

LETTER

Engineering multi-cellular logic in bacteria with metabolic wires

by

Rafael Silva-Rocha[§] and Víctor de Lorenzo**Systems Biology Program, Centro Nacional de Biotecnología CSIC, Cantoblanco-Madrid, 28049 Spain***Running Title:** Metabolic wires in bacterial logic gates**Keywords:** Regulatory networks, regulator-inducer specificity, logic gates, *Pseudomonas*, biodegradation, TOL system

*Correspondence to: Víctor de Lorenzo
Centro Nacional de Biotecnología-CSIC
Campus de Cantoblanco, Madrid 28049, Spain
Tel.: 34- 91 585 45 36; Fax: 34- 91 585 45 06
E-mail: vdlorenzo@cnb.csic.es

[§]Current address: FMRP - University of São Paulo, Ribeirao Preto, SP, Brazil.

1 SUMMARY

2
3 Aromatic biodegradation pathways of environmental bacteria are vast sources of matching trios of
4 enzymes, substrates and regulators that can be refactored to run logic operations through cell-to-cell
5 communication. As a proof of concept, the connection between two *Pseudomonas putida* strains using
6 benzoic acid as the wiring molecule is presented. In this system, a *sender* strain harbouring the TOL
7 pathway for biodegradation of aromatics processed toluene as input and generated benzoate as the
8 output signal. Diffusion of such metabolic intermediate to the medium was then sensed by a second
9 strain (the *receiver*) that used benzoate as input for a new logic gate producing a visual output (i.e.
10 light emission). The setup was functional irrespective of whether sender and receiver cells were in
11 direct contact or in liquid culture. These results highlight the potential of environmental metabolic
12 pathways as sources of building blocks for the engineering of multi-cellular logic in prokaryotic
13 systems.

14

15

16 INTRODUCTION

17

18 One of the most fascinating potential applications of synthetic biology is the re-programming of cells to
19 perform sophisticated electronic-like computations^{1,2}. Most research in this area focuses on the design
20 and implementation of regulatory logic gates as the building blocks for the assembly of more complex
21 circuits³⁻⁶. Consequently, all 16 Boolean logic gates for two-inputs have been implemented in living
22 cells through a number of different experimental setups⁶⁻⁸. While much progress has been made with
23 these relatively simple circuits, the real challenge is to engineer complex synthetic circuits for
24 applications of biotechnological value^{1,2}. Technical difficulties stems from the interconnection of many
25 different logic gates in one organism, both in terms of the propagation of noise during circuit operation⁹,
26 ¹⁰ and by the lack of sufficiently different regulatory parts (i.e. promoters and transcription factors) to
27 assemble the system of interest^{11,12}. A way to overcome this state of affairs is the engineering of new
28 regulatory elements with well-defined, orthogonal activities suitable for circuit implementation, as
29 recently described^{13,14}. An alternative approach is based on the construction of multi-cellular synthetic
30 circuits implemented such that parts of the system are split between different host strains^{7,15}. In these
31 systems, signal computation from the initial strains (those that sense the system inputs) are

1 transmitted to other strains via production of a signalling compound (the *wire*), which is then further
2 processed in the cascade. Diffusible wires used to date include bacterial quorum-sensing molecules⁷
3 and yeast pheromones¹⁵. This division of labour-type approach could in principle allow the engineering
4 of very sophisticated synthetic cellular programs for biotechnological and biomedical uses.

5
6 As multi-cellular circuit engineering requires the *wiring* of the host cells harbouring the system
7 components, the clear problem is the limited number of well-characterised signalling molecules
8 currently available¹⁶. There is thus a need to expand the number of *molecular wires* available for
9 engineering logic circuits. In this context, we pondered the value of small molecules unrelated to
10 quorum sensing as potential vehicles for assembling multi-cellular logic gates. Specifically, we focused
11 on the intermediary, diffusible metabolites originating from catabolic pathways for aromatic compounds
12 borne by environmental bacteria¹⁷⁻¹⁹. Complete genome sequences of such microorganisms (e.g. the
13 Gram-negative soil bacterium *Pseudomonas putida*) have revealed a large repertoire of genes
14 encoding pathways for utilization of aromatic compounds as carbon sources²⁰. Diverse substrate-
15 responsive transcription factors are required for expression of these catabolic pathways, each of them
16 with different DNA- and ligand-binding specificity^{18,21,22}. Additionally, biodegradation intermediates of
17 aromatic compounds are known to diffuse and be catabolized by different members of the bacterial
18 community²³ such that communication mediated by metabolic signals has precedents in natural
19 scenarios.

20
21 In this work, we report the engineering of a simple cell-to-cell communication device in *Pseudomonas*
22 *putida* using the pathways for toluene and benzoate (*Bz*) degradation encoded in this organism. Using
23 a series of promoter-reporter fusions, we showed that the extracellular diffusion of *Bz* during toluene
24 catabolism could be sensed as an input by a reporter strain engineered with a *Bz*-responsive promoter.
25 The receiver strain was able to further metabolise the released *Bz* and trigger a second reporter
26 system based on the metabolic intermediate *cis,cis*-muconate (*2cM*). This approach can be expanded
27 to many other characterised catabolic systems.

28
29 RESULTS AND DISCUSSION

30

1 As a proof of concept for the utilisation of metabolic wires, we generated a simple system based on
2 two different strains. The *sender* strain senses the input and generates an output, and the *receiver*
3 takes the output from the first strain as its input. The general strategy is depicted in **Fig. 1a**. As shown
4 in the figure, input A is processed by the logic circuit (formed by the promoter P_1 and regulator R_1)
5 existing in the *sender* strain to generate output B, which then diffuses to the extracellular medium.
6 Once in the medium, this compound is perceived by the *receiver* strain as an input to its logic circuit
7 (composed by the promoter P_2 and regulator R_2). The outcome of this system, output C, can be a
8 reporter protein as implemented here or could be another signalling molecule that could be further
9 connected to additional strains. The theoretical limitation for the number of steps an entry compound
10 could generate would depend on the number of metabolic intermediates of the pathway that can be
11 sensed by transcription factors. For experimental validation of the system, we focused on catabolic
12 pathways for toluene and *Bz* from *P. putida*^{24,25}. *P. putida* mt-2 is a versatile environmental bacterium
13 that completely metabolises toluene to generate TCA cycle intermediates²⁶. This task is performed by
14 two pathways known as *upper* and *meta* that are encoded in the large catabolic plasmid pWW0
15 (TOL²⁴). The *upper* enzymes perform the first step of toluene metabolism, generating *Bz* as a
16 metabolic intermediate (**Fig. 1b**). *Bz* is transformed into TCA intermediates by the *meta* pathway²⁴. In
17 the TOL system, induction of the *upper* pathway is activated by the regulator XylR in response to
18 toluene, while induction of *meta* genes is triggered by XylS bound to *Bz*^{27,28}. Additionally, most
19 *Pseudomonas* species have a specific metabolic route for *Bz* metabolism known as the *ortho*
20 pathway^{25,29}. **Fig. 1c** shows the main steps of such *ortho* route as found in *P. putida* KT2440, the
21 pWW0-cured variant of *P. putida* mt-2³⁰. A set of three operons (*ben*, *cat* and *pca*) are involved in *Bz*
22 degradation. These operons are under the regulation of three transcription factors, BenR, CatR and
23 PcaR²⁹. Each of these regulators senses specific intermediates (*Bz*, 2cM and β KA) depicted in **Fig. 1c**.
24 Thus, BenR activates *Pb* (*ben* promoter) in response to *Bz*, while CatR triggers *Pc* (*cat* promoter) in
25 the presence of 2cM^{25,29}. Finally, PcaR triggers the induction of the *pca* promoter (*Ppca*) in response
26 to β -keto adipate (β KA^{25,29})

