagur-Kroll, Shimshon Belkin, Shantanu Roy & Jan Roelof van der Meer

The original version of this Article contained an error in the title of the paper, where the word “*Escherichia*” was incorrectly given as “*Escherchia*”. This has now been corrected in both the PDF and HTML versions of the Article.