Metabolic coupling in bacteria

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ABSTRACT

Symbiotic associations have radically shaped the diversity and complexity of life on earth. Many known symbioses represent physiological fusions of previously independent organisms, in which metabolites are traded between interacting partners in intricate ways. The first steps leading to this tight entanglement, however, remain unknown. Here we demonstrate that unidirectional cross-feeding of essential amino acids between two bacterial cells can already couple their metabolisms in a source-sink-like relationship. Auxotrophic recipients used intercellular nanotubes to derive amino acids from other bacterial cells. Removal of cytoplasmic amino acids in this way increased the amino acid production of donor cells by delaying feedback inhibition of the corresponding amino acid biosynthetic pathway. Strikingly, even though donor cells produced all the focal amino acids recipients required to grow, this additional metabolic burden did not incur detectable fitness costs. Our results demonstrate that one loss-of-function mutation is sufficient to couple the metabolic networks of two organisms, thus resulting in a functional fusion of two previously independent individuals.

KEY WORDS: cross-feeding, nanotubes, source-sink mechanism, inter-cellular coordination

INTRODUCTION

Life on Earth has produced a bewildering diversity of forms and physiologies. Understanding the evolution of this complexity in organismal design is a fundamental problem in biology. Major leaps in biological complexity have resulted from evolutionary

transitions, during which previously independent organisms were functionally integrated to form a new, higher-level entity[1-3]. Eminent examples of such symbiotic associations involve transformative events such as the origin of the eukaryotic cell[1,4,5] or the emergence of plastids from a cyanobacterial progenitor[6].

Selective advantages resulting from a cooperative division of labour among the constituent lower-level units likely fuelled the emergence of these associations[7,8]. By interacting with individuals that feature novel traits, microorganisms could significantly extend their metabolic repertoire[9-11]. In this way, ecological strategies and evolutionary trajectories became available to the newly emerged consortium that otherwise would be inaccessible to individual organisms.

To function as a cohesive whole, the interacting partners need to coordinate their cellular activities. In derived symbiotic systems, this usually involves a sophisticated chemical communication between cells via an exchange of e.g. hormones[12], ions[13], or sugars[14]. However, it remains unclear how primitive symbiotic associations that lack coevolved regulatory machinery can maintain their functional integrity. At early phases of a symbiotic transition, the ability to coordinate functions among cells likely represents a major hurdle that determines the evolutionary fate of the incipient symbiotic association.

Here we used the experimental tractability of bacteria to study the simplest kind of a metabolic interaction: the unidirectional transfer of metabolites from a producer to a recipient cell. Our main goal was to identify whether two bacteria that engage in such a one-way cross-feeding interaction, already display some primitive form of regulation to coordinate their combined metabolism.

For this, we took advantage of a set of bacterial mutants that have been previously used to study fitness consequences of obligate amino acid cross-feeding[15]. Deletion of one biosynthetic gene rendered the growth of the resulting mutant (hereafter: *auxotroph*) dependent on an external supply of amino acids, while deletion of another gene caused an overproduction of one or more amino acids (hereafter: *overproducer*). By combining both deletion alleles in one genetic background, '*cross-feeder*' genotypes were created, which reciprocally exchanged essential amino acids in coculture. Surprisingly, coculturing two of these double-deletion mutants with complementary amino acid requirements provided the cross-feeding consortium with a significant growth advantage relative to the metabolically autonomous (i.e. prototrophic) wild type cells – even when both types directly competed against each other[15]. This observation suggested that cross-feeding genotypes benefitted from dividing their metabolic labour. Moreover, loss of genes that are essentially involved in amino acid biosynthesis triggered the formation of intercellular nanotubes, which auxotrophic bacteria used to obtain cytoplasmic amino acids from other bacterial cells[16]. However, it remains unclear how cross-feeding bacteria coordinate metabolite production and consumption despite the lack of derived regulatory mechanisms.

We addressed this question using a unidirectional exchange of essential amino acids between two genotypes of *Escherichia coli*. These one-way cross-feeding interactions were established by matching amino acid donors with auxotrophic recipients that obligately required the corresponding amino acid for growth. Utilizing genetically engineered single gene deletion mutants for this purpose ruled out pre-existing traits that arose as a consequence of a coevolutionary history among both interaction

93 partners. Moreover, a focus on unidirectional cross-feeding excluded confounding
94 effects that may occur in reciprocal interactions such as e.g. self-enhancing feedback
95 loops[17]. Taking advantage of intracellular reporter constructs allowed analysing both
96 internal amino acid pools as well as their production levels in real-time under *in-vivo*
97 conditions.

98 Our results show that the two bacterial genotypes exchange amino acids via
99 intercellular nanotubes. By lowering cytoplasmic amino acid-concentrations in donor
100 cells, auxotrophic recipients delayed the feed-back inhibition of the donor's biosynthetic
101 pathway, thus increasing overall production levels of the focal amino acid. In other
102 words, a nanotube-mediated exchange of cytoplasmic amino acids coupled the
103 metabolism of two interacting partners in a source-sink-like relationship. Our results
104 show the ease with which mechanisms emerge that regulate the metabolic exchange
105 between two symbiotic associates. By reducing conflicts of interests in this way, this
106 mechanism likely helps to stabilise incipient symbiotic associations, thus contributing to
107 the widespread distribution of metabolic cross-feeding interactions in nature.

108

109 **RESULTS**

110 **Construction and characterisation of uni-directional cross-feeding interactions**

111 To establish unidirectional cross-feeding interactions within *Escherichia coli*, five
112 different genotypes served as amino acid donors: Two single gene deletion mutants
113 (Δ*mdh* and Δ*nuoN*) that produce increased amounts of several different amino acids[15],
114 two deletion mutants that produce increased amounts of either histidine or tryptophan
115 (Δ*hisL* and Δ*trpR*)[18], as well as unmanipulated *E. coli* WT cells. Three genotypes served

as recipients, which were auxotrophic for the amino acids histidine (Δ*hisD*), lysine (Δ*lysR*), and tryptophan (Δ*trpB*) (Fig. 1, Supplementary table 1) and thus essentially required an external source of these metabolites to grow[19].