27

28 Taking into account the regulatory and metabolic relationships explained above, we implemented a
29 cell-to-cell communication system where *Bz* was the metabolic wire between the *sender* and *receiver*
30 strains. In our setup, *P. putida* mt-2 was the *sender* strain, and toluene was the first input to the
31 system. Toluene was sensed by *P. putida* mt-2 and triggered the expression of the *Pu* (*upper*

1 promoter) TOL pathway. The *upper* enzymes converted toluene into *Bz*. While this compound can be
2 further metabolised by the *meta* pathway, part of the product diffused to the extracellular medium;
3 there it could be sensed by the *receiver* strain (*P. putida* KT *Pb::lux*) harbouring the BenR-*Pb-lux*
4 reporter system, which generated a light signal in response to *Bz* (**Fig. 2d**). **Fig. 2a** highlights the main
5 logic interactions in TOL system of the *sender* cell³¹ during the response to toluene as well as the
6 receiver cell logic circuit controlling the production of the reporter output (i.e. light). The truth table of
7 the AND logic gate used to construct the representations is shown in **Fig. 2b**, (a systems
8 representation of the two logic circuits is shown in Supplementary Fig. S1).

9
10 For the construction of the metabolically wired logic system, we used two different *receiver* strains as
11 shown in **Fig. 3a-b**. The first was the above-mentioned BenR-*Pb-lux* reporter strain that directly
12 sensed *Bz*³² (**Fig. 3a**). For the second system, we used a reporter strain based on the CatR-*Pc* system
13 (*P. putida* KT *Pc::lux*; **Fig. 3b**) that is triggered by the metabolic intermediate *2cM* produced during *Bz*
14 degradation by the *ortho* pathway (**Fig. 1c**). This strain only produces output signal if *Bz* is
15 metabolised by enzymes encoded by the *ben* genes. The use of two different receiver strains was
16 important to ensure that the levels of *Bz* released to the medium could induce expression of enough
17 *ben* enzymes capable to convert this metabolite to *2cM*, which in turn could trigger the CatR-*Pc*
18 system. To evaluate the response of these two sensor bacteria to the inducer *Bz*, we assayed the
19 promoter activity in liquid medium where each strain was exposed separately to 1 mM of this
20 compound. Overnight cultures were diluted in fresh medium with the inducer and incubated for several
21 hours in a plate reader. At 30-min intervals, light emission and optical density at 600 nm (OD₆₀₀) were
22 recorded and used to calculate the promoter activity as described in the Methods section. Uninduced
23 cultures were used as controls to calculate basal promoter activities. As shown in **Fig. 3c-d**, both
24 reporter systems were highly responsive to *Bz*. The BenR-*Pb*-based strain showed a higher induction
25 than its CatR-*Pc* counterpart (152.5- vs. 9.8-fold, respectively). This result was mainly due to the
26 higher basal activity observed for *P. putida* KT *Pc::lux* (**Fig. 3d**). We next investigated the sensitivity of
27 *P. putida* KT *Pb::lux* in low concentrations of *Bz*. *P. putida* KT *Pb::lux* was assayed as before except
28 that cells were exposed to *Bz* concentrations ranging from 0.5 to 25 μ M. As shown in **Fig. 3e**, strain *P.*
29 *putida* KT *Pb::lux* was highly sensitivity to the inducer; as little as 1.25 μ M triggered 7.5-fold promoter
30 induction. The highest concentration resulted in 43-fold induction. Taken together, these results

1 revealed the high sensitivity of the *P. putida* KT *Pb::lux* reporter strain at micromolar concentrations of
2 the inducer *Bz*, making it a suitable for the cell-to-cell wired system.

3
4 After characterisation of the individual receiver strain, we validated the synthetic approach for
5 metabolic wiring by mixing *P. putida* sender and receiver strains and exposing the cells to toluene. As
6 sender strains, we used either *P. putida* mt-2 or *P. putida* KT2440, a variant of the former lacking the
7 TOL plasmid. Because *P. putida* KT2440 is unable to metabolise toluene, it serves as a control where
8 the sender and receiver strains do not communicate. The receiver strains were *P. putida* KT *Pb-lux*
9 and *P. putida* KT *Pc-lux*, described above. Initially, we assayed the wiring between the strains in solid
10 media. Each individual strain was grown as described in the Material and Methods. After pre-growth,
11 10 μ L of the cultures were added to the surface of 1.6% agar plates in the following four combinations
12 of senders/receivers: (i) *P. putida* KT2440/*P. putida* KT *Pb-lux*; (ii) *P. putida* KT2440/*P. putida* KT *Pc-*
13 *lux*; (iii) *P. putida* mt-2/*P. putida* KT *Pb-lux*; and (iv) *P. putida* mt-2/*P. putida* KT *Pc-lux*. These
14 combinations are represented in **Fig. 4a**. Toluene was entered in medium in the form of saturating
15 vapours as described in the Methods section. The plates were sealed air-tight and incubated for 4
16 hours. After incubation, bioluminescence was analysed using a CCD camera in a VersaDoc Imaging
17 System (Bio-Rad). As shown in **Fig. 4b**, after exposure to toluene vapours, both *P. putida* KT *Pb-lux*
18 and *Pc-lux* receiver strains mixed with the *P. putida* mt-2 sender generated higher light emission than
19 the controls where *P. putida* KT2440 was used as the sender, while control conditions (where no
20 toluene was added) failed to enhance luminescence of the sensors. This result showed that *Bz*
21 production from toluene by upper enzymes was indeed detected by the reporter strains.

