Mechanistic and metabolic basis of bacterial cross-feeding

Dissertation

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Chapter 2: Summary

In their natural habitat, microorganisms interact with a variety of micro- as well as macro-organisms. Such interactions result in either positive or negative effects on the growth and survival of involved species. Negative effects on growth, can be mostly attributed to competition for limited resources and space, while positive effects on growth are challenging to justify. Metabolite cross-feeding is one such interaction that describes the transfer of primary or secondary metabolites from one organism to another. Considering that metabolites are costly and impose a significant energetic cost to the cell producing them, it is intriguing to know how the process of cross-feeding is favourable.

Bacteria employ different mechanisms to carry out the exchange of metabolic by-products, intermediates, and electrons between each other during the process of cross-feeding. Contact-dependent mechanisms of exchange (such as direct cell-cell contact, type secretion systems, and pili), provide the following advantages: (i) protection of the exchanged product from environmental degradation or modification, (ii) provision of the product in a concentrated form, and (iii) prevention of uptake of the product by unintended recipients. The role of contact-dependent mechanisms in the transfer of genetic material (bacterial conjugation) and toxins (contact-dependent inhibition or killing) has been studied for years. However, the importance of similar contact-dependent mechanisms during the cross-feeding of essential nutrients is not fully understood.

This thesis aimed at identifying a contact-dependent mechanism for amino acid cross-feeding in bacteria. The main questions were,

(i) Can a transfer of essential nutrients take place between bacteria in an environment that is not conducive for diffusion-based exchange?
(ii) Which characteristic of the interacting partners influences the mechanism of transfer? Does the nutritional status of the cell play a role?
(iii) What are the effects of nutrient exchange on the biosynthesis of these nutrients in interacting partners?
(iv) Are other factors like cell adhesion, motility, and chemotaxis, also relevant for contact-dependent cross-feeding of nutrients?

A synthetically engineered cross-feeding system in bacteria was employed to answer these questions. Cross-feeding genotypes of *Escherichia coli* and *Acinetobacter baylyi* were co-cultivated, harbouring mutations that impart the following phenotype: (i) a dependency on an external source of amino acid (auxotrophy), and (ii) increased levels of a specific amino acid (over-production). A complementary pair of cross-feeding genotypes was able to support the growth of either partner in minimal medium without an external supply of amino acids. Both species are genetically tractable and easy to culture in the lab, thus reducing the complications of studying the dynamics of cross-feeding in a natural consortium. The genetically modified bacterial system was ideal to study the genetic, metabolic and transcriptional aspects of a nutrient cross-feeding interaction.

Nanotube mediated exchange of cytoplasmic contents in bacteria. Cross-feeding genotypes of *E. coli* exhibit membrane-derived tubular structures that connect neighbouring cells in a shaken liquid environment. Differential fluorescent labelling of the cytoplasm of two complementary cross-feeding genotypes, indicated a transfer of cytoplasmic content both within and between species. Cytoplasmic exchange between cells appeared to be dependent on the turbulence of the surrounding medium, since double-labelled cells were found exclusively in shaken liquid cultures as compared to stagnant liquid cultures. The formation of tubular connections, denoted as nanotubes, between cross-feeding genotypes was found to be dependent on the nutritional status of the cell as well. Auxotrophic genotypes that relied on an external source of amino acid did not form nanotubes in a medium supplemented with excess of focal amino acid.

Metabolic complementarity of cross-feeding genotypes. The metabolic network of bacteria consists of a series of step-wise reactions, which degrade organic substrates to precursor metabolites (catabolism) and convert these precursor metabolites to building blocks (anabolism). These biosynthetic pathways are often under end-product mediated feedback control which helps to maintain optimal levels of all metabolites in the cell's cytoplasm. Amino acid biosynthesis pathways are also under feedback regulation, imposed by the level of free amino acids in the cytoplasm. A cell-internal biosensor was used to track the changes in internal amino acid level in the context of cross-feeding. Periodic measurements of internal amino acid levels in an auxotrophic recipient provide a direct evidence of amino acid exchange during cross-feeding. The presence and uptake of amino acid by an auxotroph resulted in fluctuations in the level of amino acid in the cytoplasm of the donor cell (*E. coli* wildtype). Furthermore a reduction in the amino acid level in the donor cytoplasm triggered an increased transcription of the promoter for the amino acid biosynthetic gene present in the donor cell. These results provide a source-sink-like model for explaining how metabolism of two interacting cells can be coupled through delaying of the feedback inhibition system

Transcriptional changes due to amino acid cross-feeding. A loss-of-function mutation in amino acid biosynthesis (auxotrophy) was known to trigger the formation of nanotubes. However, the cellular effects of this nutritionally dependent lifestyle on nanotube structure and function were unclear. To get an overview of changes in gene expression during the process of cross-feeding, a transcriptomic analysis of a coculture of *E. coli* cross-feeding genotypes was performed. Gene ontology and pathway analysis revealed the putative role of genes regulating, (i) cell adhesion (*ppdA*, *ydeQ*, *yhjH*, *dgcM*), (ii) motility (*ycgR*), and (iii) vesicle production (*degP*, *nlpA*, *nlpI*). Targeted deletions of some of these genes in the auxotrophic genotype revealed significant effects on growth of the auxotroph in coculture with a donor (over-producer genotype). Furthermore a role of vesicles in amino acid cross-feeding was identified in this study through a combination of techniques comprising (i) scanning electron microscopy, (ii) use of mutants depicting varying levels of vesicle production, and (iii)

addition of vesicle fractions to different cocultures of auxotroph and donor (WT or over-producer). Vesicles potentially act as cell material for establishing inter-cellular connections between auxotrophs and neighbouring cells.

Taken together the results of this thesis provide a connected view of bacterial communities as opposed to a population of autonomously replicating bacterial cells. Auxotrophic genotypes may employ either (i) a nanotube-mediated mechanism, or (ii) vesicle-chain-like structures, to obtain nutrients from neighbouring cells especially during a scarcity of nutrients in the environment. This study shows that the establishment of a cytoplasmic bridge between cross-feeding cells results in a coupling of the metabolism of interacting partners. A division of metabolic labour in this way leads to a growth benefit for the cross-feeding partners rendering this strategy beneficial in terms of fitness. Hence a bacterial community may be considered as a large, inter-connected, metabolic network with biosynthetic pathways being divided among individual members.

Chapter 3: Zusammenfassung

Mikroorganismen interagieren in ihrem natürlichen Lebenslauf mit einer Vielfalt anderer Mikro- als auch Makroorganismen. Diese Interaktionen können entweder positive oder negative Effekte auf das Wachstum und schließlich die Überlebensfähigkeit der involvierten Spezies haben. Der Ursprung für negative Effekte liegt dabei überwiegend in der Konkurrenz um limitierte Ressourcen und begrenzten Raum begründet. Positive Effekte sind hingegen oft wesentlich schwerer zu erklären. Der Austausch von Metaboliten (`*cross-feeding*`) repräsentiert eine solche positive Interaktion, welche den Transfer von Primär,- oder Sekundärmetaboliten von einem Organismus zu einem anderen beschreibt. Da die Biosynthese von Metaboliten allerdings meist mit energetischen Kosten für den Produzenten verbunden ist, stellt sich die Frage, inwiefern der Austausch von Metaboliten vorteilhaft sein kann.

Bakterien besitzen eine Reihe von Mechanismen um Elektronen, sowie Zwischen,- und Endprodukte des Stoffwechsels effizient untereinander auszutauschen. Einige dieser Mechanismen basieren auf den physischen Kontakt zwischen Zellen (z.B. Sekretionssysteme und Pili) und bieten den interagierenden Organismen folgende Vorteile: (i) Der transportierte Metabolit wird vor dem Abbau oder der Modifizierung durch Umwelteinflüsse geschützt. (ii) Die Substanz kann lokal in höherer Konzentration zur Verfügung gestellt werden. (iii) Der Konsum der Substanz durch andere Organismen wird verhindert.
Die Relevanz kontaktabhängiger Mechanismen bei dem Austausch von genetischem Material (Konjugation) und Toxinen (kontaktabhängige Inhibierung oder Eliminierung) wird bereits seit einem längeren Zeitraum erforscht. Die Bedeutung von Mechanismen, die den Zellkonktakt ermöglichen, für den wechselseitigen Transfer von Metaboliten ist hingegen nicht vollständig aufgeklärt.

