Supplementary information for

Nanotube-mediated cross-feeding couples the metabolism of interacting bacterial cells

Shraddha Shitut[1,2], Tobias Ahsendorf[3,4], Samay Pande[1†], Matthew Egbert[5], Christian Kost[1,2*]

[1] Experimental Ecology and Evolution Research Group, Department of Bioorganic Chemistry, Max Planck Institute for Chemical Ecology, Jena, 07745, Germany

[2] Department of Ecology, School of Biology/Chemistry, University of Osnabrück, Osnabrück, 49076, Germany

[3] Deutsches Krebsforschungszentrum, Heidelberg, Baden-Württemberg, D-69120, Germany

[4] Department of Systems Biology, Harvard Medical School, Boston, MA 02115, USA

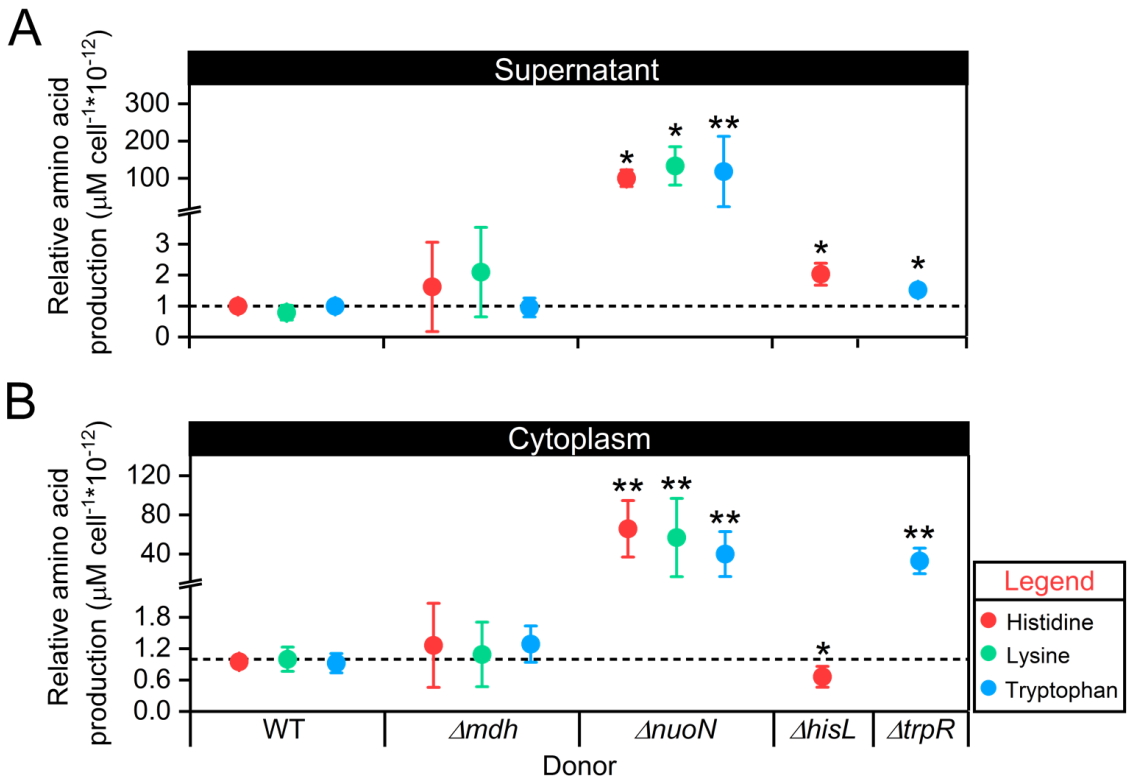
[5] Department of Computer Science, University of Auckland, Auckland, 1010, New Zealand