22
23 To further characterise the transmission of *Bz* from the sender to the receiver, quantitative
24 experiments were performed in liquid media. Overnight cultures were diluted 1:10 in 1X PBS buffer in
25 similar combinations as in the solid media experiments (**Fig. 4c**). Mixed cells were exposed to toluene
26 vapors for 20 min and loaded onto a plate reader. At 1-hour intervals, bioluminescence and OD₆₀₀
27 were measured and used to calculate promoter activity. As shown in **Fig. 4d**, promoter activity in the
28 receiver strains was highly stimulated by toluene when *P. putida* mt-2 was used as the sender strain.
29 In contrast, when *P. putida* KT2440 was used as the sender, the receiver strains only produced low-
30 level basal promoter activity. These results confirmed that *Bz* produced by *P. putida* mt-2 was

1 specifically sensed by the *receiver* strains and was sufficient to induce the *Pb* and *Pc* promoters in
2 these hosts.

3
4 In conclusion, these results accredit catabolic pathways for aromatic compounds as reliable sources of
5 synthetic wiring devices between different bacterial strains. We thus advocate their exploitation for
6 engineering multi-cellular logic circuits. Furthermore, metabolically-wired cells could be in principle
7 connected to additional strains, through linking the generation of the output C (**Fig. 1a**) to the
8 production of e.g. quorum sensing molecules, which could use these compounds as inputs. Also,
9 well-characterized regulatory elements (such as LacI, TetR, AraC, etc.) could be merged to such
10 metabolic devices from catabolic pathways for implementing complex circuits in the same cell. Note
11 that when placed in a different host many of such pathways are altogether alien to the endogenous
12 metabolism^{18, 20, 33} and, therefore, the logic gates composed of small molecule-enzyme-regulator trios
13 that could be built upon them would be orthogonal. This is because catabolic pathways for recalcitrant
14 and xenobiotic compounds used to reside in specific types of organisms^{18,22}, so that no crosstalk would
15 be expected with the endogenous metabolic networks of distant hosts. In combination with standard
16 tools available for circuit engineering in bacteria^{34,35}, we anticipate that a number of environmentally
17 relevant logic circuits could be easily assembled in different hosts of interest. On these bases we
18 encourage adoption of similar approaches for circuit engineering with other well characterised
19 catabolic pathways of environmental microorganisms (such as the *nah*, *tfd*, *bph*, *tod*, etc.^{18,20})

21 METHODS

22
23 **Bacterial strains and growth conditions.** *E. coli* strain CC118 was used as the host organism for
24 plasmid constructs³⁶. *E. coli* strain HB101 (pRK600) was utilized as a helper strain for tri-parental
25 mating, which was performed as described³⁶. *P. putida* KT2440³⁰ and *P. putida* mt-2²⁶ were used as
26 *sender* strains. Unless otherwise indicated, *E. coli* cells were grown in Luria-Bertani (LB) medium at
27 37°C, while *P. putida* strains were grown in M9 minimal medium³⁷ supplemented with 2 mM MgSO₄
28 and 10 mM of succinate as the sole carbon source. When required, kanamycin (Km, 50 µg/mL) or
29 chloramphenicol (Cm, 30 µg/mL) was added to the media. The aromatic compounds used as inducers
30 (toluene and benzoate) were all purchased from Sigma-Aldrich.

31

1 **Construction of the receiver strains.** We constructed reporter fusions in receiver strains by cloning
2 the target promoter in a broad-host range plasmid pSEVA226 (a RK2 derivative with a Km resistance
3 marker) that harbours the *luxCDABE* operon³⁸ downstream of a pUC18-like multiple cloning site³⁴.
4 Briefly, PCR reactions were performed using *P. putida* KT2440 DNA, *Pfu* DNA polymerase (Promega),
5 and primers for the *Pb* promoter as follows: PBF (5'-TGG ATG AAT TCG ACA GTA CCC TCC-3') and
6 PBR (5'-GCG CGG ATC CGG CCA GGG TCT CCC TTG-3'). For *Pc* promoter amplification, primers
7 PCF (5'-GAG AGA ATT CAG GCC CAG TTC CAG CTC G-3') and PCR (5'-GCG CGG ATC CTG TTG
8 CCA GGT CCC GTC AG-3') were used. These primers introduced *EcoRI* and *BamHI* sequences at
9 the 5' and 3' ends, respectively (restriction sites are underlined in the primer sequences). After
10 purification of the PCR products, fragments were digested with *EcoRI/BamHI* enzymes (New England
11 Biolabs) and ligated into a pSEVA226 vector that was previously digested with the same enzymes.
12 Ligations were used to transform chemically competent *E. coli* CC118 cells, and the resulting Km^R
13 clones were selected. After confirmation of the correct insertion of the promoters, the resulting
14 plasmids were named pSEVA226-*Pb* and pSEVA226-*Pc*. Cloned promoters were verified by DNA
15 sequencing. The reporter constructs were transferred to *P. putida* KT2440 by tri-parental mating³⁶,
16 generating strains *P. putida* KT *Pb::lux* (*P. putida* KT2440 with pSEVA226-*Pb*) and *P. putida* KT
17 *Pc::lux* (*P. putida* KT2440 with pSEVA226-*Pc*), which were used as *receivers* in the experiments
18 below.

19
20 **Promoter activity assays.** For testing promoter induction in solid media, overnight cultures of *sender*
21 and *receiver P. putida* strains were mixed in different combinations as shown in **Fig. 4a**, spotted on
22 M9/succinate agar plates and exposed to saturating vapours of 1 M toluene dissolved in DMSO.
23 Plates were sealed with parafilm and incubated for 4 hours. After induction, non-disruptive monitoring
24 of promoter output was carried out with a VersaDoc™ Imaging System (BioRad), and results were
25 processed with ImageJ software (<http://rsbweb.nih.gov/ij/>). To analyse promoter activity quantitatively,
26 single colonies of *P. putida* were used to inoculate 5 mL of M9 minimal medium supplemented with 10
27 mM of succinate. Cultures were grown for 16 h and diluted 1:20 into fresh minimal media containing
28 different effectors. To quantify promoter responses in different inducer concentrations, benzoate was
29 used at 0.5, 1.25, 2.5, 5, 12.5 and 25 µM. For single concentration experiments, benzoate was used
30 at a 1 mM final concentration. Diluted cells were placed in 96-well microplates (Optilux™, BD Falcon)
31 and analysed in a WallacVictor II 1420 Multilabel Counter (Perkin Elmer). Every 30 min, the optical

1 density at 600 nm (OD_{600}) and the bioluminescence were recorded. Strains harbouring an empty
2 vector (pSEVA226) were used as a control, and background production of the lux genes was
3 subtracted from the assayed promoters. To assay receiver induction in the wiring experiments,
4 overnight cultures were mixed as in **Fig. 4c** in 1X PBS buffer at 1:10 dilutions. Mixed cultures were
5 exposed to saturating vapours of toluene for 20 min. After this pre-exposure, cells were transferred to
6 96-well microplates (Optilux™, BD Falcon), which were loaded in a plate reader. Every hour, the
7 optical density at 600 nm (OD_{600}) and the bioluminescence were recorded. Promoter activities were
8 calculated in relative units (RU, bioluminescence/ OD_{600}) by normalizing bioluminescence to cell
9 density.