As a first step, we quantified the amounts of amino acids the five donor strains produced in monoculture during 24 hours of growth. Analysing culture supernatant and cytoplasm of the focal donor populations using tools of analytical chemistry revealed Δ*nuoN* produced significantly increased amounts of histidine, lysine, and tryptophan in both fractions relative to the WT (Mann Whitney U-test: $P<0.05$, $n=4$, Supplementary figure 1), while the production levels of the Δ*mdh* mutant did not differ significantly from WT-levels (Mann Whitney U-test: $P>0.05$, $n=4$, Supplementary figure 1). Similarly, both the intra- and extracellular concentrations of tryptophan in the Δ*trpR* mutant were significantly elevated over WT-levels (Mann Whitney U-test: $P<0.05$, $n=4$, Supplementary figure 1). In contrast, Δ*hisL* released twice as much of histidine into the growth medium as was released by the WT (two sample Mann Whitney test: $P<0.05$, $n=4$, Supplementary figure 1), while it contained much lower levels of histidine in its cytoplasm than the WT.

Intercellular transfer of amino acids is contact-dependent

Capitalizing on the set of well-characterised genotypes, we addressed the question whether donor and recipient cells exchange amino acids in coculture and if so, whether this interaction is contact-dependent. To this end, populations of donor and recipient cells were cocultured in a device (i.e. *Nurmikko cell*), in which both partners can either be grown together in the same compartment or separated by a filter membrane that

allows passage of small molecules, yet prevents direct interactions among bacterial cells[16]. Inoculating donor and recipient strains in different combinations revealed in all tested cases growth of auxotrophic recipients when they were not physically separated from donors (Fig. 2A-C). Auxotrophic recipients grew significantly better when cocultured with amino acid overproducers (Δ*mdh*, Δ*nuoN*, Δ*hisL*, and Δ*trpR*) than with the WT (Dunnett's T3 post hoc test: $P<0.05$, $n=4$). However, physically separating donor and recipient cells by introducing a filter membrane, effectively eliminated growth of recipients in all cases. Surprisingly, this treatment did not affect growth of donor populations (Fig. 2A-C). Three main insights result from this experiment: First, producing the amino acids required by the auxotrophs for growth did not incur detectable fitness costs to the donor strain (Dunnett's T3 post hoc test: $P>0.05$, $n=4$). Second, the total productivity of the coculture involving amino acid overproducers as donors (Δ*mdh*, Δ*nuoN*, Δ*hisL*, and Δ*trpR*) was significantly increased when cells were cocultured in the same environment as compared to the situation when they were physically separated by a filter membrane (Mann Whitney U-test: $P<0.05$, $n=4$, Fig. 2A-C). Third, physical contact between donor and recipient cells was required for a transfer of amino acids between cells.

Cytoplasmic constituents are transferred from donor to recipient cells

The observation that metabolite cross-feeding among cells was contact-dependent suggested that separating cells with a physical barrier prevented the establishment of structures required for amino acid exchange. A possible explanation for this could be intercellular nanotubes, which would allow direct transfer of cytoplasmic amino acids

from donor to recipient cells[16]. This hypothesis was verified by differentially labelling the cytoplasm of donor and recipient cells with plasmids that express either red or green fluorescent proteins. Quantifying the proportion of recipient cells that contained both cytoplasmic markers after 24 hours of growth in coculture using flow cytometry allowed us to determine the exchange of cytoplasmic materials between cells under our experimental conditions. Finding that all cocultures analysed comprised a significant proportion of auxotrophic cells containing both fluorescent proteins simultaneously confirmed that cytoplasmic materials such as protein and free amino acids have been transferred from donor to recipient cells (Fig. 2D). However, it has been previously shown that the presence of the amino acid, auxotrophic genotypes require for growth, prevents the formation of nanotubes[16]. Uncoupling the obligate dependency by supplementing the growth medium with saturating concentrations of the focal amino acid provided no evidence for a significant increase in double-labelled auxotrophs (Fig. 2D), thus linking the establishment of these structures to the physiological requirement for amino acid cross-feeding.

Auxotrophic recipients derive amino acid from cocultured donor cells

One hypothesis that could explain why recipients were able to grow in donor-recipient cocultures (Fig. 2A-C) is that the physical contact between cells increased amino acid production rates of donors. Amino acid production is energetically and metabolically very costly to the bacterial cell[20-22]. To minimize production costs, bacteria tightly regulate their amino acid biosynthesis, for example by end product-mediated feedback mechanisms that reduce production rates when cytoplasmic amino acid concentrations

exceed critical thresholds[23,24]. In our case, recipient cells removed amino acids from the cytoplasm of donors using nanotubes. This decrease in the cell-internal amino acid pools could delay feedback inhibition in the donor cell, thus increasing its overall amino acid production (Fig. 3). Quantifying the amount of free amino acids in the cytoplasm of donor cells in both the absence and presence of an auxotrophic recipient would allow testing the delayed-feedback inhibition hypothesis.

To determine cytoplasmic concentrations of free amino acids in real-time, we used the lysine riboswitch as a cell-internal biosensor. When free lysine binds to the riboswitch, it undergoes a conformational change, thus down-regulating expression of a downstream reporter gene, in our case *gfp*[25]. Introducing the plasmid-borne reporter construct (hereafter: *Lys-riboswitch*, Supplementary figure 2) into the lysine auxotroph Δ*lysR* and exposing the resulting cells to different concentrations of lysine validated the utility of this biosensor: A strong negative correlation between the cells' cytoplasmic amino acid concentrations as quantified via LC/MS/MS analysis of lysed cells and their fluorescence emission ($r=-0.68$, $P=0.003$, Supplementary figure 3) corroborated that this construct allowed indeed determining levels of free lysine in the cytoplasm of living *E. coli* cells by simply quantifying their GFP emission.

Accordingly, introducing the lys-riboswitch into the lysine auxotrophic recipient (Δ*lysR*) and growing the resulting strain in lysine-supplemented media revealed consistently elevated levels of cytoplasmic lysine throughout the experiment (Fig. 4B). In contrast, when the same recipient cells were grown in the absence of lysine, cell-internal lysine levels were significantly reduced (FDR-corrected paired sample t-tests: $P<0.005$, $n=4$, Fig. 4B), indicating amino acid starvation of auxotrophic cells.