* Correspondence to: christiankost@gmail.com

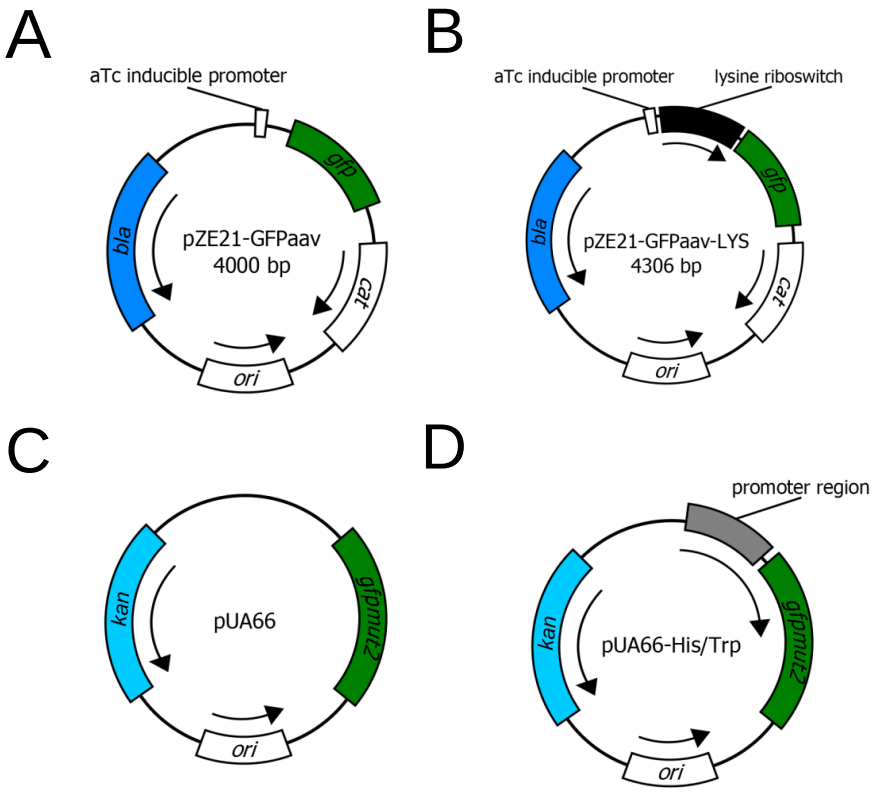
†Current address- Department of evolutionary biology, Swiss Federal Institute of Technology (ETH), Zurich, Switzerland

Table of contents **Page**

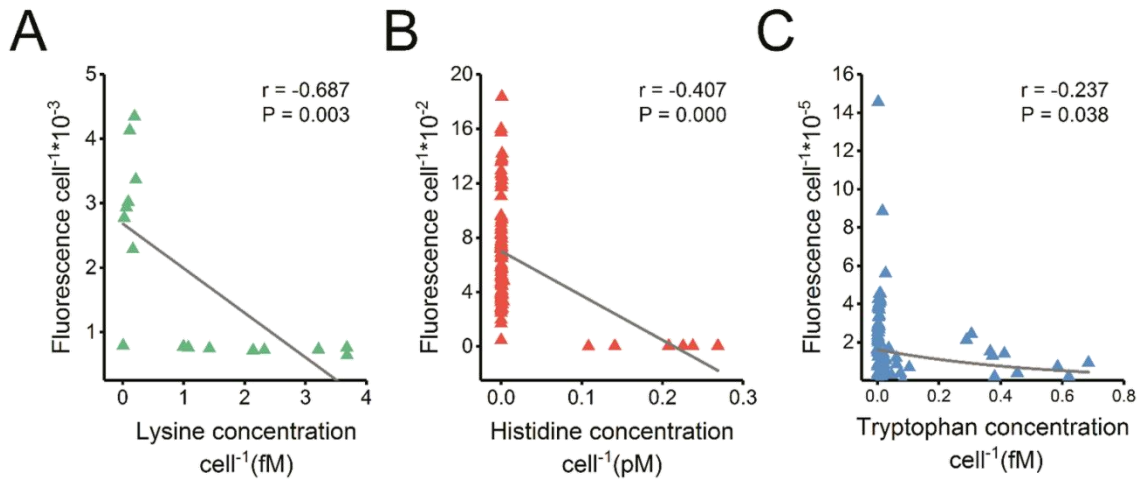
Supplementary figures



Supplementary Figure 1. Amino acid production levels of different donor genotypes as determined by LC/MS/MS analysis. Values represent mean (± 95% CI) concentrations of histidine (red), lysine (green), and tryptophan (blue) per cell (i.e. number of CFUs) after 24 h of growth. Amino acid concentrations in (**A**) the cell-free culture supernatant or (**B**) the cytoplasm of different donor genotypes (i.e. WT and four overproducers) are displayed. Asterisks indicate significant differences to WT levels (dashed line, independent-sample t-test: * $P < 0.05$, ** $P < 0.01$, n=8).