Das Hauptziel der vorliegenden Dissertation war die Identifikation von kontaktabhängigen Mechanismen, die den Austausch von Stoffwechselprodukten, hier Aminosäuren, zwischen Bakterien ermöglichen. Folgende Fragestellungen wurden behandelt:

(i) Inwiefern können essentielle Nährstoffe zwischen Bakterien unter Umweltbedingungen ausgetauscht werden, welche ungeeignet für die Diffusion und die anschließende Aufnahme von Metaboliten sind?
(ii) Welche Eigenschaften der interagierenden Partner bestimmen den Austauschmechanismus? Spielt die aktuelle Versorgung der Zelle mit Nährstoffen in diesem Kontext eine Rolle?
(iii) Wie beeinflusst der Austausch von Metaboliten die Biosynthese dieser in den jeweiligen Partnern?
(iv) Sind andere Faktoren, beispielsweise die Adhäsion, die Mobilität, oder die Chemotaxis von Zellen, relevant für den kontaktabhängigen Austausch von Stoffwechselprodukten?

Um diese Fragen zu beantworten wurde zunächst ein Modellsystem mit genetisch modifizierten Bakterien auf der Basis von wechselseitigem Aminosäureaustausch etabliert. Dafür wurden Mutationen in die Genome von *Escherichia coli* und *Acinetobacter baylyi* eingeführt, welche zum einen die Abhängigkeit von externer Aminosäurezufuhr (Auxotrophie) und zum anderen die Überproduktion anderer Aminosäuren zur Folge hatten. In Folge dessen war ein komplementäres Paar von auxotrophen Überproduzenten in Minimalmedium, das keine Aminosäuren enthält, in der Lage essentielle Aminosäuren auszutauschen und demzufolge zu wachsen. Ein solches Modellsystem bietet eine Reihe von Vorteilen gegenüber einer metabolischen Wechselbeziehung eines natürlichen Konsortiums: Die Laborstämme sind unter definierten Wachstumsbedingungen einfacher zu kultivieren, leichter zu unterscheiden und ihr Metabolismus und Genom sind weitreichend beschrieben. Außerdem stehen etablierte Plattformen zur Genom,- und Transkriptomanalyse zur Verfügung, wodurch das bakterielle Modellsystem ideal zur Erforschung einer metabolischen Wechselbeziehung geeignet ist.

Der Austausch cytoplasmatischer Bestandteile durch Nanotubes. In geschüttelten Kulturen von *E. coli* konnten röhrenförmige Strukturen nachgewiesen werden, welche aus Membranbestandteilen bestehen und benachbarte Zellen verbinden. Bemerkenswerterweise wurden diese „Nanotubes“ ausschließlich von auxotrophen Überproduzenten gebildet. Durch die Markierung mit unterschiedlichen Fluoreszenzproteinen wurden Hinweise auf den Transport von cytoplasmatischen Komponenten zwischen verschiedenen Genotypen von *E.coli*, aber auch zwischen beiden Modellspezies gefunden. Hierbei war der Austausch von Fluoreszenzproteinen und somit die Entstehung doppelt markierter Zellen abhängig von der Kultivierung. Nur in geschüttelten Kulturen, jedoch nicht in statischen Kulturen, fand ein Austausch statt.

Metabolische Komplementarität von interagierender Genotypen. Die Stoffwechselwege von Bakterien beruhen auf einer Reihe von nacheinander ablaufenden Reaktionen, entweder zum Abbau organischer Substanzen (Katabolismus), oder um aus Vorprodukten schließlich alle zellulären Komponenten zu synthetisieren (Anabolismus). Endprodukte dieser Biosynthesewege aktivieren häufig eine Feedback-Regulierung, welche zur optimalen Versorgung der Zelle mit dem betreffenden Metaboliten beitragen. Dies trifft auch auf die Aminosäurebiosynthese zu. Da die internen Konzentrationen von Aminosäuren während des beiderseitigen Austausches von Zytoplasma besonders relevant für das Verständnis von *cross-feeding* sind, wurden interne Biosensoren zur Überwachung dieser eingesetzt. Die Bestimmung der Konzentrationen in regelmäßigen Abständen erlaubt einen direkten Beweis von Aminosäuretransfer von Produzenten zu Rezipienten. Selbst im Wildtyp von *E.coli*, in diesem Fall der Produzent, schwankten die internen Aminosäurekonzentrationen bei Anwesenheit eines auxotrophen Genotypen. Hierbei korrelierte die Reduzierung der internen Konzentrationen an Aminosäure mit einer Erhöhung der Transkription der zugehörigen Biosynthesegene. Diese Beobachtungen deuten auf ein dynamisches Verhalten hin, welches durch Inaktivieren der Feedback-Inhibierung und der Kopplung des Metabolismus zweier Genotypen entsteht.

Aminosäureaustausch verändert das Transkriptom. Wie zuvor beschrieben kann ein durch Mutation hervorgerufener Funkltionsverlust zur Biosynthese einer Aminosäure die Bildung von Nanotubes auslösen. Jedoch sind die involvierten Gene nach wie vor unbekannt. Rückschlüsse darauf lieferte die Transkriptionsanalyse in einer Kultur von auxotrophen Überproduzenten von *E.coli*, wodurch Veränderungen der Genexpression durch den Transfer von Aminosäuren identifiziert werden konnten. Die potentiellen Gene sind in die Regulation folgender Prozesse involviert: (i) Zelladhäsion (*ppdA*, *ydeQ*, *yhjH*, *dgcM*), (ii) Mobilität (*ycgR*) und (iii) Vesikelproduktion (*degP*, *nlpA*, *nlpI*). Durch Knockouts dieser Gene in auxotrophen Genotypen konnten anschließend signifikante Effekte auf das Wachstum in Anwesenheit eines Überproduzenten festgestellt werden. Außerdem konnte der Einfluss extrazellulärer Vesikel durch die Kombination verschiedener Techniken festgestellt werden. Diese Techniken umfassten Rasterelektronenmikroskopie, den Einsatz von Mutanten mit verschiedenen Ausmaßen an Vesikelproduktion, und die Supplementierung von Vesikeln zu Kulturen verschiedener auxotropher Genotypen und Produzenten von Aminosäure. Dadurch konnten die Vesikel als potentielle Bausteine zur Herstellung einer Verbindung zwischen auxotrophen und benachbarten Zellen identifiziert werden.

Zusammengefasst ermöglichen die Ergebnisse dieser Arbeit einen Einblick in mögliche Interaktionen innerhalb einer bakteriellen Gemeinschaft im Kontrast zu einer Population von autonom replizierenden Einzelzellen. Die Beobachtungen deuten darauf hin, dass auxotrophe Genotypen in der Lage sind entweder röhrenförmige Strukturen (Nanotubes), oder kettenähnliche Strukturen aus Vesikeln auszubilden, mit dem Zweck fehlende Ressourcen aus der näheren Umgebung zu akquirieren. Des Weiteren wurde gezeigt, dass interagierende Bakterien aufgrund des Austausches von Aminosäuren durch eine zytoplasmatische Brücke einen gekoppelten Metabolismus aufweisen können. Eine solche Arbeitsteilung hinsichtlich der Biosynthese von Aminosäuren resultiert schließlich in ein Erhöhung der Fitness. Daher könnte eine bakterielle Gemeinschaft als ein großes kohärentes metabolisches Netzwerk betrachtet werden, in welchem essentielle Biosynthesewege auf einzelne Mitglieder aufgeteilt wird und deren Produkte zwischen Zellen ausgetauscht werden.