10

11 **AUTHOR INFORMATION**

12

13 **Corresponding Author:** E-mail: vdlorenzo@cnb.csic.es14 **Author Contributions:** R S-R performed experiments and drafted the manuscript. VdL
15 directed the project and wrote the paper.16 **Notes:** The authors declare no conflict of interest

17

18 **ACKNOWLEDGMENTS**

19

20 This study was supported by the BIO and FEDER CONSOLIDER-INGENIO program of the Spanish
21 Ministry of Science and Innovation, the MICROME, ST-FLOW and ARYSIS Contracts of the EU, and
22 the PROMT Project of the Autonomous Community of Madrid.

23

24 **REFERENCES**

25

- 26 1. Andrianantoandro, E., Basu, S., Karig, D. K., and Weiss, R. (2006) Synthetic biology: new
-
- 27 engineering rules for an emerging discipline,
- Mol Syst Biol*
- 2, 2006 0028.
-
- 28 2. Purnick, P. E., and Weiss, R. (2009) The second wave of synthetic biology: from modules to
-
- 29 systems,
- Nat Rev Mol Cell Biol*
- 10, 410-422.
-
- 30 3. Hermsen, R., Tans, S., and ten Wolde, P. R. (2006) Transcriptional regulation by competing
-
- 31 transcription factor modules,
- PLoS Comput Biol*
- 2, e164.

- 1 4. Silva-Rocha, R., and de Lorenzo, V. (2008) Mining logic gates in prokaryotic transcriptional
2 regulation networks, *FEBS Lett* 582, 1237-1244.
- 3 5. Zhan, J., Ding, B., Ma, R., Ma, X., Su, X., Zhao, Y., Liu, Z., Wu, J., and Liu, H. (2010) Develop
4 reusable and combinable designs for transcriptional logic gates, *Mol Syst Biol* 6, 388.
- 5 6. Hunziker, A., Tuboly, C., Horvath, P., Krishna, S., and Semsey, S. (2010) Genetic flexibility of
6 regulatory networks, *Proc Natl Acad Sci USA* 107, 12998-13003.
- 7 7. Tamsir, A., Tabor, J. J., and Voigt, C. A. (2011) Robust multicellular computing using genetically
8 encoded NOR gates and chemical 'wires', *Nature* 469, 212-215.
- 9 8. Siuti, P., Yazbek, J., and Lu, T. K. (2013) Synthetic circuits integrating logic and memory in living
10 cells, *Nat Biotechnol* 31, 448-52.
- 11 9. Ghosh, B., Karmakar, R., and Bose, I. (2005) Noise characteristics of feed forward loops, *Phys*
12 *Biol* 2, 36-45.
- 13 10. Pedraza, J. M., and van Oudenaarden, A. (2005) Noise propagation in gene networks, *Science*
14 307, 1965-1969.
- 15 11. Sprinzak, D., and Elowitz, M. B. (2005) Reconstruction of genetic circuits, *Nature* 438, 443-448.
- 16 12. Voigt, C. A. (2006) Genetic parts to program bacteria, *Curr Op Biotech* 17, 548-557.
- 17 13. Mutalik, V. K., Guimaraes, J. C., Cambray, G., Mai, Q. A., Christoffersen, M. J., Martin, L., Yu, A.,
18 Lam, C., Rodriguez, C., Bennett, G., Keasling, J. D., Endy, D., and Arkin, A. P. (2013)
19 Quantitative estimation of activity and quality for collections of functional genetic elements, *Nat*
20 *Methods* 10, 347-353.
- 21 14. Bonnet, J., Yin, P., Ortiz, M. E., Subsoontorn, P., and Endy, D. (2013) Amplifying genetic logic
22 gates, *Science* 340, 599-603.
- 23 15. Regot, S., Macia, J., Conde, N., Furukawa, K., Kjellen, J., Peeters, T., Hohmann, S., de Nadal, E.,
24 Posas, F., and Sole, R. (2011) Distributed biological computation with multicellular engineered
25 networks, *Nature* 469, 207-211.
- 26 16. Waters, C. M., and Bassler, B. L. (2005) Quorum sensing: cell-to-cell communication in bacteria,
27 *Annu Rev Cell Dev Biol* 21, 319-346.
- 28 17. van der Meer, J. R., and Sentchilo, V. (2003) Genomic islands and the evolution of catabolic
29 pathways in bacteria, *Curr Op Biotech* 14, 248-254.
- 30 18. Tropel, D., and van der Meer, J. R. (2004) Bacterial transcriptional regulators for degradation
31 pathways of aromatic compounds, *Microbiol Mol Biol Rev* 68, 474-500.