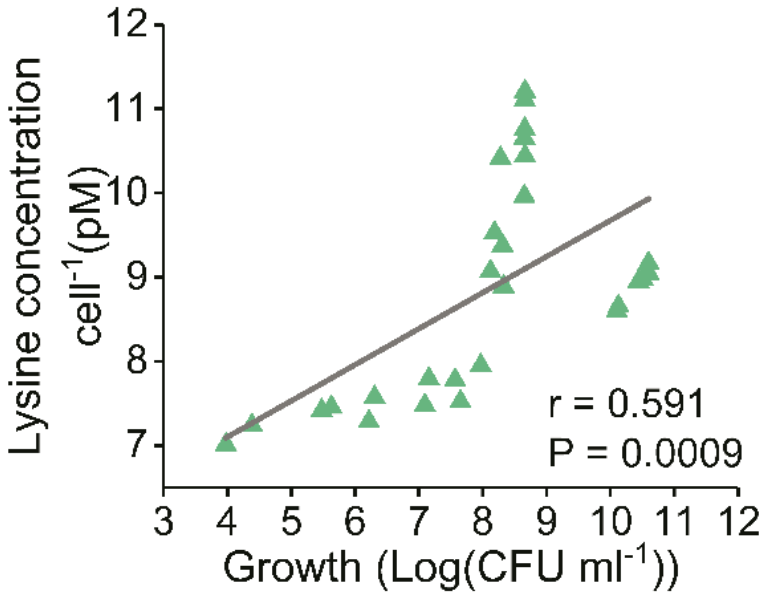
Supplementary figure 2: Plasmids used in this study. (**A**) The pZE1-GFPaav plasmid was used as the backbone for construction of the lysine riboswitch plasmid as well as to control for basal fluorescence emission levels. (**B**) The lysine riboswitch plasmid (pZE21-GFPaav-LYS) was modified from the original pZE21-GFPaav to include the lysine riboswitch gene as well as an ampicillin resistance gene (*bla*) in place of the original kanamycin resistance gene (*kan*). (**C**) The promoter-less plasmid (pUA66) that gives rise to basal fluorescence levels served as a control for the promoter-GFP-fusion plasmid. (**D**) The promoter-GFP-fusion plasmid (pUA66-His or pUA66-Trp) containing a specific promoter region (*hisL* or *trpL*) upstream the fluorescent reporter gene (*gfpmut2)* was used to quantify changes in the transcriptional activity of histidine or tryptophan biosynthesis genes.



55

56 **Supplementary Figure 3. Characterization of reporter constructs**. The
57 responsiveness of cell-internal reporter constructs to cytoplasmic concentrations of
58 lysine, histidine, or tryptophan was verified by analyzing the statistical relationship
59 between the concentration of intracellular amino acids and the cells' GFP emission
60 levels. Fluorescence levels are given in arbitrary units. The concentration of cytoplasmic
61 amino acids was determined via LC/MS/MS. Both measures were normalized per
62 number of cells (i.e. colony-forming units). (**A**) Negative correlation between cytoplasmic
63 lysine (green) levels and fluorescence emitted from Δ*lysR* cells harboring the lysine
64 riboswitch plasmid (pZE21-GFPaav-Lys). (**B, C**) Negative correlation between
65 cytoplasmic (**B**) histidine (red) and (**C**) tryptophan (blue) levels and fluorescence emitted
66 from donor cells (i.e. WT, Δ*mdh*, Δ*hisL*/ Δ*trpR*) harboring the promoter-GFP-fusion
67 plasmid (pUA66-His/ Trp). Grey lines are fitted linear regressions and the r- and P-
68 values of the corresponding Pearson's correlation coefficient are shown.

69



Supplementary Figure 4. Growth of lysine auxotrophs strongly depends on the amount of lysine they obtain from cocultured donor cells. Shown is the statistical relationship between cytoplasmic lysine levels (in µM) of Δ*lysR* cells harboring the lysine riboswitch plasmid (pZE21-GFPaav-Lys) in coculture with different donor cells (i.e. WT, Δ*mdh*, and Δ*nuoN*) and the growth of Δ*lysR* cells. Growth of the recipient is displayed as a logarithm of the difference in number of CFUs reached at 0 h and 24 h. The grey line is a fitted linear regression and the r- and P-value of the corresponding Pearson's correlation coefficient are shown.

Supplementary tables.

Table 1. Strains and plasmids used in this study. AA = amino acid.