Interestingly, when recipient cells were grown in the presence of one of the three donor genotypes, their lysine levels resembled that of lysine-starved auxotrophs until 18 hours of cocultivation, after which lysine levels increased back to the level of lysine-supplemented cells (FDR-corrected paired sample t-tests: $P<0.04$, $n=4$, Fig. 4B). Prior to these coculture experiments, auxotrophs had to be pre-cultured in lysine-containing medium. Thus, the lysine levels measured in auxotrophs under coculture conditions likely reflected the fact that these cells first used up internal residual lysine pools before switching to other sources, in this case the cytoplasmic lysine of donor cells. Consistent with this interpretation is the observation that the presence of donor cells that provided this amino acid allowed lysine auxotrophs to grow (Fig. 4A). A strongly positive correlation between the growth of lysine auxotrophs and their cell-internal lysine levels corroborates that the lysine auxotrophic recipients obtained from cocultured donor cells limited their growth ($r=0.625$, $P=0.003$, Supplementary figure 4).

The presence of auxotrophic recipients increases cytoplasmic amino acid concentrations in donor cells

To test the delayed-feedback inhibition hypothesis, the lys-riboswitch was introduced into the three donors WT, Δ*mdh*, and Δ*nuoN*. Each of these donor genotypes were then grown in monoculture as well as in coculture with the lysine-auxotrophic strain Δ*lysR*. In these donor-recipient pairs only the donor contained the reporter plasmid.

The amino acid biosynthesis of WT cells is most stringently controlled, thus preventing accumulation of free lysine in its cytoplasm. In contrast, the cytoplasm of the Δ*nuoN* strain was characterized by generally increased amino acid levels

(Supplementary figure 1). Similarly, deletion of the malate dehydrogenase gene caused an accumulation of citric acid cycle intermediates and thus a dysregulated amino acid biosynthesis in the Δ*mdh* mutant[15]. Hence, removing lysine from the cytoplasm of WT cells is expected to trigger the strongest increase of cytoplasmic lysine levels. In contrast, higher concentrations of lysine or its biochemical precursors in the cytoplasm of the Δ*mdh* and the Δ*nuoN* strain likely prevent a lowering of the lysine concentration below the critical threshold that triggers a further production.

We tested these predictions by monitoring changes in intracellular lysine levels of donor cells using the lys-riboswitch. In monocultures, lysine levels unveiled a steady increase over time (Fig. 4C). This pattern, however, changed in the presence of the auxotrophic recipient. When *E. coli* WT cells were used as donor, their cytoplasmic lysine levels first increased significantly over the levels WT cells reached in monoculture (FDR-corrected paired sample t-tests: $P<0.03$, $n=4$, Fig. 4C). After that lysine levels dropped significantly before increasing back to monoculture levels (Fig. 4C). The observed fluctuations in the lysine levels of the donor's cytoplasm are consistent with a nanotube-mediated cell attachment that is contingent on the nutritional status of the receiving cell. In contrast, when Δ*mdh* and Δ*nuoN* were cocultured as donor strains together with the auxotrophic recipient, their cytoplasmic lysine levels did not differ significantly from the levels reached under monoculture conditions (Fig. 4C). Thus, these observations are in line with the above expectations and confirm indeed that an auxotroph-mediated removal of amino acids from the donor's cytoplasm was sufficient to prompt an increased amino acid biosynthesis levels in donor cells. Conversely, lysine-auxotrophic recipients displayed significantly increased lysine levels when

cocultured with one of the donor genotypes relative to lysine-starved monocultures. Both observations together suggest a unidirectional transfer of amino acids from donor to recipient cells that in turn results in an intercellular regulation of amino acid biosynthesis. Hence, these findings concur with the delayed-feedback inhibition hypothesis (Fig. 3).

The presence of auxotrophic recipients increases transcription of biosynthesis genes in donor cells

Bacterial cells use feedback inhibition to maintain homeostasis of certain metabolites in their cytoplasm. Once metabolite levels drop below a certain threshold, production levels are increased to allow optimal growth[26,27]. In the case of amino acid biosynthesis, the promoter elements that control transcription of biosynthetic pathways are frequently highly sensitive to intracellular levels of the synthesized amino acid[24], thus enhancing transcription of the operon when the amino acid is scarce. As soon as amino acid concentrations reach optimal levels, further transcription is blocked enzymatically[28] or by direct binding of the amino acid to the operon[29].

Taking advantage of this principle, we employed plasmid-borne promoter-GFP-fusion constructs (Supplementary figure 2) to identify transcriptional changes in amino acid biosynthesis genes. These reporter constructs have been previously shown to accurately measure promoter activity with a high temporal resolution[30]. For analysing the focal cross-feeding interactions, the fusion constructs for *hisL* and *trpL* were selected, which sense the cytoplasmic concentration of histidine[31] and tryptophan[29,32], respectively. Correlating GFP emission levels with the cytoplasmic concentration of the

277 corresponding amino acid as quantified chemically via LC/MS/MS revealed a
278 significantly negative relationship for both histidine (r=-0.407, P<0.001, Supplementary
279 figure 3) and tryptophan (r=-0.237, P=0.038, Supplementary figure 3), confirming the
280 link between transcription of metabolic genes and the cytoplasmic concentration of the
281 corresponding amino acids.

282 These promoter-GFP-fusion constructs were introduced into donor cells (i.e. WT,
283 Δ*mdh*, Δ*hisL*, and Δ*trpR*), which were then cultivated for 24 hours in the absence or
284 presence of the Δ*hisD* or Δ*trpB* auxotrophic recipient cells. In line with expectations,
285 donor strains WT, Δ*hisL*, and Δ*trpR* displayed a starkly increased transcription of the
286 respective biosynthetic operon in the presence of auxotrophic recipients as compared to
287 donors growing in monoculture (FDR-corrected paired t-tests: P<0.05, n=4, Fig. 5).
288 Together, these results demonstrate that the presence of auxotrophic recipients
289 significantly increased the amino acid production of donor cells. By withdrawing amino
290 acids from the cytoplasm of donor cells, auxotrophic recipients prompted donor cells to
291 readjust their amino acid levels by up-regulating the transcription of the corresponding
292 amino acid biosynthesis genes.