- 1 19. Galvao, T. C., Mohn, W. W., and de Lorenzo, V. (2005) Exploring the microbial biodegradation
2 and biotransformation gene pool, *Trends Biotechnol* 23, 497-506.
- 3 20. Diaz, E., Jimenez, J. I., and Nogales, J. (2012) Aerobic degradation of aromatic compounds, *Curr*
4 *Op Biotech* 24, 431-42
- 5 21. Diaz, E., and Prieto, M. A. (2000) Bacterial promoters triggering biodegradation of aromatic
6 pollutants, *Curr Op Biotech* 11, 467-475.
- 7 22. Carbajosa, G., Trigo, A., Valencia, A., and Cases, I. (2009) Bionemo: molecular information on
8 biodegradation metabolism, *Nucleic Acids Res* 37, D598-602.
- 9 23. Katsuyama, C., Nakaoka, S., Takeuchi, Y., Tago, K., Hayatsu, M., and Kato, K. (2009)
10 Complementary cooperation between two syntrophic bacteria in pesticide degradation, *J Theor*
11 *Biol* 256, 644-654.
- 12 24. Ramos, J. L., Marques, S., and Timmis, K. N. (1997) Transcriptional control of the *Pseudomonas*
13 TOL plasmid catabolic operons is achieved through an interplay of host factors and plasmid-
14 encoded regulators, *Ann Rev Microbiol* 51, 341-373.
- 15 25. Jimenez, J. I., Minambres, B., Garcia, J. L., and Diaz, E. (2002) Genomic analysis of the aromatic
16 catabolic pathways from *Pseudomonas putida* KT2440, *Env Microbiol* 4, 824-841.
- 17 26. Williams, P. A., and Murray, K. (1974) Metabolism of benzoate and the methylbenzoates by
18 *Pseudomonas putida (arvilla)* mt-2: evidence for the existence of a TOL plasmid, *J Bacteriol* 120,
19 416-423.
- 20 27. Spooner, R. A., Lindsay, K., and Franklin, F. C. (1986) Genetic, functional and sequence analysis
21 of the *xyIR* and *xyIS* regulatory genes of the TOL plasmid pWW0, *J Gen Microbiol* 132, 1347-
22 1358.
- 23 28. Inouye, S., Nakazawa, A., and Nakazawa, T. (1987) Expression of the regulatory gene *xyIS* on
24 the TOL plasmid is positively controlled by the *xyIR* gene product, *Proc Natl Acad Sci USA* 84,
25 5182-5186.
- 26 29. Harwood, C. S., and Parales, R. E. (1996) The beta-ketoadipate pathway and the biology of self-
27 identity, *Ann Rev Microbiol* 50, 553-590.
- 28 30. Bagdasarian, M., Lurz, R., Ruckert, B., Franklin, F. C., Bagdasarian, M. M., Frey, J., and Timmis,
29 K. N. (1981) Specific-purpose plasmid cloning vectors. II. Broad host range, high copy number,
30 RSF1010-derived vectors, and a host-vector system for gene cloning in *Pseudomonas*, *Gene* 16,
31 237-247.

- 1 31. Silva-Rocha, R., and de Lorenzo, V. (2013) The TOL network of *Pseudomonas putida* mt-2
2 processes multiple environmental inputs into a narrow response space, *Env Microbiol* 15, 271-
3 286.
- 4 32. Cowles, C. E., Nichols, N. N., and Harwood, C. S. (2000) BenR, a XylS homologue, regulates
5 three different pathways of aromatic acid degradation in *Pseudomonas putida*, *J Bacteriol* 182,
6 6339-6346.
- 7 33. Diaz, E. (2004) Bacterial degradation of aromatic pollutants: a paradigm of metabolic versatility,
8 *Int Microbiol* 7, 173-180.
- 9 34. Silva-Rocha, R., Martinez-Garcia, E., Calles, B., Chavarria, M., Arce-Rodriguez, A., de Las Heras,
10 A., Paez-Espino, A. D., Durante-Rodriguez, G., Kim, J., Nickel, P. I., Platero, R., and de Lorenzo,
11 V. (2013) The Standard European Vector Architecture (SEVA): a coherent platform for the
12 analysis and deployment of complex prokaryotic phenotypes, *Nucleic Acids Res* 41, D666-675.
- 13 35. Shetty, R. P., Endy, D., and Knight, T. F., Jr. (2008) Engineering BioBrick vectors from BioBrick
14 parts, *J Biol Eng* 2, 5.
- 15 36. de Lorenzo, V., and Timmis, K. N. (1994) Analysis and construction of stable phenotypes in
16 gram-negative bacteria with Tn5- and Tn10-derived minitransposons, *Meth Enzymol* 235, 386-
17 405.
- 18 37. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular cloning: A laboratory manual*, Cold
19 Spring Harbor, New York.
- 20 38. Choi, K. H., Gaynor, J. B., White, K. G., Lopez, C., Bosio, C. M., Karkhoff-Schweizer, R. R., and
21 Schweizer, H. P. (2005) A Tn7-based broad-range bacterial cloning and expression system, *Nat*
22 *Methods* 2, 443-448.

23

24

25

26

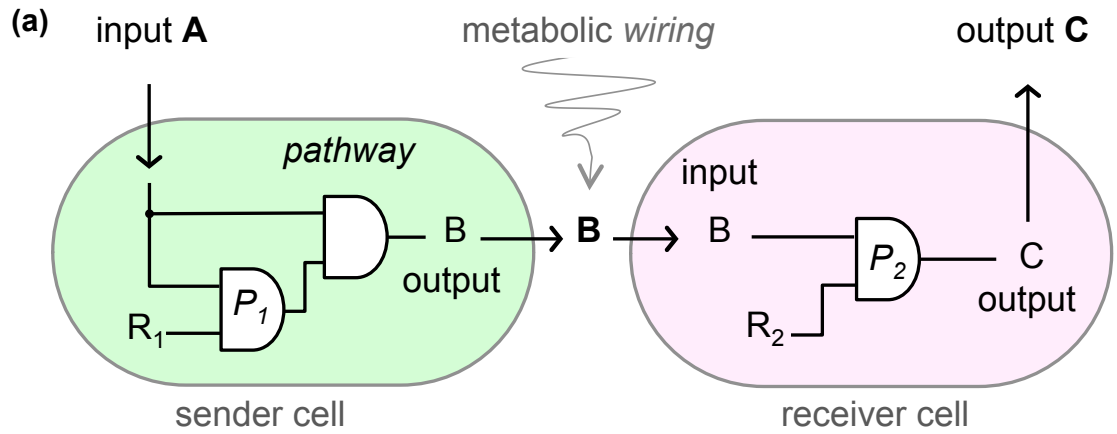
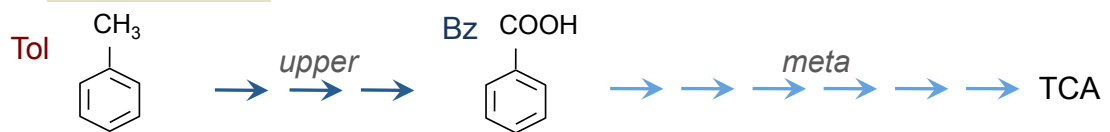
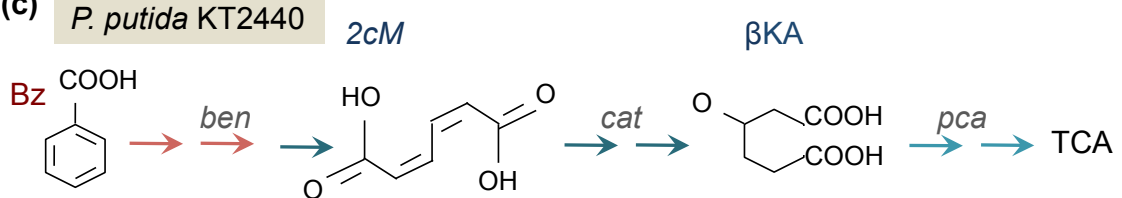
27