Strain/Plasmid	Genotype	Phenotype	Reference
Escherichia coli BW25113	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), λ^-, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*	WT	(Baba *et al* 2006)
Δmdh	WT, Δ*mdh*::kan^R	AA overproducer	(Pande *et al* 2014)
ΔnuoN	WT, Δ*nuoN*::kan^R	AA overproducer	(Pande *et al* 2014)
ΔhisL	WT, Δ*hisL*::kan^R	AA overproducer	(Pande *et al* 2015)
ΔtrpR	WT, Δ*trpR*::kan^R	AA overproducer	(Pande *et al* 2015)
ΔhisD	WT, Δ*hisD*::kan^R	AA auxotroph	(Pande *et al* 2014)
ΔlysR	WT, Δ*lysR*::kan^R	AA auxotroph	(Pande *et al* 2014)
ΔtrpB	WT, Δ*trpB*::kan^R	AA auxotroph	(Pande *et al* 2014)
Lysine riboswitch plasmid (pZE21-GFPaav-Lys)	ColE1*ori*, *bla*, *cat*, lysine riboswitch, *gfpmut3*	Ampicillin resistance, chloramphenicol resistance	This study
Promoter-GFP fusion plasmid (pUA66-His)	SC101*ori*, *kan*, *hisL*, *gfpmut2*	Kanamycin resistance	(Zaslaver *et al* 2006)
Promoter-GFP fusion plasmid (pUA66-Trp)	SC101*ori*, *kan*, *trpL*, *gfpmut2*	Kanamycin resistance	(Zaslaver *et al* 2006)
Promoter-less plasmid (pUA66)	SC101*ori*, *kan, gfpmut2*	Kanamycin resistance	(Zaslaver *et al* 2006)

Table 2: Primers used in this study.

Target gene	Amplicon size (bp)	Direction	Sequence (5'-3')	Melting temperature (°C)
Lysine riboswitch	306	Forward	TTTTGGTACCGTACTACCTGCGCTAGCG	73.7
		Reverse	TTTTGGTACCAACTACCTCGTGTCAGGGG	74.6
Beta lactamase (*bla*)	1,000	Forward	TTTTCTCGAGCTTTTCGGGGAAATGTGCGCGGAACCCCTATTTG	87
		Reverse	TTTTACTGTTGATCTTTTCTACGGGGTCTGACGCTC	76.4
Chloramphenicol acetyltransferase (*cat*)	1,000	Forward	TTTTAAGCTTAAAGAGGAGAAATACTAGATGGAGAAAAAAATCACTGGATATAC	74.2
		Reverse	TTTTCCCGGGTTACGCCCCGCCCTGCCACTCATC	88.3

Supplementary methods

Plasmid construction

The plasmid containing the lysine riboswitch (pZE21-GFPaav-Lys) was constructed using the pZE21 plasmid (supplementary figure 2) (Lutz and Bujard 1997). This plasmid contains a gene encoding a variant of the fluorescent reporter (*gfp*), which emits fluorescence within 5 minutes of transcription, has a low toxicity, and negligible degradation(Cormack *et al* 1996). The plasmid also contains two genes encoding ampicillin- (*bla*) and chloramphenicol resistance (*cat*). The lysine riboswitch was amplified from the upstream region of *lysC* in *E*. *coli* MG1655 using the primers mentioned in table S2 and inserted into pZE21 at the *KpnI* restriction site (New England Biolabs GmbH (NEB), Frankfurt am Main, Germany) downstream of an anhydrotetracycline-inducible promoter. The kanamycin resistance gene was replaced with an ampicillin resistance gene, which has been amplified from the plasmid pSB1A2 using the primers mentioned in supplementary table 2. The restriction enzymes used for removing the kanamycin cassette were *XhoI* and *SpeI* (NEB, Frankfurt am Main, Germany). The final plasmid additionally contained a chloramphenicol resistance gene (*cat*), which was amplified from pSB1C3 (iGEM registry) using primers mentioned in supplementary table 2 and restriction enzymes *HindIII* and *XmaI* (NEB, Frankfurt am Main, Germany). The promoter activity of *hisL* and *trpR* was measured with the help of plasmids pUA66-His and pUA66-Trp, respectively (Zaslaver et al 2006) (supplementary figure 2). These promoter-GFP-fusion plasmids contain the promoter region of the corresponding gene cloned upstream of the *gfpmut2* gene, which codes for a variant of GFP that emits fluorescence within 5 minutes of transcription initiation, is highly stable, and non-toxic to *E. coli* (Cormack et al 1996). The same plasmid without a promoter region (plasmid pUA66) was used as a control to measure the basal *gfpmut2* expression level (supplementary figure 2). All plasmids were transformed into *E. coli* cells using the calcium chloride method followed by a heat shock treatment (Wood 1983).