293

294 **DISCUSSION**

295 Our study demonstrates for the first time that the deletion of a single metabolic gene
296 from a bacterial genome can be sufficient to couple the metabolism of two previously
297 independent bacterial cells. Auxotrophic cells that had lost the ability to autonomously
298 produce a certain amino acid established intercellular nanotubes to derive the amino
299 acid they required for growth from other cells in the environment. Quantifying cell-

internal amino acid levels revealed a primitive form of intercellular regulation of amino acid biosynthesis between donor and recipient cells in a source-sink-like manner. This relationship emerged as a consequence of feedback-based control mechanisms in the biosynthetic pathways of individual cells. The metabolic network of a cell provides and maintains specific levels of the building block metabolites that are required for growth[33]. An excess or deficit of metabolites within cells can disturb the cell-internal equilibrium and thus cause stress[34]. Our results show how the removal of metabolites from the donor's cytoplasm translates into increased production levels of the metabolite. Strikingly, this source-sink-like relationship between donor and recipient did not impose detectable fitness costs on the donor, but instead increased growth of the whole bacterial consortium.

Obligate metabolic interactions are common in natural microbial communities[35,36]. When certain metabolites are sufficiently available in the environment, bacteria that lose the ability to produce these metabolites autonomously (e.g. by a mutational deactivation of the corresponding biosynthetic gene) gain a significant growth advantage of up to 30% relative to cells that produce these metabolites[37,38]. As a consequence, auxotrophic genotypes rapidly increase in frequency by deriving the focal metabolites from both environmental sources and other cells in the vicinity. The results of our study help to explain this tremendous fitness advantage: by selectively upregulating only those biosynthetic pathways that enhance growth of the symbiotic consortium, cells only invest resources into those metabolites that help the respective interaction partner to grow. If the exchange is reciprocal, groups of cross-feeding cells gain a significant fitness advantage relative to metabolically autonomous types, even when both parties

are directly competing against each other in the same environment[15]. Thus, the type of intercellular regulation discovered in this study minimizes the amount of resources each interaction partner needs to invest into the corresponding others. From this emerges a metabolic division-of-labour, in which the benefit that participating cells gain is more, than the costs incurred by the interaction. This effect reduces conflicts of interests within consortia of cross-feeding cells, thus providing a mechanistic explanation for the widespread distribution of this type of interaction in nature.

Nutritional stress or starvation in a cell is known to induce an aggregative lifestyle in bacteria[16,39,40]. In many cases, this physical contact is followed by an exchange of cytoplasmic contents between interacting cells[16,39,41]. Structurally similar connections between cells are known to be involved in short- and long-distance communication in many multicellular organisms[42,43]. In both cases, networks of interacting cells are challenged with the question of how to optimally organize transport within the network such that all cells involved derive sufficient amounts of the traded signal or molecule. While the intercellular communication within tissues of eukaryotic organisms is notoriously difficult to study, our focal system provides a paradigmatic case to experimentally study the constraints and rules that determine the assembly and structure of intercellular communication networks. In this context, the results of our study suggest that the distribution of metabolites within networks of interacting bacterial cells mainly results from local interactions among neighbouring cells.

A metabolic relationship that is remarkably similar to the one studied here has been described for the obligate association between aphids, *Acyrthosiphon pisum*, and their endosymbiotic bacteria *Buchnera aphidicola*. In this system, the aphid host regulates

the amino acid production levels of its symbionts by changing its intracellular precursor concentrations[44]. This functional link is afforded by a mutational elimination of feedback control in the corresponding biosynthetic pathway of the bacterial symbionts. Thus, similar to the results of our study, manipulation of the biosynthetic pathway in the host led to an efficient coupling of the metabolism of host and symbiont. An intimate coordination such as this enabled the symbionts to function as an extension of the host's metabolic network.

Our work highlights the ease, with which two previously independent organisms can form a physiologically integrated whole: the mutational deactivation of a biosynthetic gene is sufficient to trigger the establishment of this kind of metabolic interaction. Given that a loss of seemingly essential biosynthetic genes is very common in bacteria[37] and that a nanotube-mediated exchange of cytoplasmic materials is known to also occur between different bacterial species[16], it is well conceivable how a reductive genome evolution of coevolving bacteria can result in the formation of a multicellular metabolic network. Once a biosynthetic gene is lost, the resulting auxotrophic genotype is more likely to lose additional genes than to regain the lost function via horizontal gene transfer[45]. Given that dividing metabolic labour in this way can be highly advantageous for the interacting bacteria[15] relative to metabolic autonomy, bacteria in their natural environment may exist within networks of multiple bacterial cells that reciprocally exchange essential metabolites rather than as functionally autonomous units.

METHODS

Strains and plasmids used in the study

Escherichia coli BW25113 was used as wild type, from which mutants that overproduce amino acids *(*Δ*mdh,* Δ*nuoN,* Δ*hisL,* and Δ*trpR)* and mutants that are auxotrophic for histidine (Δ*hisD*), lysine (Δ*lysR*), or tryptophan (Δ*trpB)* were obtained by a one-step gene inactivation method[15,16] (supplementary table 1). Deletion alleles were transferred from existing single gene deletion mutants (i.e. the Keio collection[46]) into *E. coli* BW25113 using the phage P1. The cytoplasm of all donor and recipient strains was labelled by introducing one of the two plasmids pJBA24-*egfp* or pJBA24-*mCherry.* The plasmids constitutively express the ampicillin resistance gene (*bla*) as well as either the fluorescent protein EGFP (*egfp*) or mCherry (*mCherry*). Two reporter constructs were used: (i) lys-riboswitch (pZE21-GFPaav-Lys) for measuring internal amino acid levels (lysine) and (ii) promoter fusion plasmids (pUAA6-His and pUA66-Trp) for measuring the transcriptional activity of the promoters *hisL* and *trpR* respectively (see supplementary experimental procedures for plasmid construction and characterization of reporter constructs).

Culturing methods and general procedures

Minimal media for *Azospirillum brasiliense* (MMAB)[47] without biotin and with fructose (5 gl^{-1}) instead of malate as a carbon source served as the growth media in all experiments. The required amino acids (histidine, lysine, and tryptophan) were supplemented individually at a concentration of 100 µM. Cultures were incubated at a temperature of 30 °C and shaken at 220 rpm for all experiments. All strains were precultured in replicates by picking single colonies from lysogeny broth (LB)[48] agar plates and incubated for 18 hours. The next morning, precultures were diluted to an

optical density (OD) of 0.1 at 600 nm as determined by a Tecan Infinite F200 Pro platereader (Tecan Group Ltd, Switzerland). 10 µl of these precultures were inoculated into 1 ml of MMAB. In case of cocultures, donor and recipient were mixed in a 1:1 ratio by co-inoculating 5 µl of each diluted preculture. To cultivate strains containing the lys-riboswitch, ampicillin was added at a concentration of 100 µg ml^{-1} and kanamycin was added at 50 µg ml^{-1} in case of strains containing the promoter-GFP-fusion constructs. Anhydrotetracycline (aTc) (Biomol GmbH, Hamburg, Germany) was added at a concentration of 42 ng ml^{-1} to induce expression of the lys-riboswitch.