1 FIGURES

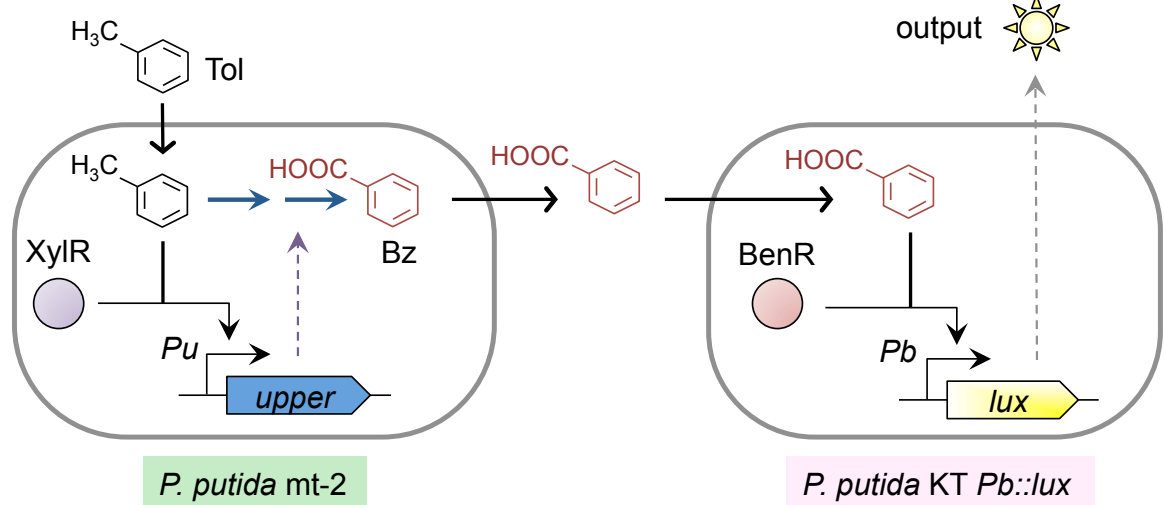
2

3 Figure 1. Overall strategy of metabolic wiring.

4

(b) *P. putida* mt-2(c) *P. putida* KT2440

(d)



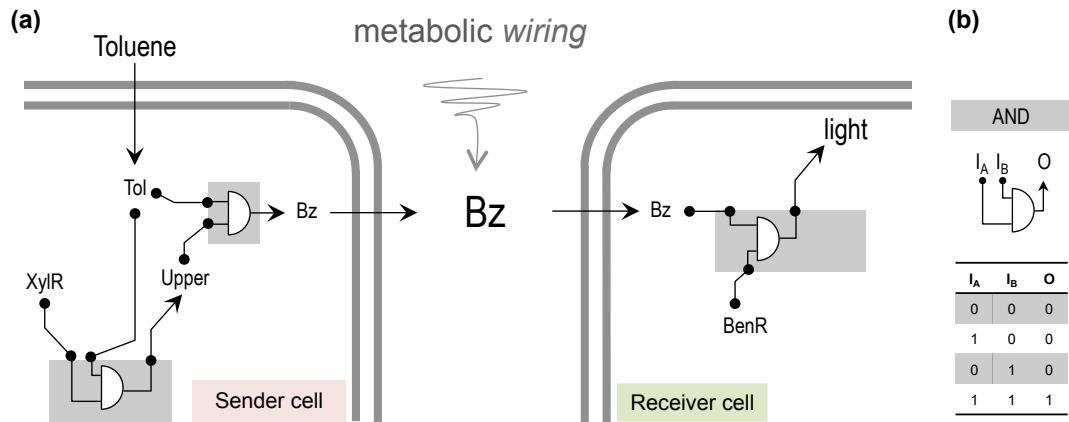
5

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25

(a) In this system, an entry input A controls a set of logic interactions in the *sender* strain. P_1 and P_2 represent promoters regulated by transcriptional factors R_1 and R_2 . These regulators are the sensors of inputs A and B. As a result of the computation performed by this circuit, input A is converted to B, which diffuses to the extracellular media. Once this compound accumulates in the outside, it is sensed by the *receiver* strain, where it serves as an input signal for next logic circuit. In this way, the two logic circuits are wired through the intermediate metabolite. (b) In *P. putida* mt-2, toluene is degraded in two steps via the *upper* and *meta* pathways of the TOL network²⁴. In the first step, toluene (Tol) is converted to benzoate (*Bz*) by the *upper* enzymes. The enzymes are expressed from the *Pu* promoter when activated by XylR in response to toluene. Next, *Bz* is metabolised to generate TCA cycle intermediates through the action of the *meta* enzymes. In this case, the *meta* operon is expressed from the *Pm* promoter when activated by XylS bound to the inducer *Bz*. (c) In the *ortho* pathway of *P. putida* KT2440, three sets of enzymes (*ben*, *cat* and *pca*) are necessary to completely break down *Bz*. The compound *cis,cis*-muconate (labelled as *2cM*) generated during this process acts with CatR to stimulate the activation of the *cat* operon, while β -keto adipate (β KA) is sensed by the PcaR regulator to trigger production of the *pca* enzymes. (d) In the implemented system, *P. putida* mt-2 works as the *sender* strain and *P. putida* KT *Pb::lux* as the receiver. When toluene is present in the media, it is sensed by *P. putida* mt-2, which activates the complex TOL network³¹. One of the outcomes of this circuit is production of *upper* enzymes that convert toluene to *Bz*. As the *upper* enzymes start to produce high amounts of *Bz* from toluene, the compound diffuses to the extracellular medium and is then sensed by the receiver strain, where it triggers the activation of the BenR regulator. Active BenR binds the *Pb* promoter and stimulates the production of the *lux* operon, leading to bioluminescent emission.