Amino acid analysis

Amino acid levels in donor strains (WT, Δ*mdh*, Δ*nuoN*, Δ*hisL,* and Δ*trpR*) as well as the cytoplasmic lysine levels of Δ*lysR* carrying the lysine riboswitch plasmid (pZE21-GFPaav-Lys) were determined using the following protocols. The extracellular fraction (containing amino acids secreted into the supernatant) and intracellular fraction (containing cytoplasmic amino acids) of cultures grown for 24 h was collected and subsequently subjected to a liquid chromatography-mass spectrometry (LC/MS/MS) analysis.

Extraction of extracellular amino acids. The amounts of amino acids that were present in the extracellular environment (i.e. culture supernatant) were quantified from cultures that have been grown for 24 h in 1 ml MMAB in deep-well plates (Eppendorf, Germany). The next day, cultures were centrifuged (Sigma 3-18K, Germany) at 3,800 rpm for 15

minutes. After centrifugation, 400 µl of the supernatant were filter-sterilized (0.2 µm) and analyzed by LC/MS/MS.

Extraction of cytoplasmic amino acids. To quantify intracellular concentrations of amino acids, cells were extracted following a previously published protocol (Borner et al 2007). In a nutshell, monocultures of donor or recipient cells were grown in 1 ml MMAB in deep-well plates (Eppendorf, Germany) for 24 h and subsequently centrifuged (Sigma 3-18K, Germany) at 3,800 rpm for 15 minutes followed by washing with 0.8% sodium chloride solution. Cell pellets were resuspended in an ethanol-ribitol solution (400 µl ethanol + 16 µl of 0.2 mg ml^{-1} ribitol) and sonicated (Sonorex RK102H, Germany) at 70 °C for 15 minutes to lyse cells. Next, the polar phase (containing cell debris and proteins) was extracted by adding 400 µl water and 250 µl of chloroform followed by mixing and centrifugation at 3,800 rpm for 15 minutes. Afterwards, 400 µl of the hydrophilic phase (containing water-soluble amino acids) was transferred to a fresh deep-well plate and dried in a glass desiccator under vacuum for 18 h. The dried extract was resuspended in 400 µl MMAB and subjected to further analysis.

Amino acid quantification by LC/MS/MS. The analysis of amino acids in the cells' cytoplasm and culture supernatant was focused on the three amino acids histidine, lysine, and tryptophan. For the tryptophan analysis, samples were diluted 1:1 in borate buffer (pH 8), while for histidine and lysine quantification samples were diluted 1:1 in borate buffer containing a ^{13}C, ^{15}N-labelled amino acid mix (Isotec, Miamisburg, USA). Labeled amino acids were added as an internal standard at a concentration of 10 µg of the mix ml^{-1}. All samples were directly analyzed via LC/MS/MS using a modification of a method described previously[16]. Chromatography was performed on an Agilent 1200 HPLC system (Agilent Technologies, Böblingen, Germany). Separation was achieved on a Zorbax Eclipse XDB-C18 column (50 x 4.6 mm, 1.8 µm, Agilent Technologies, Germany). Formic acid (0.05%) in water and acetonitrile were employed as mobile phases A and B, respectively. The elution profile was: 0-1 min, 3% B in A; 1-2.7 min, 3-100% B in A; 2.7-3 min 100% B, and 3.1-6 min 3% B in A. The mobile phase flow rate was 1.1 ml min^{-1}. Column temperature was maintained at 25 °C. The liquid chromatography was coupled to an API 3200 tandem mass spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with a turbospray ion source operated in positive ionization mode. The ion spray voltage was maintained at 5.5 keV. The turbo gas temperature was set at 700 °C. Nebulizing gas was set at 70 psi, curtain gas at 35 psi, heating gas at 70 psi and collision gas at 2 psi. Multiple reaction monitoring (MRM) was used to monitor analyte parent ion → product ion. Both Q1 and Q3 quadrupoles were maintained at unit resolution. Analyst 1.5 software (Applied Biosystems, Darmstadt, Germany) was used for data acquisition and processing.