Chapter 4: Overview of manuscripts

4.1 Manuscript I

Title: Metabolic cross-feeding via intercellular nanotubes among bacteria.

Authors: Shraddha Shitut (SS), Samay Pande (SP), Lisa Freund (LF), Martin Westermann (MW), Felix Bertels (FB), Claudia Colesie (CC), Ilka B. Bischofs (IB) and Christian Kost (CK).

Status: Published (Nature Communications, doi: 10.1038/ncomms7238, February 2015).

Summary: This study describes a mechanism of contact-dependent nutrient exchange between two bacterial species, *Escherichia coli* and *Acinetobacter baylyi*. These strains were genetically modified to cross-feed amino acids upon coculture in a shaken liquid medium not supplemented with external amino acids. A single loss-of-function mutation rendering the *E. coli* strain auxotrophic for a specific amino acid was sufficient to induce the formation of nanotubes. A combination of fitness experiments, fluorescent labeling, lipid staining and microscopy was used to identify the structures connecting cross-feeding genotypes. Transport of metabolites (amino acid) through nanotubes potentially avoids the loss of this metabolite to the environment and protects the metabolite from degradation or chemical modification.

Description	**Author contribution**
Conceived the project	SP (50%), CK (50%)
Designed the experiments	SS (30%), SP (35%), CK (35%)
Performed the experiments	SS (35%), SP (35%), LF (30%)
Fluorescence microscopy	SS (30%), SP (30%), IB (40%)
Electron microscopy (TEM, cryo-TEM, SEM)	SP (20%), CC (20%), MW (60%)
Construction of plasmids	FB (100%)
Data analysis	SS (20%), SP (40%), CK (40%)
Manuscript preparation	SS (20%), SP (20%), CK (60%)

4.2 Manuscript II

Title: Metabolic coupling in bacteria.

Authors: Shraddha Shitut (SS), Samay Pande (SP), Tobias Ahsendorf (TA), Matthew Egbert and Christian Kost (CK).

Status: Under revision (The ISME Journal, May 2017), preprint on biorxiv (doi:10.1101/114462, 2017).

Summary: Here the question of how cellular metabolism deals with the process of metabolite exchange between cross-feeding cells is answered. Single gene deletion mutants of *E. coli* were used that render them auxotrophic for amino acid (recipient) or producers of amino acid (donor). A coculture of the recipient and donor illustrated a contact-dependent exchange of amino acid between the two partners. Using a combination of, (i) cell internal amino acid sensors and (ii) transcriptional activity sensors, changes in the cytoplasm of both partners were quantified over time. The results from this study provide a biochemical explanation for the initiation and establishment of metabolite exchange between organisms in nature.

Description	**Author contribution**
Conceived the project	SS (20%), SP (20%), CK (60%)
Designed the experiments	SS (60%), CK (40%)
Performed the experiments	SS (100%)
Construction of plasmids	TA (30%)
Data analysis	SS (60%), CK (40%)
Manuscript preparation	SS (40%), SP (10%), CK (50%)

4.3 Manuscript III

Title: Transcriptional insights from metabolite cross-feeding bacteria

Authors: Shraddha Shitut (SS), Ramya Ganesan (RG), Muhammad Atiqur Rehman (MR), Martin Westermann (MW), Heiko Vogel (HV) and Christian Kost (CK).

Status: Under preparation (June 2017)

Summary: The transfer of nutrients between cells through nanotubes requires more than just an auxotrophy mutation. Several gene products and the coordination of different pathways potentially results in cross-feeding. This study shines light on the transcriptional changes arising in *E. coli* cross-feeding genotypes resulting from, (i) amino acid starvation and (ii) the exchange of cytoplasmic contents through nanotubes. A combination of gene expression analysis and targeted gene deletions in different genotypic backgrounds followed by growth quantification was used. The results of this study indicate the involvement of c-di-GMP signaling in the process of cross-feeding in *E. coli*. Furthermore the role of outer membrane vesicles as building blocks for nanotubes has been investigated.

Description	**Author contribution**
Conceived the project	SS (40%), CK (60%)
Designed the experiments	SS (80%), CK (20%)
Performed the experiments	SS (60%), RG (40%)
Bioinformatics analysis	SS (40%), MR (60%)
Electron microscopy	SS (20%), MW (80%)
Data analysis	SS (50%), CK (50%)
Manuscript preparation	SS (50%), CK (50%)

Chapter 5: General Introduction

Henry Osborn, the American paleontologist writes (in "The origin and evolution of life upon the earth") that "a bacteria-less ocean and a bacteria-less earth would be uninhabitable for either plants or animals" (Osborn 1916). He tries to emphasize here how bacterial growth, metabolism and activity prepared the water and earth for higher, dependent forms of life. Since the appearance of bacteria about 3.5 billion years ago, they have colonized a range of ecological niches on earth. Most notable is the presence of microbial life in extreme environments like hydrothermal vents, sulfur springs and the Antarctic glaciers. Bacteria are hence ubiquitous in the true sense (Karl 1995). Different environments on earth were characterized by differences in their biotic components (competing species, predators) and abiotic components (nutrients, temperature, viscosity, pH). Out of these abiotic and biotic components, the type of nutrients present (i.e. sources of carbon, nitrogen and energy) greatly influence the diversity of bacteria (Perry and Staley 1997, Rinke *et al.,* 2013, Vos *et al.,* 2013). As each bacterial species inhabits the environment, the cell generates chemical changes in its immediate surrounding (microenvironment) that get amplified in the larger connected ecosystem. Chemical changes are mainly introduced by nutrient cycling i.e. the uptake of complex organic matter for degradation, the breakdown and subsequent secretion of by-products of metabolism into the environment. It is these biochemical modifications resulting from bacterial activity that also made the oceans and land habitable for higher organisms. For example, cyanobacteria are thought to have played a role in the Great Oxidation Event (GOE) that resulted in increased oxygen levels in the atmosphere on account of oxygenic photosynthesis (Schirrmeister *et al.,* 2013). Higher organisms that grew in this pre-conditioned atmosphere were constantly faced with microorganisms and thus developed interactions with bacteria (Faust and Raes 2012, Little *et al.,* 2008, Srivastava and Srivastava 2003, Wintermute and Silver 2010). Interactions within microorganisms as well as between micro and macro-organisms have a significant impact on our ecosystem (section 5.1). Interactions within bacteria can be further characterized according to the effect of an interaction on the growth of the interacting partners (section 5.2), as well as by the process in which these interactions are carried out (section 5.3). As mentioned above major changes in the earth's atmosphere resulted from bacterial metabolic activity. In order to envisage the far-reaching effects of nutrient uptake and subsequent bacterial growth on the environment and the organisms in it, we need to understand the general metabolic network of bacteria (section 5.4).

5.1 Significance of microbial interactions

The biological activity of microbes often involves the production of building block molecules after degradation of organic matter. During metabolic processes, there is a release of chemical compounds like sugars, amino acids or gases into the surrounding space. Owing to the high abundance of bacteria in nature, the released compounds when considered

collectively, results in a change in the environment as well as the organisms inhabiting the environment (Fig. 1).