1 **Figure 2.**

2



3

4

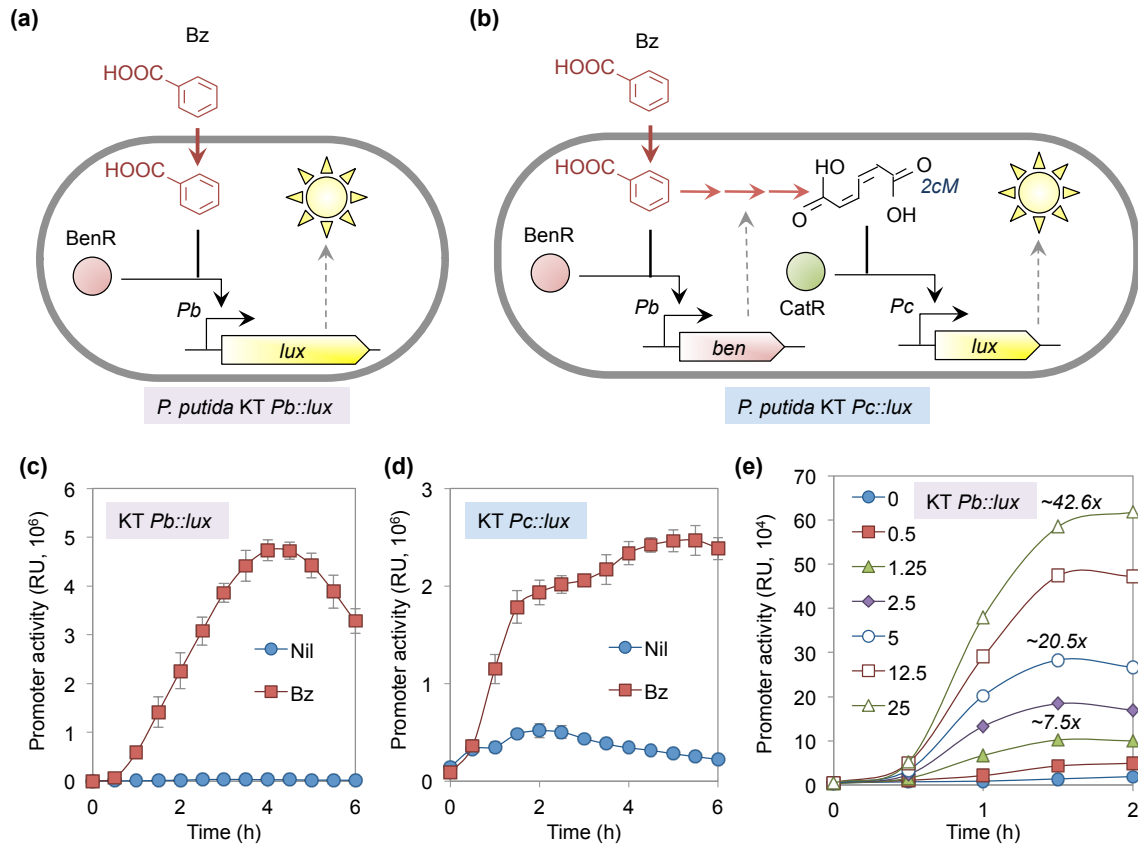
5 Logic circuits of *P. putida* sender and receiver strains. (a) To the left, the logic circuit that converts
 6 toluene (input) into Bz (output) is composed of the regulatory *xylR* gene and the *upper* metabolic
 7 operon. To the right, the logic interactions involving the receiver strain are shown. In this case, the
 8 transcriptional factor BenR and one external signal (Bz) are used as inputs for the control of light
 9 emission. (b) The AND logic gate used to construct the circuits along with its truth table.

10

11

1 **Figure 3.** Individual characterisation of the transcriptional response of receiver strains.