Characterization of reporter constructs

Two reporter constructs were used. The lysine riboswitch plasmid (pZE21-GFPaav-Lys), which indicated changes in cytoplasmic lysine levels, and two promoter-GFP-fusion plasmids (pUA66-His and pUA66-Trp), which quantified changes in the transcriptional activity of the two genes *hisL* and *trpL*. To characterize the lysine riboswitch plasmid, the construct was introduced into the auxotrophic recipient *ΔlysR*. The resulting strain was then cultured for 24 h in MMAB, which has been supplemented

with different concentrations of lysine (i.e. 0 µM, 50 µM, 100 µM, and 200 µM) and which did or did not contain aTc for induction of the riboswitch gene. An aliquot of the resulting culture was used for measuring fluorescence intensity (see below) and the rest was subjected to chemical analysis of cytoplasmic amino acid concentrations as well as cell number determination. The fluorescence intensity obtained for cultures grown without aTc induction was used as control to determine basal fluorescence emission levels of these cultures. In case of the promoter-GFP-fusion plasmid, the plasmid (pUA66-His and pUA66-Trp) and the control plasmid (pUA66) was individually introduced into donors (WT, Δ*mdh*, Δ*hisL*/ Δ*trpR*), which were cultured for 24 h in MMAB containing increasing concentrations of the amino acids histidine or tryptophan (0 µM, 50 µM, 100 µM, and 200 µM). An aliquot of the culture was used for measuring the intensity of GFP fluorescence and the rest was subjected to chemical analysis of cytoplasmic amino acid concentrations and cell number determination.

Promoter activity measurements

To determine biosynthesis levels of histidine and tryptophan *in vivo*, the promoter activity of the corresponding biosynthetic genes (i.e. *hisL* and *trpL*) was quantified using the promoter-GFP-fusion plasmids (pUA66-His/Trp). To this end, the plasmids were first introduced into the donor genotypes (i.e. WT, Δ*mdh*, and Δ*hisL*/Δ*trpR*). Plasmid-containing donors were then either paired with recipients (i.e. Δ*hisD* or Δ*trpB*) or cultured alone for 24 h. At five selected time points (i.e. 9 h, 12 h, 15 h, 18h, and 24 h), a sample was taken to measure GFP fluorescence intensity and the number of CFUs. Fluorescence values were normalized by dividing with the CFU number of plasmid-containing cells. Normalized fluorescence values of controls (i.e. cells carrying the promoter-less plasmid pUA66) were averaged and subtracted from the values of cells carrying the promoter-GFP-fusion plasmid (pUA66-His/Trp). Transcriptional activity at a given time point was calculated by computing the time derivative of the above control-subtracted fluorescence values (i.e. [d((pUA66-His/Trp) – pUA66)/dT]) (Zaslaver et al 2006).

Supplementary references

Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M *et al* (2006). Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Sys Biol* **2:** 2006.0008-2006.0008.

Borner J, Buchinger S, Schomburg D (2007). A high-throughput method for microbial metabolome analysis using gas chromatography/mass spectrometry. *Anal Biochem* **367:** 143-151.

Cormack BP, Valdivia RH, Falkow S (1996). FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* **173:** 33-38.

Lutz R, Bujard H (1997). Independent and tight regulation of transcriptional units in Escherichia coli via the LacR/O, the TetR/O and AraC/I1-I2 regulatory elements. *Nucleic Acids Res* **25:** 1203-1210.

Pande S, Merker H, Bohl K, Reichelt M, Schuster S, de Figueiredo LF *et al* (2014). Fitness and stability of obligate cross-feeding interactions that emerge upon gene loss in bacteria. *ISME J* **8:** 953-962.

Pande S, Shitut S, Freund L, Westermann M, Bertels F, Colesie C *et al* (2015). Metabolic cross-feeding via intercellular nanotubes among bacteria. *Nat Commun* **6**.

Sambrook J, Fritsch E, Maniatis T. (1989). Molecular cloning: a Laboratory Manual. Cold Spring Harbor Press: New York, pp 545.

Zaslaver A, Bren A, Ronen M, Itzkovitz S, Kikoin I, Shavit S *et al* (2006). A comprehensive library of fluorescent transcriptional reporters for *Escherichia coli*. *Nat Meth* **3:** 623-628.