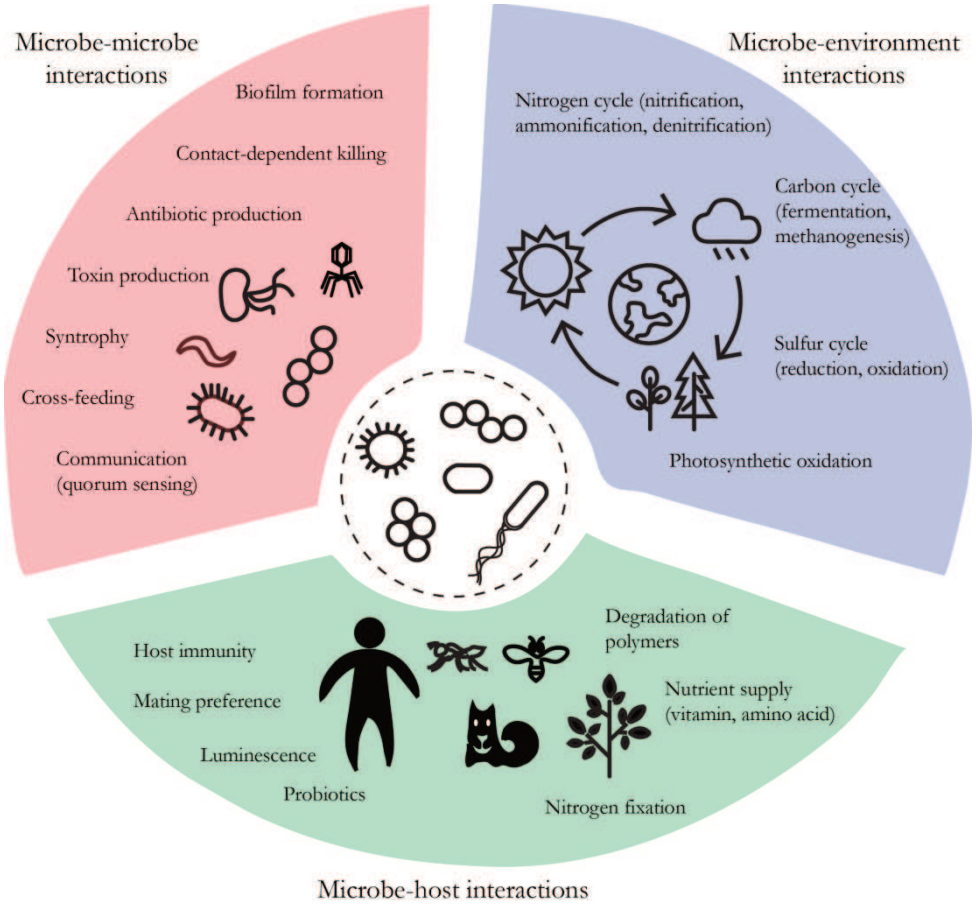

Figure 1: **Overview of interactions involving microbes in an ecosystem**. Microorganisms ensure cycling of intermediate compounds and gases of the biogeochemical cycles through metabolic activity (blue). Microorganisms in associations with plants, insects and animals mainly provide essential nutrients that are unattainable or not produced by higher organisms (green). Microorganisms within microbial communities depict interactions that promote or inhibit the growth of other members of the microbial community (red).

5.1.1 Microbes influence the biogeochemical cycles

Microorganisms are often considered as the biogeochemical engineers of life on earth (Falkowski *et al.,* 2008). The growth of bacteria requires breakdown of complex organic matter to intermediate compounds that serve as building blocks like amino acid, nucleotides, lipids and fatty acids for increasing cell biomass. During the degradation of complex compounds and subsequent growth, various by-products are released into the surrounding. By-products like methane, nitric oxide, carbon dioxide and hydrogen sulfide, then re-enter the respective elemental cycle (carbon, nitrogen or sulfur cycle). The gaseous forms of the basic elements are then fixed by microorganisms and made available to plants and animals

ultimately increasing organic matter. Microbes are hence important catalysts in the functioning of the carbon, nitrogen and sulfur cycles (Fig. 1).

Interestingly, all biochemical transformations are carried out by a coordinated metabolism of multiple bacteria. Thus, the energy and nutrient-cycling in the environment are basically being driven by communities or consortia of microorganisms (Morris *et al.,* 2013, Paerl and Pinckney 1996). For instance in organic matter decay, fermentation and methanogenesis are two processes working in tandem. Primary fermenters break down carbohydrates into simple sugars, acetate, hydrogen and CO_2. These fermentation products are further converted to methane and CO_2 by methanogens, which are anaerobic archaebacteria (McInerney *et al.,* 2008). The entire process is possible due to an exchange of carbon compounds between two different groups of microorganisms with different growth requirements. Such an association is often called a syntrophy and similar interactions can be observed in the nitrogen cycle (Galloway 1998), the oxygen cycle (Dismukes *et al.,* 2001), and the sulfur cycles (Fenchel and Blackburn 1979) as well. The absence of such syntrophic consortia in the environment would lead to accumulation of intermediates (methane, CO_2, ammonia and hydrogen sulfide), generated as a result of primary fermentation. The atmosphere would consequently become toxic and unfavorable for the survival of higher organisms without a coordinated microbial activity.

5.1.2 Microbes support growth of higher organisms in the ecosystem

Microbes have preceded animals and plants in the time line of earth's evolutionary history (Battistuzzi *et al.,* 2004, Margulis 1981). All higher organisms have evolved in the presence of microbes and have encountered them in every ecosystem. The plant rhizosphere that consists of plant roots, associated mycorrhizal fungi and bacteria, is estimated to harbor about 10^9 microbial cells per gram of root (Egamberdieva *et al.,* 2008). Bacteria in a rhizosphere referred to as plant growth promoting rhizobacteria (PGPR), are either associated directly with the plant root or with mycorrhizal fungi. These microbes provide, (i) nutrients (like fixed nitrogen and phosphorous) or (ii) phytohormones to the plant (Bais *et al.,* 2006, Van Der Heijden *et al.,* 2008, Xie *et al.,* 1996), (iii) they prevent the colonization of plant root by pathogenic bacteria (Doornbos *et al.,* 2012) and (iv) modulate plant immunity (Van der Ent *et al.,* 2009). *Pseudomonas putida* for instance is known to produce the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase. The ACC deaminase converts ACC to ammonia and alpha-ketobutyrate thus preventing the plant cells from converting ACC to ethylene. An absence of ethylene in the plant root leads to increased root growth and nodulation (Glick *et al.,* 1998). Higher nodulation allows for increased colonization of the mycorrhizal fungi which results in improved plant growth.

Microbes are also closely associated with insects and animals, both outside and inside the body. The fungus farming *Attine* ants are known for cultivating specific strains of fungi that are used as a food source for growing larvae (Cherrett *et al.,* 1989, Mueller *et al.,* 1998). The queen also transports a pellet of the fungus as a seed for farming at the new location thus helping to maintain a constant food supply. On the other hand, the plant-based diet of many

insects which is composed of complex polymers (cellulose, hemicellulose, lignin, pectin) cannot be digested by the insect due to absence of enzymes for degradation. This polymer-based diet is also nutritionally imbalanced due to the absence of essential amino acids, vitamins and co-factors. Symbiotic microbes present within the host assist in digesting the polymers as well as supplementing nutrients like vitamins and amino acids (Douglas 2009, Engel and Moran 2013, Flint *et al.,* 2012, Van Soest 1994). Symbionts are also known to detoxify compounds produced by a plant upon a herbivore attack resulting in protection of the insect-host (Clay 2014, Hammer and Bowers 2015). Unique characteristics of assisted growth and survival imparted by symbionts have led to them being considered as drivers for the insect host to adapt to new ecological niches (Farrell and Mitter 1994, Moran 2007, Sudakaran *et al.,* 2017).

5.1.3 Microbes support growth of other microbes in the community

It is well known that only 1-2% of bacteria on earth can be cultured as isolates in vitro (Curtis and Sloan 2004, Hugenholtz *et al.,* 1998, Nichols 2007). There is a significant difference in microscopic cell counts versus colony counts on artificial media. This phenomenon of lower cell counts on the plate is called the great plate count anomaly (Staley and Konopka 1985). The reasons hypothesized for this anomaly are largely influenced by abiotic and biotic factors (Vartoukian *et al.,* 2010). Abiotic factors include insufficient growth conditions in the lab especially while growing fastidious microbes that require specific pH, temperature, oxygen levels, long incubation times and specific nutrient concentrations (Pham and Kim 2012, Stewart 2012). The biotic components, on the other hand, include support of microbial growth through interactions with other microorganisms.