2



3

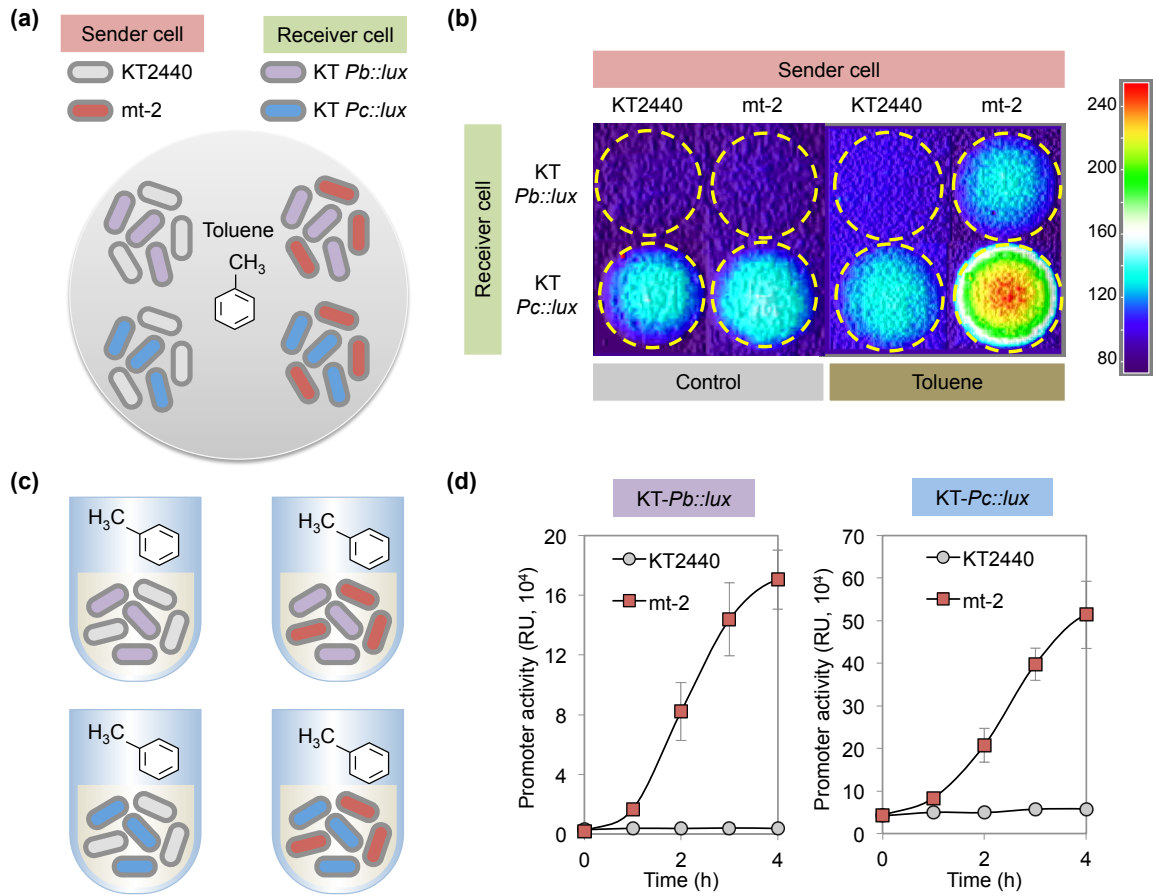
4

5 **(a)** *P. putida* KT *Pb::lux* with the regulatory system controlling *lux* expression. **(b)** In *P. putida* KT
 6 *Pc::lux*, Bz induces expression of the *ben* enzymes that convert this compound to *cis,cis*-muconate
 7 (2cM). This compound is then sensed by CatR to activate the *Pc::lux* reporter fusion. **(c)**
 8 Transcriptional response of *Pb::lux* fusion induced with 1 mM Bz. Briefly, overnight cultures were
 9 diluted 1:20 in fresh minimal media supplemented with or without (control; Nil) the inducer. Samples
 10 were loaded into a plate reader, and at 30-min intervals, the bioluminescence and the OD₆₀₀ were
 11 measured. RU are relative units calculated as bioluminescence/OD₆₀₀ at each time point. Vertical bars
 12 are the standard deviation (SD) calculated from at least four technical replicates. **(d)** Transcriptional
 13 response of *Pc::lux* fusion induced with 1 mM Bz. Experiments were performed as in **(c)**. Induction
 14 kinetics of *Pb::lux* fusion in response to micromolar concentrations of Bz. For these experiments,
 15 overnight cultures were diluted 1:20 in fresh minimal media supplemented without (control; Nil) or with
 16 different concentrations of Bz (0.5, 1.25, 2.5, 5, 12.5 and 25 μM). Promoter activities were assayed as
 17 in **(c)**. In each experiment, the calculated SD was less than 15% (not shown). In the graph, the
 18 changes in expression at concentrations of 1.25 (7.5x), 5 (20.5x) and 25 μM (42.6x) are shown.

19

1 **Figure 4.** Implementation of multi-cellular circuits through metabolic wiring.

2



3

4

5 **(a)** Solid media experiments. *P. putida* KT2440 or *P. putida* mt-2 were used as *sender* strains, while *P.*
6 *putida* KT *Pb::lux* or *P. putida* KT *Pc::lux* were the receivers. Equal amounts of overnight cultures were
7 mixed in agar plates in the indicated *sender/receiver* combinations. Cells were exposed to toluene
8 vapours phase and incubated for 4 hours. After induction, light emission from each mixture was
9 analysed using a CCD camera as described in Material and Methods. **(b)** Bioluminescence of mixed
10 cells after 4 hours of induction. Cells were incubated in the absence (control) or presence of toluene.
11 **(c)** Liquid media experiments. Overnight cultures were diluted 1:20 in 1X PBS buffer in the
12 combinations indicated. Cells were exposed to saturating amounts of toluene for 20 min, after which
13 samples were loaded into a plate reader and assayed for bioluminescence emission. **(d)** Promoter
14 activity of mixed cultures in liquid media. RU refers to relative units calculated as
15 bioluminescence/OD₆₀₀ at each time point. Vertical bars are the standard deviation (SD) calculated
16 from at least four technical replicates.

17

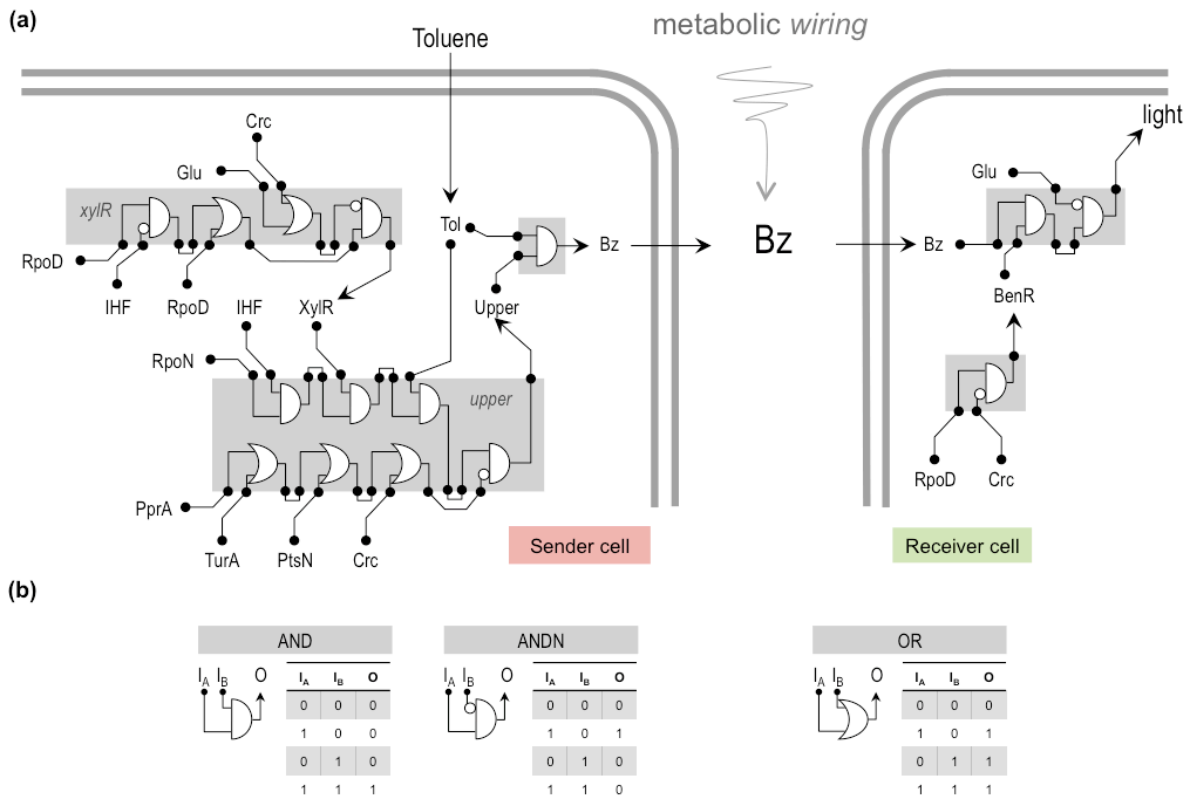
18

Engineering multi-cellular logic in bacteria with metabolic wires

Rafael Silva-Rocha[§] and Victor de Lorenzo*

Supplementary Information

Figure S1. Complete logic circuits of *P. putida* sender and receiver strains.



(a) To the right, the logic circuit of the *xylR* gene and the *upper* TOL operon shows the main internal and external inputs sensed by the system as reported previously²⁸. This system is composed of several proteins (RpoD, RpoN, Crc, PtsN, TurA, PprA, IHF and XylR) and external stimuli (e.g. Glu: glucose). The final outcome of this system is the conversion of toluene into Bz. To the left, the logic interactions involving the receiver strain. In this case, a few proteins (RpoD, Crc and BenR) and external stimuli (Bz and Glu) are used as inputs for the control of light emission. (b) The logic gates used to construct the circuits in are shown along with their respective truth tables.