Contact-dependent exchange of amino acids

To determine if physical contact between cells is required for an exchange of amino acids between donor and recipient cells, a previously described method was used[16]. In brief, each donor (i.e. WT, Δ*mdh*, Δ*nuoN*, Δ*hisL,* and Δ*trpR*) was individually paired with each recipient (i.e. Δ*hisD*, Δ*lysR,* and Δ*trpB*) and every combination was inoculated together into a Nurmikko cell that allows cultivation of both populations either together in the same compartment or separated by a membrane filter (0.22 µm, Pall Corporation, Michigan, USA). The filter allows passage of free amino acids in the medium, but prevents direct interaction between cells. After inoculating 4 ml of MMAB, the apparatus was incubated for 24 h. Bacterial growth after 24 h was determined as colony forming units (CFU) per ml culture volume by plating the serially-diluted culture on MMAB agar plates that did or did not contain ampicillin or kanamycin for selection. The increase in cell number was calculated as the logarithm of the difference between the CFU counts determined at the onset (0 h) of the experiment and after 24 h. Each donor-recipient

415 combination was replicated 4-times for both experimental conditions (i.e. with and
416 without filter).
417
418 **Flow cytometric analysis of cytoplasmic protein transfer**
419 A previously established protocol was applied to identify a transfer of cytoplasmic
420 material from donor to recipient genotypes[16]. For this, pairs of donor and recipient cells
421 with differentially labeled cytoplasms (i.e. containing EGFP or mCherry) were co-
422 inoculated into 1 ml MMAB. At the beginning of the experiment (0 h) and after 24 h of
423 growth, the sample was analyzed in a Partec CyFlow Space flow cytometer (Partec,
424 Germany). In the flow cytometer, cells were excited at 488 nm with a blue solid-state
425 laser (20 mV) and at 561 nm with a yellow solid-state laser (100 mV). Green (*egfp*) and
426 red (*mCherry*) fluorescence emission was detected at 536 nm and 610 nm, respectively.
427 *E. coli* WT devoid of any plasmid was used as a non-fluorescent control. The number of
428 single- and double-labeled cells in a population was quantified at both time points. Data
429 analysis and acquisition was done using the FlowMax software (Partec GmbH,
430 Germany). The experiment was conducted by coculturing eGFP-labelled donor with
431 mCherry-labelled recipient gentoypes and *vice versa* in all possible combinations (i.e.
432 each donor paired with each recipient, except in case of Δ*hisL* and Δ*trpR*, which were
433 only paired with Δ*hisD* and Δ*trpB,* respectively) for 24 h. Each combination was
434 replicated 4-times.
435
436 **Fluorescence measurement**

The fluorescence levels of cells containing the lys-riboswitch or the promoter-GFP-fusion constructs were measured by transferring 200 µl of the culture into a black 96-microwell plate (Nunc, Denmark) and inserting the plate into a Tecan Infinite F200 Pro platereader (Tecan Group Ltd, Switzerland). The plate was shaken for 5 seconds prior to excitation at 488 nm followed by emission detection at 536 nm. Fluorescence values were always recorded together with a cognate control measurement. In case of the lys-riboswitch, the uninduced plasmid-containing culture served this purpose, while in case of the promoter fusion constructs, the promoter-less plasmid (pUA66) was used as control. See supplementary methods for plasmid reporter characterization and promoter activity measurements.

Statistical analysis

Normal distribution of data was assessed using the Kolmogorov-Smirnov test and data was considered to be normally distributed when $P > 0.05$. Homogeneity of variances was determined using the Levene's test and variances were considered homogenous if $P > 0.05$. One-way ANOVA followed by a Dunnett's T3 post hoc test was used to compare growth differences in the contact-dependent growth analysis. Differences in the fluorescence emission levels of donor cells in the presence and absence of a recipient were assessed with paired sample t-tests. The same test was used to compare the number of recipient (Δ*lysR*) CFUs at the start and at the end of the coculture experiments to detect donor-enabled growth. The False Discovery Rate (FDR) procedure of Benjamini et al. (2006) was applied to correct P values after multiple testing. Pearson product moment correlation provided identification of the statistical

relationship between cytoplasmic amino acid levels and fluorescence emission as well as between cytoplasmic lysine level and growth of the Δ*lysR* recipient.

AUTHOR CONTRIBUTIONS

SS and CK conceived the study, SS, CK, and SP designed the study. SS performed all experiments. SS and CK interpreted and analyzed the data. TA generated some plasmids for the study. SS and CK wrote the manuscript, all authors amended the manuscript.

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FIGURES

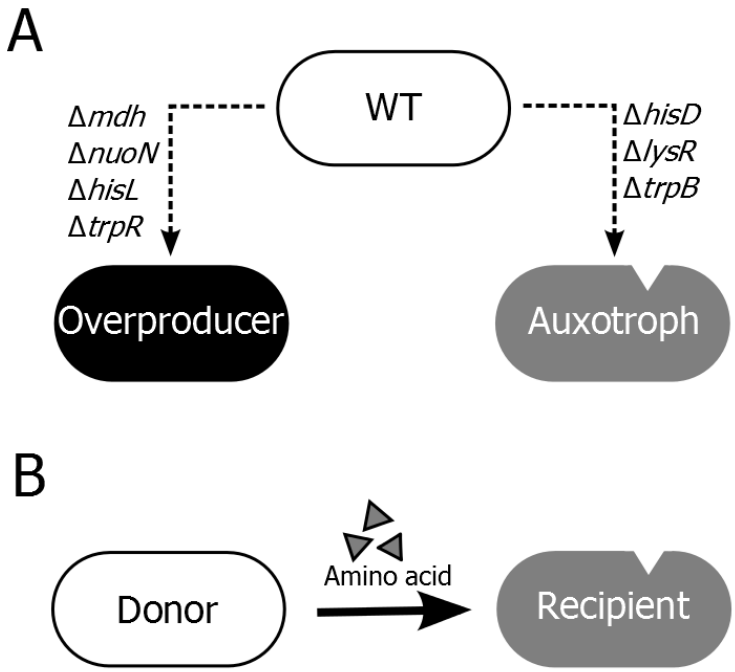

Figure 1: Experimental system used. (**A**) Design of genotypes. Single genes were deleted from *E. coli* BW25113 wild type (WT) to generate mutants that produce increased amounts of amino acids (overproducer, black) as well as mutants that essentially require a certain amino acid to grow (auxotroph, grey). (**B**) Coculturing an amino acid donor (i.e. WT or overproducer) together with an auxotrophic recipient results in a one-way cross-feeding interaction that is obligate for the recipient, but not the donor.