Microbial interactions within a community, in the context of unculturable natural isolates, can be of two types based on the role of the neighbor: (i) removal of harmful by-products or (ii) removal of electrons from the system. Removal of harmful by-products by another cell in the surrounding results in maintaining sub-inhibitory concentration of the compound for the producer cell. For instance, the co-enrichment of *Hyphomicrobium* sp. with methanotrophs is based on the removal of methanol by *Hyphomicrobium* sp. (Moore 1981) Methanotrophic bacteria oxidize methane to methanol which when present at high concentrations inhibits the growth of methanotrophs in monoculture. However, when grown in coculture with *Hyphomicrobium* sp. the growth constraint on the methanotroph is relieved. Alternatively, neighboring cells could remove certain electrons from the system like in the classic case of ethanol metabolism by a mixed culture of "the S organism" and *Methanobacterium bryantii* (Bryant *et al.,* 1967). Ethanol is fermented to acetate and hydrogen in the primary step, released hydrogen is utilized by *M. bryantii* to convert CO_2 to methane. The removal of hydrogen by *M. bryantii* maintains it in low concentrations which otherwise would lead to end-product inhibition of ethanol degradation by the primary fermenter. Testing growth of different species in co-cultures or mixed cultures as in the above examples have revealed a lot about the chemical exchanges and communication taking place between microorganisms in nature (Garcia *et al.,* 2015, Kato *et al.,* 2008, Morris *et al.,* 2013, Vartoukian *et al.,* 2010).

Metabolic activity of microbes has an impact on different levels of the ecosystem i.e. within microbial communities, with higher organisms and with the environment. Microbial interactions are essential to our ecosystem and so is the need to study them.

5.2 Types of microbial interactions

In their natural habitat, microorganisms are present as members of a community. Within these communities, a number of interactions are taking place predominantly through an exchange of molecules or chemical signals. Microorganisms show associations, both transient and permanent, with other members based on the exchanged chemical signal. Microbial associations are often compared to human interactions that are observed in the social context of a complex society. Moreover, as microorganisms have been increasingly studied under the lens of sociobiology and evolutionary biology (Crespi 2001, Tarnita 2017, West *et al.,* 2007), their interactions have been explained using anthropomorphic terms like partner choice, policing, altruism and so on (Davies 2010). However, there are disagreements among biologists regarding the use of these terms in categorizing the types of microbial interactions.

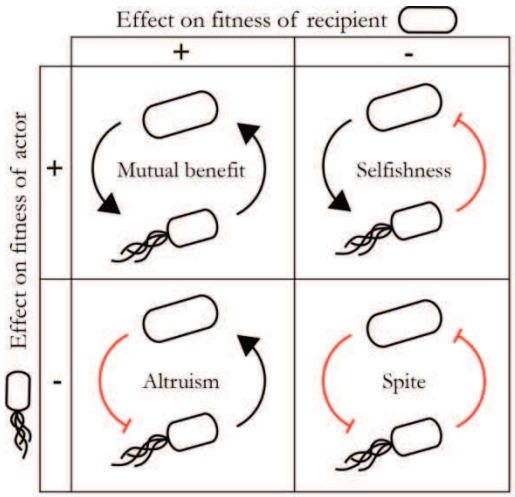

Figure 2: Characterization of bacterial interactions based on effect on the fitness of involved individuals. Consider two types of cells, one that carries out a function (actor) and the other that is affected by this function (recipient). (+/+) the actor and recipient both provide beneficial functions to each other which have a positive effect on fitness, referred to as mutual benefit or cooperation. (-/+) the actor provides a beneficial function to the recipient which is costly for the actor, referred to as indirect benefits, cooperation or altruism. (+/-) the actor provides a function that is harmful to the recipient but beneficial to the actor, known as selfishness. (-/-) both actor and recipient carry out functions that negatively affect the other, referred to as spite.

A simplified method for defining microbial interactions was developed based on the effect of an interaction on the growth of interacting partners (Hamilton 1964). According to Hamilton, all interactions can be classified through this method to give two main categories of interactions: positive and negative (Fig. 2).

5.2.1 Negative interactions

When the growth of either one or both partners is negatively affected by the interaction it is termed a negative or competitive interaction (Fig. 2, -/+ and -/-). Competition between different species is usually for resources and space. In cases where different species present in the community utilize different substrates as their food source then both species grow equally well. However, microorganisms universally depend on an external supply of inorganic nutrients like nitrogen (N) and phosphorous (P) (Smith 2002). Evidence shows a reduced

growth and activity of microorganisms in marine and terrestrial ecosystems due to limited N and P levels (Hobbie and Vitousek 2000, Rivkin and Anderson 1997). Microorganisms also compete for space in a given environment. This phenomenon is most often observed in the context of bacterial biofilms wherein the initial attachment of a species can influence successive adhesion events. For instance, in the gut of humans and animals, a few *Lactobacillus* species have been found to produce a protein after attaching to the intestinal wall (Bernet *et al.,* 1994, Todoriki *et al.,* 2001). This protein further prevents pathogenic strains from attaching to the gut surface (Bernet *et al.,* 1994, Todoriki *et al.,* 2001).

In order to succeed in such competitive environments microorganisms often produce antimicrobial compounds or toxins. These compounds deter the growth of neighboring strains resulting in more resources and space for the focal species. Some soil-dwelling *Burkholderia* sp. are known to enhance antibiotic production in the presence of quorum sensing systems, thus resisting invasion by other species (Duerkop *et al.,* 2009). Competing strains may alternatively employ a contact-dependent killing protein (type I-VI secretion systems (TSS)) and toxin-antitoxin system (*mazE/mazF* genes, *pem/parD* loci) for eliminating other genotypes not producing the same killing protein or the anti-toxin (Basler *et al.,* 2013, Russell *et al.,* 2014). Such strategies of reducing the fitness[5] of specific individuals are also known to help maintain diversity in certain environments (Foster and Bell 2012, Hibbing *et al.,* 2010, Kerr *et al.,* 2002, Leinweber *et al.,* 2017). Negative interactions are also drivers of species diversity microbial communities.

5.2.2 Positive interactions

When either one or both of the interacting partners invest their own resources to benefit the other, it is termed as a positive or cooperative type of interaction. The positive in this case refers to the effect on the fitness of the recipient and the actor (Fig. 2, +/- and +/+). Positive interactions can be further divided as illustrated (Fig. 2). Examples of the +/- consequence are termed as altruistic, cooperative or indirect benefits type of interaction. Examples include the production of costly metabolites or enzymes as public goods like siderophores or extracellular enzymes. Siderophores are produced by a number of bacteria, *E. coli* (enterobactin), *Vibrio cholerae* (vibiorbactin), *Pseudomonas aeruginosa* (pyoverdin and pyochelin), *Acinetobacter calcoaceticus* (acinetobactin) (Buckling *et al.,* 2007, Crosa and Walsh 2002, Diggle *et al.,* 2007, Griffin *et al.,* 2004, Krewulak and Vogel 2008, Visca *et al.,* 2007, West and Buckling 2003). These small, iron-chelating compounds are released into the environment in response to iron-limiting conditions (Ratledge and Dover 2000). The iron-bound siderophores are available to all cells for uptake in the neighborhood. Similarly, an extracellular enzyme like invertase produced by *Saccharomyces cerevisiae* for the breakdown of sucrose to glucose and fructose is considered an altruistic act and has been used as a model system to study the evolution of cooperation (Gore *et al.,* 2009, Greig and Travisano 2004).