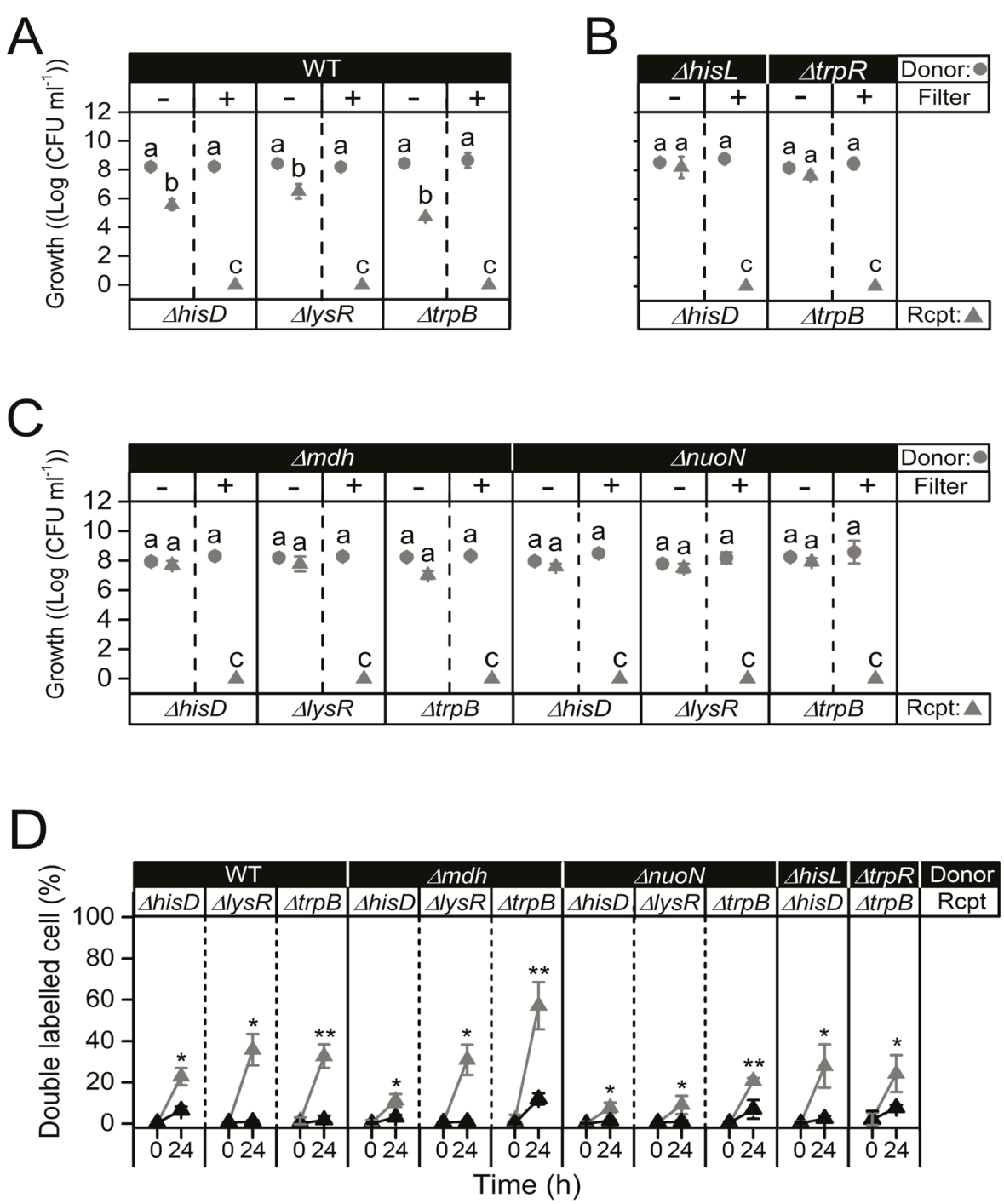

Figure 2: Contact-dependent exchange of cytoplasmic amino acids. (**A-C**) Amino acid exchange is contact-dependent. Amino acid donors (circles) were cocultured with

auxotrophic recipients (Rcpt, triangles) either together in the same compartment (- Filter) or separated by a filter membrane (+ Filter) that allows passage of free amino acids, but prevents direct physical contact among cells. Growth over 24 h was determined as number of colony-forming units (CFU) per ml by subtracting the value at 0 h from the one reached at 24 h. Different letters indicate significant differences (Dunnett's T3 post hoc test: $P<0.05$, $n=4$). (**D**) Cells exchange cytoplasmic material. The cytoplasm of donors and recipients were differentially labelled with the fluorescent proteins EGFP and mCherry, respectively. Quantifying the proportion of double-labelled auxotrophs containing both cytoplasmic markers after 0 h and 24 h of coculture allowed assessing an exchange of cytoplasm between bacterial cells. The experiment was conducted in the absence (grey triangles) and presence (black triangles) of the focal amino acid (100 µM). Asterisks indicate significant differences (paired t-test: ** $P<0.001$, * $P<0.05$, $n=4$). In all cases, mean (±95% confidence interval) are shown.