The second category of positive interactions is the two-way or mutually beneficial interaction (Fig. 2, +/+). Here both actor and recipient benefit positively from the interaction referred to as cooperation or mutualism. A classic example is that of the wrinkly spreader phenotype

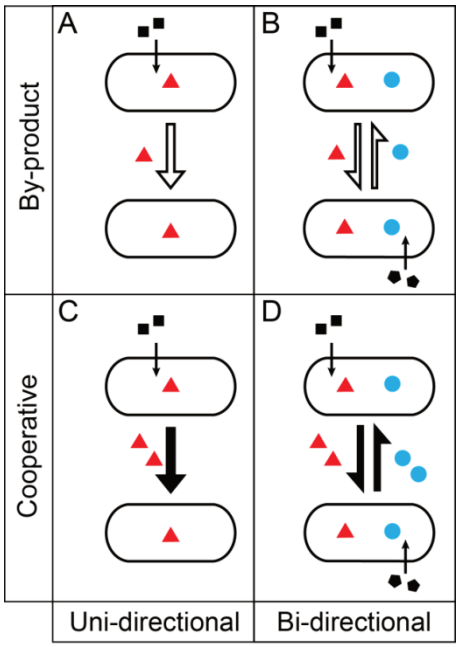

Figure 3: Types of cross-feeding interactions. Characterized first, on the basis of the direction of exchange (A,C vs B,D) and then by the cost of exchange (A,B vs C,D). (A) Unidirectional by-product cross-feeding - one partner benefits by taking up a molecule/metabolite the other does not need. (B) Bi-directional by-product cross-feeding - both partners exchange molecules that are not required by the individual partner. (C) Unidirectional cooperative cross-feeding - one partner bears a cost for producing a molecule/metabolite for the other. (D) Bi-directional cooperative cross-feeding - both partners produce and exchange costly molecules/metabolites required for growth.

observed in *Pseudomonas fluorescens* during experimental evolution. When an ancestral genotype of *P. fluorescens* is propagated in a spatially structured environment (stable liquid media), there is rapid diversification in which the wrinkly spreader is a dominant phenotype (Rainey and Rainey 2003). This wrinkly spreader mutant produces a higher amount of cellulosic polymer which is expressed at the cell poles (Spiers *et al.,* 2002). When neighboring cells both produce this extracellular polymer it promotes cell adhesion and consequently the formation of a mat of cells at the liquid-air interface. By growing in the form of a mat/biofilm the population as a whole benefits from increased access to oxygen, despite the cost to individual cells in producing increased amounts of the polymer.

5.2.2.1 Metabolic cross-feeding

Cross-feeding is an interaction where the product of one cell benefits another and is considered a type of positive interaction (Belenguer *et al.,* 2006, Estrela *et al.,* 2012, Harcombe 2010, Reinheimer 1921, Seth and Taga 2014). The exchanged products usually found in cross-feeding communities are metabolites, co-factors, ions, reduced and oxidized compounds (Mee *et al.,* 2014, Morris *et al.,* 2013, Phelan *et al.,* 2012, Schink 2002, Seth and Taga 2014). Cross-feeding interactions can be categorized into by-product and cooperative (based on the cost to the interacting partners) or uni- and bidirectional (based on the transfer of metabolites) (Fig. 3). By-product cross-feeding (Fig. 3A-B), is the release of intermediates from a cell due to overflow metabolism or membrane permeability. The cell secreting the by-product does not invest additional resources to produce increasing amounts of this by-product for another individual. This type of cross-feeding was observed during the evolution of polymorphic phenotypes in batch cultures of *E.coli* utilizing glucose as a carbon source (Pfeiffer *et al.,* 2004, Treves *et al.,* 1998). The initial flux of glucose into the tricarboxylic acid cycle resulted in increased production and release of acetate into the medium. Acetate, the by-product, was then utilized as a substrate by another cell giving rise to polymorphism as a result of cross-feeding.

Alternatively, in the case of cooperative cross-feeding (Fig. 3B-D), a cell either produces a metabolite only in presence of the partner or increases production of a metabolite in presence of the partner. In either condition the producing cell faces a cost for the production of this metabolite and this cost may affect its growth. Cooperatively cross-feeding strains

were experimentally evolved in a consortium of *Salmonella enterica* ser. Typhimurium and *E. coli* (Harcombe 2010). *S. enterica* provided methionine for *E. coli* during co-culture prior to the evolution experiment due to a mutational change in *S. enterica*. Interestingly within a few generations of co-evolving *S. enterica* and *E. coli*, the methionine production by *S. enterica* significantly increased and was observed only in the presence of the *E. coli* partner strain. The above examples of by-product and cooperative cross-feeding underline the fact that cross-feeding interactions can easily evolve in laboratory conditions. The study of cross-feeding interactions is hence of significance in the context of the evolution of microbial communities.

In conclusion, we see that all types of interactions are observed in microbial populations. Positive interactions that promote the growth of either one or both interacting partners also include cross-feeding of metabolites between bacteria. It is intriguing what mechanisms are developed and used by bacteria for the exchange of such essential and costly metabolites.

5.3 Mechanisms of metabolic cross-feeding

Cross-feeding interactions entail a transfer of a molecule or compound between interacting individuals. The nature of this transfer between individuals can be influenced by various factors like the physical and chemical characteristics of the molecule (size, stability, sensitivity) along with the environment in which the interaction takes place. The mechanisms for exchange of metabolites between bacteria can be broadly divided into two classes: (i) contact-independent and (ii) contact- dependent mechanisms.

5.3.1 Contact-independent mechanisms

Mechanisms of molecule transfer that are devoid of any physical connections between cells are denoted as contact-independent mechanisms. This is especially true for cases in which the molecule is small and freely diffusible through the cell membrane like formate or hydrogen ions, when an environmentally-mediated exchange between cells is possible (Fig. 4a). Mixed cultures of *Syntrophomonas wolfei* and *Methanobacterium formicium* depict exchange of hydrogen and formate ions through diffusion in anaerobic digestors (Boone *et al.,* 1989). Hydrogen is said to be at the "heart of syntrophy" and hence passive diffusion of this molecule during cross-feeding is a common phenomenon in nature. Apart from the size, the chemical property of the molecules also affects its mode of transfer, for example, its hydrophobic or hydrophilic nature. The bacterial cytoplasmic membrane is a phospholipid bilayer that consists of hydrophilic head groups on the outside and hydrophilic tail regions on the inside. The arrangement of the amphpathic phospholipids makes it permeable only to hydrophobic molecules. The well-studied quorum sensing molecule, acyl homoserine lactones (AHL) in most forms is amphipathic and hence can easily diffuse through membranes of gram-negative cells (Fuqua *et al.,* 2001). Molecules may also be actively transported from the cell cytoplasm to the external environment via membrane spanning

transporters. Corrinoids, which are pyrrole ring based cyclic compounds, are often exchanged via active transport (Fig. 4b) due to their large size (Degnan *et al.,* 2014, Seth and Taga 2014). Vitamin B12 is such a corrinoid that requires active transport during cross-feeding within the gut microbial community.

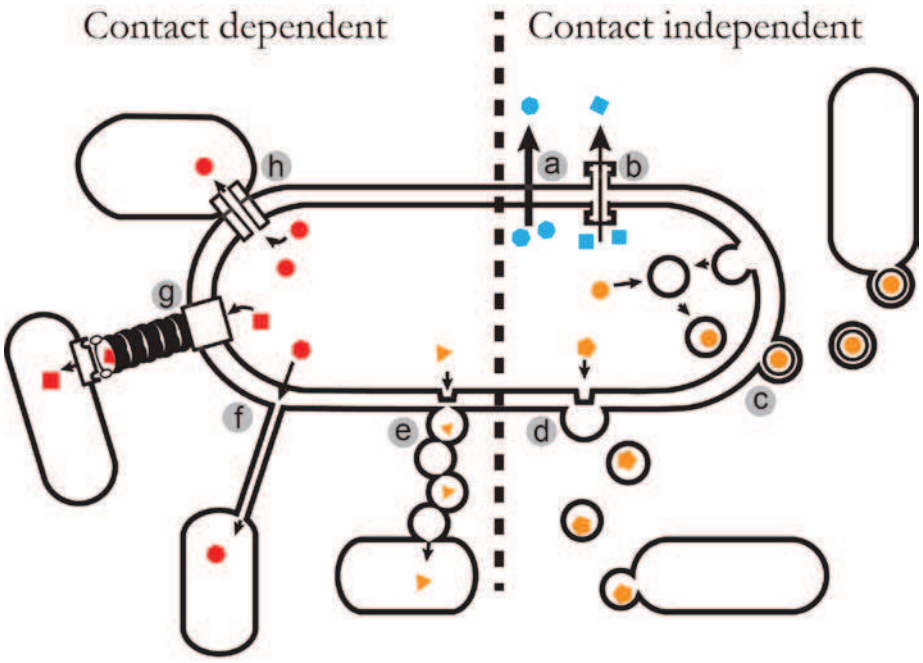

Figure 4: Mechanisms for cross-feeding. Contact independent (a to d) and contact dependent (e to h) processes of cross-feeding in bacteria. (a) Diffusion of molecules across the membrane. (b) Active transport of molecules via transporters embedded in the membrane. (c) Outer-inner membrane (O-IMV) vesicle mediated transport. (d) Outer membrane vesicles (OMV) mediated transport. (e) Outer membrane vesicle chain formed by linking of multiple vesicles. (f) Nanotube-mediated exchange. (g) Type secretion systems or flagella-like-filaments. (h) Direct membrane contact between cells.