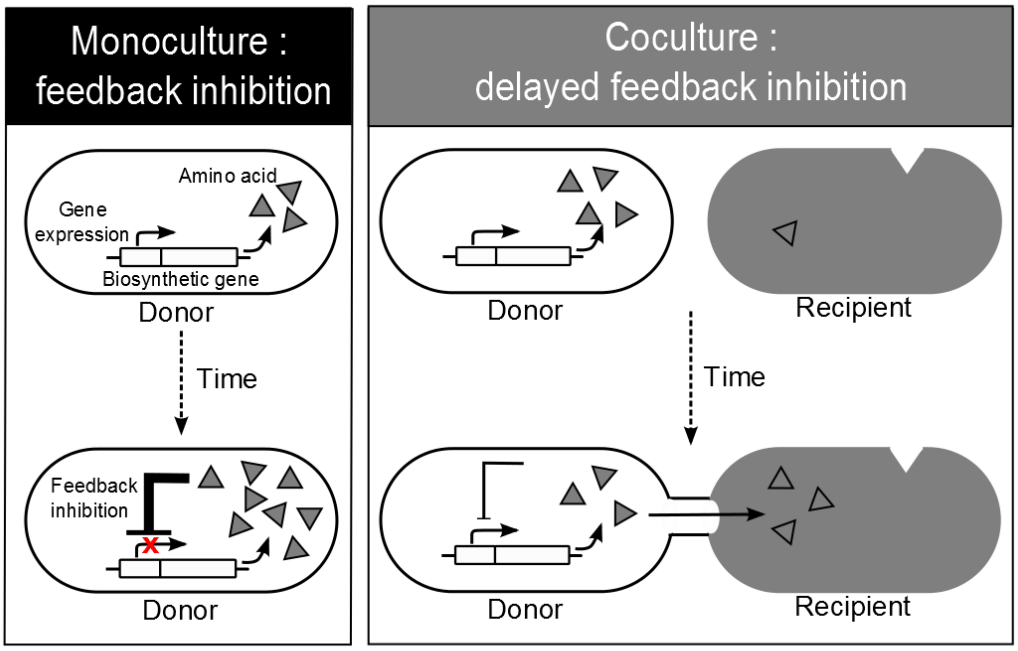

Figure 3: Delayed feedback inhibition hypothesis. In monoculture, amino acid concentrations in the cytoplasm of donor cells build up over time. When a certain concentration threshold is reached, these metabolites inhibit their own production by supressing the expression of the corresponding amino acid biosynthesis genes (i.e. end product-mediated feedback inhibition). In coculture, auxotrophic recipients reduce cytoplasmic amino acid concentrations of donor cells. As a consequence, feedback inhibition of biosynthesis genes is delayed, thus resulting in an increased amino acid biosynthesis.

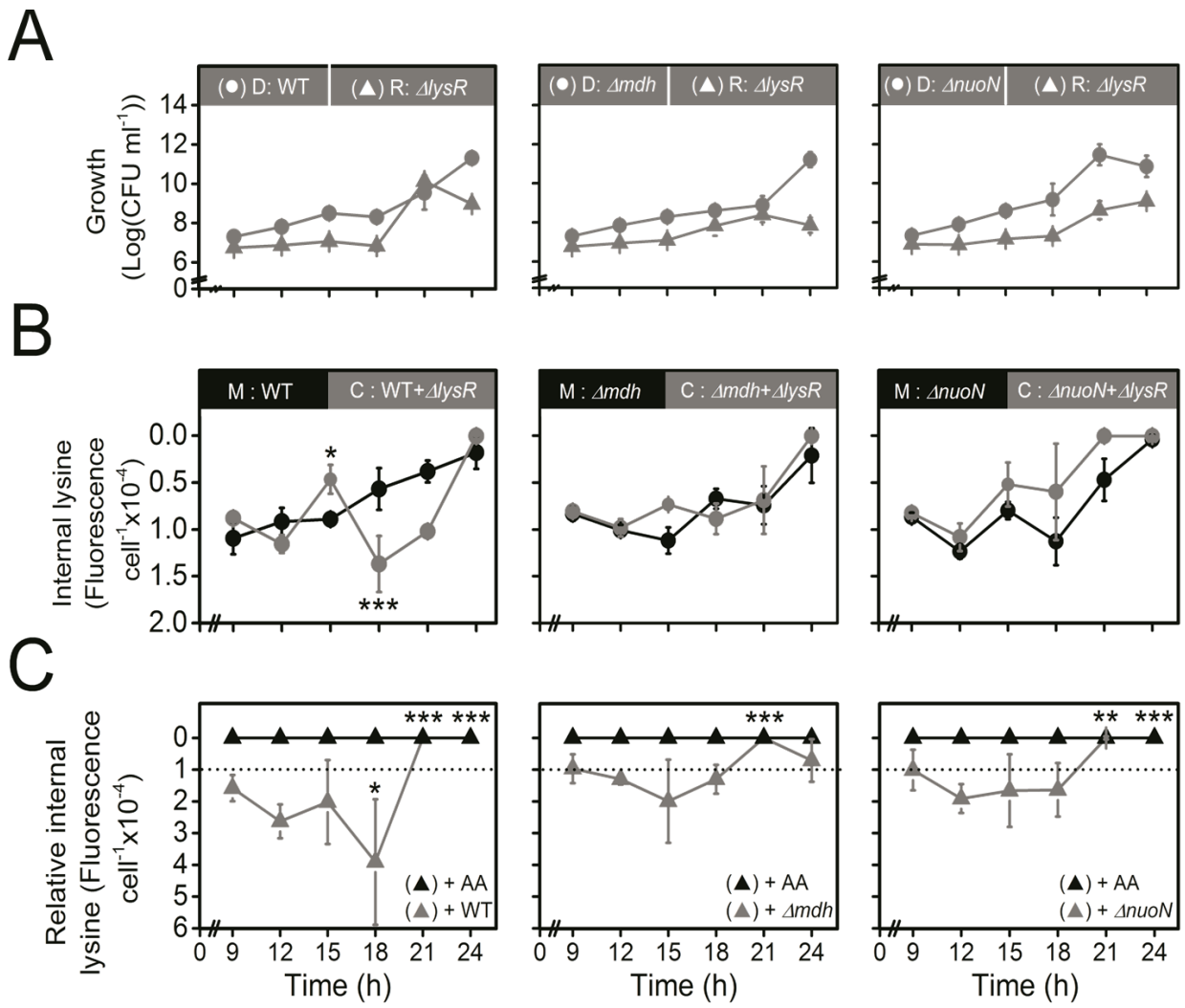

Figure 4: The presence of auxotrophs increases cytoplasmic amino acid levels in donor cells. (**A**) Growth of each partner in cocultures of donor (D, circles) and recipient (R, triangles) populations was determined as the number of colony-forming units (CFUs) ml^{-1} over 24 h. (**B, C**) Cytoplasmic lysine levels were quantified by measuring GFP fluorescence emission from a cell-internal reporter and normalized per cell containing the reporter. Low fluorescence levels indicate high lysine levels (note the inverted y-axes). (**B**) Lysine levels in lysine-supplemented monocultures (+AA) and un-supplemented cocultures were measured relative to lysine-starved monocultures