Contact-independent methods also include the exchange of metabolites via membrane vesicles (Fig. 4c-d). In this case, the cell membrane extends to form a bleb, into which cytoplasmic molecules may be transported followed by a detachment from the cell. The packaged material within this membrane vesicle remains unexposed to the environment and in a concentrated form. This method is employed by many marine microorganisms like *Prochlorococcus*, which produce vesicles that support the growth of other marine isolates like *Altermonas* and *Halomonas* (Biller *et al.,* 2014, Johnson *et al.,* 2006). Depending on the cell structure and requirement, the vesicle may be formed from the outer membrane alone (outer membrane vesicles, Fig. 4c) or from both the outer and inner membranes (outer-inner membrane vesicles, Fig. 4d). Recently outer-inner membrane vesicles (O-IMVs) were identified in multiple species- *Shewanella vesiculosa*, *Pseudomonas aeruginosa*, *Neisseria gonorrheae* and *Acinetobacter baumanii* (Pérez-Cruz *et al.,* 2015). The O-IMVs are hypothesized to play a role in the transport of DNA and toxins however the exact mechanism is unclear.

5.3.1.1 Disadvantages of contact-independent mechanisms of exchange

An exchange of molecules mediated externally as described above can have some disadvantages. In the case of passive diffusion and active transport, the metabolite is transmitted from the internal to the external environment of the cell. Once present in the extracellular space, the metabolite can undergo chemical modification or degradation which may render it ineffective for uptake by another cell (Boyer and Wisniewski-Dyé 2009). N-acyl-homoserine lactone (AHL), the auto-inducer molecule produced by bacteria during quorum sensing, is vulnerable to bond cleavage which causes the lactone ring to open at high temperature and alkaline pH (Decho *et al.,* 2009, Yates *et al.,* 2002). Alternatively, the metabolite may be carried away by movement of the surrounding liquid through a process called advection (Purevdorj *et al.,* 2002). *P. aeruginosa* biofilms show a rapid loss of AHL signal at the biofilm surface as compared to the internal region.

5.3.2 Contact-dependent mechanisms

An exchanged molecule may also be transported between interacting cells only after a physical connection is established between the cells. Such mechanisms are classified as contact-dependent mechanisms and can include either a direct cell-cell connection or linking of cells through membrane-derived structures. The former i.e. direct cell-cell contact (Fig. 4h) is seen in the syntrophic consortium, *Chrolochromatium aggregatum*, which consists of several motile, green sulfur bacteria surrounding a central, non-motile, chemotrophic bacterium. The two partners are always found associated within a cluster in which the chemotroph transfers sulfide to the peripheral sulfur bacteria (Wanner *et al.,* 2008). The transfer of sulfide and 2-oxoglutarate is hypothesized to take place through transporters in the membranes of either species. *Myxococcus xanthus* on the other hand produces a distinct extracellular structure called a membrane vesicle chain (Fig. 4e). These vesicle chains that connect neighboring cells contain sugars, lipids and proteins that promote cell-cell communication (Remis *et al.,* 2014). Owing to advancement in electron microscopy techniques (cryo-TEM, HR-SEM), there have been reports of novel structures found connecting bacterial cells. Nanotubes were found to transport plasmid DNA from a cell harboring the plasmid to a cell devoid of this plasmid in *B. subtilis* (Fig. 4f) (Dubey and Ben-Yehuda). *Shewanella oneidensis* was reported to produce nanowires in order to increase the surface area available for electron uptake in a limiting environment (Gorby *et al.,* 2006). The possible role of these novel structures (nanotubes, nanowires, vesicle chains) in the establishing contact between cross-feeding cells unclear. Moreover, the dynamics of metabolite exchange assisted by extracellular structures is also unknown.

Contact-dependent delivery systems are widely observed in the case of antagonistic interactions, which entail killing of specific bacterial cells (Fig. 4g). A well-studied category of contact-dependent killing in bacteria is the type secretion system (TSS), which has subgroups of seven different toxin delivery mechanisms (Hayes *et al.,* 2010). All seven types of toxin delivery systems are present on the cell surface. The cell-associated structures in TSS have been found to resemble different bacterial appendages; the type III secretion system

resembles flagella, the type IV secretion system resembles surface adhesins or pili, the type V secretion system is a membrane receptor complex, the type VI secretion system is similar to a phage infection system. All of these systems have three modules: (i) a base connector on the actor cell, (ii) a secretion channel, and (iii) a receptor on the target/recipient cell. The effector molecule is produced in the actor and passed through the channel to be transported into the target cell for subsequent activity. The possible role of the type secretion system in the exchange of other molecules (apart from toxins) is however unexplored.

In conclusion, bacteria have devised a myriad of ways to exchange molecules during interactions between each other. The method of exchange depends on the characteristic of the molecule as well as the interacting species. Due to the predominantly greater interest in studying competitive interactions/negative interactions within bacterial communities, the knowledge about contact-dependent exchange in the context of nutrient cross-feeding is unclear. In this thesis, I present a study of nanotube-mediated cross-feeding of amino acids in bacteria to provide insights on the genetic and metabolic requisites in a cell for carrying out contact-dependent cross-feeding.

5.4 Bacterial metabolic network

A bacterial cell growth involves using substrates from the environment to synthesize building block molecules (Fig. 5). The reactions taking place in a bacterial cell for the above purpose can be broadly classified into catabolic and anabolic pathways. Catabolic reactions refer to the degradation of complex organic matter into metabolically useful precursor metabolites like glucose 6-phosphate, phosphoenolpyruvate, acetyl CoA, pyruvate, succinyl-CoA and oxaloacetate (Fig. 5a - b). These precursor metabolites serve as the raw material for synthesis of all building blocks (amino acids, nucleotides, lipids, lipopolysaccharides, peptidoglycans, glycogen and polyamines) through anabolic reactions (Fig. 5c – d). Amino acids serve as the most abundant of these building blocks due their involvement in the formation of proteins and enzymes.

5.4.1 Amino acid biosynthesis and regulation

Biosynthesis of amino acids takes place through a series of reactions either connected in a sequential cascade or interconnected branched pathways. The precursor metabolites for all the 20 amino acids produced by bacteria are obtained through the tricarboxylic acid cycle (TCA) cycle (Fig. 5b) (Boyle 2005). These metabolites are then chemically transformed during amino acid biosynthesis resulting in free amino acid which is further processed for mRNA translation. Amino acids being one of the most abundant and highly produced of the building block molecules (Milo and Phillips 2015); amino acids have been optimized for efficient production by bacteria. Amino acid biosynthesis is hence subject to regulation through feedback inhibition pathways (Fig. 5d). Feedback for the production of a given amino acid or a group of amino acids is often mediated by the end product of the pathway which in this case is the amino acid itself (Umbarger 1978). For instance, consider the last step for synthesis of serine which is catalyzed by the 3-phosphoglycerate dehydrogenase

enzyme. High levels of serine in the cell cytoplasm lead to the binding of four serine residues to 3-phosphoglycerate dehydrogenase, thus inactivating the enzyme (Gottschalk 2012).