(dashed line). In the presence of lysine, monocultures of the recipient (black triangles) showed constantly increased cytoplasmic lysine levels. In coculture with the donor (grey triangles), lysine levels in the recipient first declined and then increased back to the level of the +AA condition. (**C**) In coculture with lysine-auxotrophic recipients, cytoplasmic lysine levels of WT donor cells were significantly increased at 15 h of growth and significantly decreased at 18 h of growth in coculture (C, grey circles) relative to monoculture conditions (M, black circles). However, in case of the overproducers Δ*mdh* and Δ*nuoN*, cell-internal lysine levels did not vary between mono- and coculture conditions. In all cases, mean (±95% confidence interval) are shown and asterisks indicate the results of FDR-corrected paired sample t-tests (*P<0.05, **P<0.01, ***P<0.001, n=4).

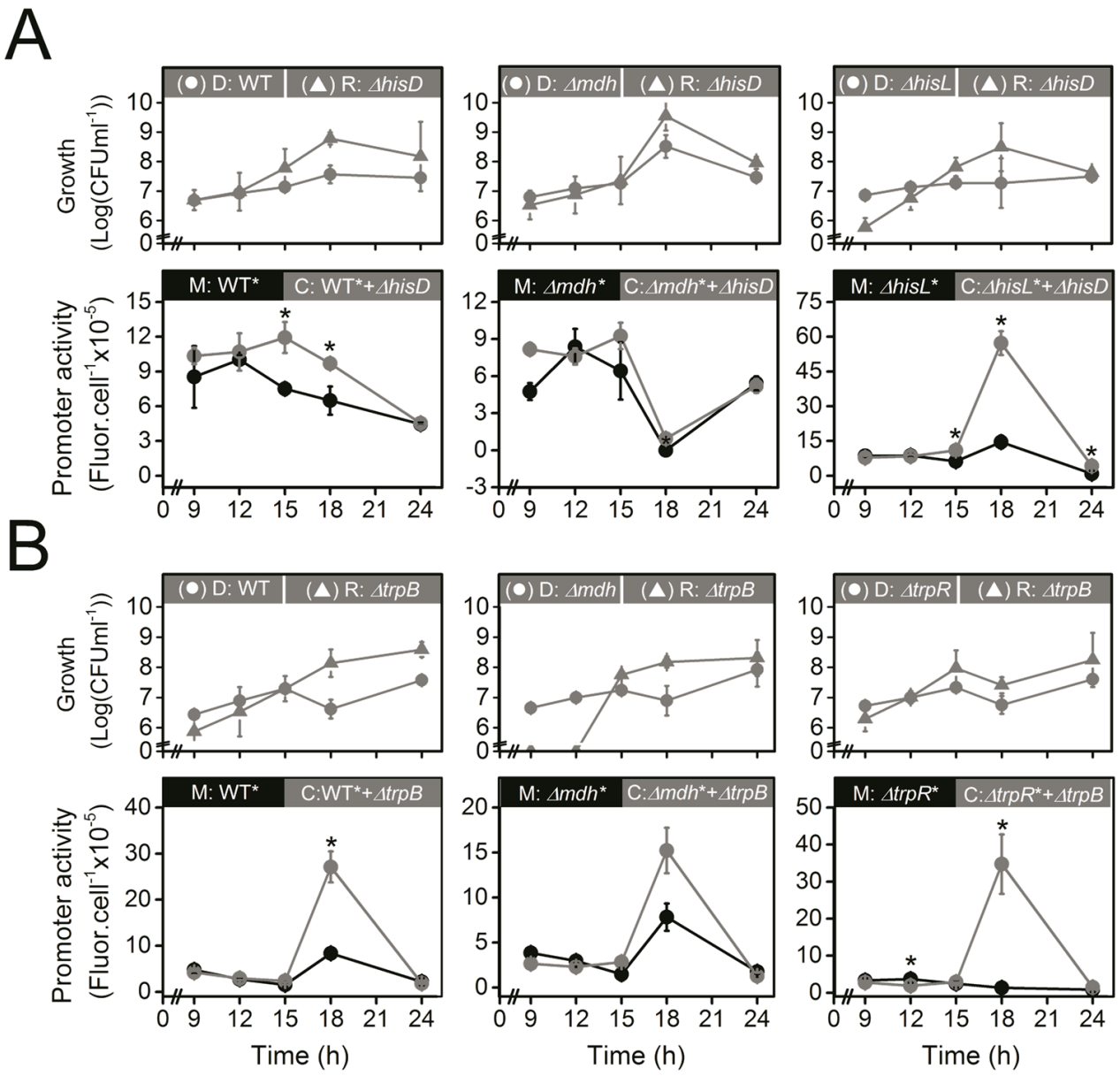

Figure 5: The presence of auxotrophs increases transcription of biosynthetic genes in the donor. (**A, B**) Top panels depict growth of donor (D, circle) and recipient (R, triangle) in coculture over time quantified as the number of colony-forming units (CFU) per ml. Bottom panels show promoter activity of the donors' amino acid biosynthesis gene in monoculture (M, black circles) and coculture with an auxotrophic recipient (C, grey circles). Promoter activity was quantified as the emission of GFP

fluorescence from a promoter-GFP-fusion construct and normalized per number of donor cells (CFUs) containing the construct. Asterisks indicate significant differences of the promoter activity of donor cells in mono- and coculture conditions (FDR-corrected paired t-test: * $P<0.05$, $n=4$). Populations of donor cells (D, circles) were grown in monoculture or cultivated together with (**A**) the histidine auxotrophic recipient (Δ*hisD*) or (**B**) the tryptophan auxotrophic recipient (Δ*trpB*). In all cases, mean (±95% confidence interval) are shown.