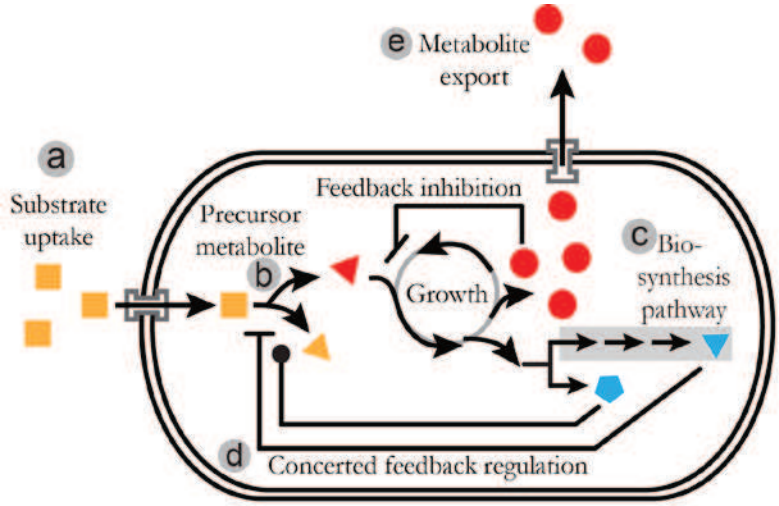

Figure 5: The bacterial metabolic network. (a) Organic matter may be degraded externally or after uptake in the cytoplasm. (b) Precursor metabolites are formed as a result of glycolysis and enter the tricarboxylic acid cycle. (c) A biosynthetic pathway for individual building blocks (amino acids, nucleotides, lipids, lipopolysaccharides) is a cascade of reactions. The result of a biosynthetic pathway is the end-product which is a metabolite that can be incorporated into cell material for growth. (d) End products can regulate the pathway by feedback inhibition. There are different types of feedback inhibition (isofunctional enzymes, cumulative feedback inhibition, sequential feedback inhibition, inhibition plus activation) depending on the branching and interconnectedness of individual pathways. (e) Cytoplasmic cell material may also be exported into the environment due to leaky membranes or through dedicated membrane transporters.

Regulatory mechanisms get complicated with the branching of biosynthetic pathways which result in groups of amino acids as end products (Fig. 5d). Consider the production of leucine, isoleucine and valine from hydroxyethyl thiamine pyrophosphate (hydroxyethyl-TPP) (Gottschalk 2012, Umbarger 1978). When hydroxyethyl-TPP reacts with α-ketobutyrate, the reaction consequently leads to the formation of isoleucine and hence is also inhibited by isoleucine. Alternatively, when hydroxyethyl-TPP reacts with pyruvate, the subsequent steps result in the formation of valine and leucine. The two reactions (hydroxyethyl-TPP + α-ketobutyrate versus hydroxyethyl-TPP + pyruvate) are competing branches in a common pathway. Interestingly valine can allosterically activate the enzyme that catalyzes the conversion of hydroxyethyl-TPP and α-ketobutyrate to isoleucine. In this way, a balance is maintained between the amounts of amino acids being synthesized from common precursor metabolites. Branched amino acid pathways also show a cumulative inhibitory effect by the end product amino acids. For instance, glutamine metabolism feeds in precursors to a number of amino acid biosynthesis pathways like tryptophan, histidine, alanine and glycine. The enzyme glutamine synthetase that catalyzes the conversion of glutamate, ammonium and ATP to glutamine, depicts reduced activity upon concerted binding of each of these amino acids (histidine, tryptophan, alanine and glycine).

5.4.2 Effect of the external environment on bacterial metabolism

Precursor metabolites as described in the previous section play a pivotal role in the biosynthesis of amino acids. In nature, these precursor metabolites are often the first products of organic matter decomposition that enter the TCA cycle. The levels of metabolites like pyruvate, glucose 6-phosphate, oxaloacetate, acetyl-CoA, control the flux of high energy molecules (ATP) and reducing agents (NADH or NADPH) through individual amino acid biosynthetic pathways. By calculating the number of ATP molecules and NADH, NADPH molecules involved in a given pathway, one can estimate biosynthetic costs of amino acids (Chubukov *et al.,* 2014, Varma and Palsson 1994). Furthermore, an estimate of amino acid biosynthetic cost obtained through flux-balance models predicts that when costly amino acids are available in the environment, bacteria prefer to take up the amino acid and shut down internal biosynthesis (Coffin 1989, Morris). This can be observed by providing ^{14}C-labeled carbon source (glycerol, fructose, glucose) along with an unlabeled amino acid to a cell (Neidhardt *et al.,* 1990). The uptake and processing of the labeled carbon should result in the ^{14}C-label being passed on to the amino acids synthesized through anabolic pathways. It was observed that all amino acids except histidine carried the ^{14}C-label since histidine was available in the non-labeled form in the medium and was taken up by the wildtype cell. This finding also indicates that when the amino acid was externally available, the cell shuts down the flux of labeled precursor metabolite through that specific amino acid biosynthesis pathway. Bacterial cells thus preferentially take up amino acids available in the environment to save production cost. Indeed natural samples depict the presence of auxotrophic bacteria which require an external supply of amino acids or vitamins or co-factors for growth.

5.4.3 Bacterial metabolism in the context of a microbial community

In their natural habitat, bacteria are faced with varying concentrations of organic matter or precursor metabolites. Different bacterial genotypes will degrade a preferred substrate. Depending on which substrate and precursor metabolites enter the TCA cycle the flux through amino acid biosynthesis pathways will be determined. Hence bacterial cells demonstrate distinct amino acid profiles in a given environment. Consider three aspects; (i) bacterial membranes are leaky i.e. they are permeable to certain molecules (Kallus *et al.,* 2016, Nikaido 2003), (ii) bacteria produce extracellular enzymes that degrade complex organic matter in the environment (Arnosti 2011) and (iii) all cells upon death result in lysis and a release of internal components (Bayles 2007, Lewis 2000). Taken together these three events would lead to the presence of building blocks (amino acids, vitamins, nucleotides, lipids) in a given environment. The availability of metabolites in the surrounding results in the saving of biosynthetic cost (section 1.5.2). This hypothesis of cells taking advantage of "free functions" which are the externally available nutrients and increasing fitness is referred to as the Black Queen hypothesis (Morris *et al.,* 2012) (Box 1).

Box 1: Black queen hypothesis

Microscopic parasites and endosymbionts are always present in the context of a host from which nutrients are derived. Interestingly these parasites and endosymbionts also depict highly reduced genomes in comparison to their free-living species (D'Souza *et al.,* 2014, McCutcheon and Moran 2012). The Black Queen hypothesis (BQH) provides a mechanistic explanation for this gene loss (Morris *et al.,* 2012, Morris). This hypothesis is based on the game of Hearts in which the aim is to score as few points as possible. The queen of spades (the black queen), is worth the highest points and hence is preferred to be lost by individual players. The hypothesis in context of microbial communities, predicts that when a costly function (e.g. amino acid production) is freely available in the environment (e.g. inside the host gut) then the loss of biosynthetic gene for the free function will be favored at the cell level. This gene loss is selected for only when fitness advantage upon gene loss outweighs the cost. This means that by deleting a gene, all the related metabolic and replication cost is saved which results in the fitness advantage should be higher than the biosynthetic cost of that free function. Apart from endosymbionts and parasites, one can also apply this hypothesis to microbial communities wherein the leakiness of cell membranes along with cell death results in the availability of free functions in the environment. Here biosynthetic gene loss would be favored when the lost function is provided either by the environment or other members of the community. Over time as selection acts on a given community, we see a presence of dependent members (beneficiaries) and autonomous "leaky" members (helpers). This type of adaptive gene loss has been observed in evolution experiments with *E. coli* indicating the ease with which it can happen in natural environments. The BQH has been discussed in the context of marine microbial communities that contain *Prochlorococcus* (Kettler *et al.,* 2007) which are sensitive to hydrogen peroxide (H_2O_2). Photo-oxidation of organic carbon in ocean water results in the formation of H_2O_2 which upon accumulation can lead to the death of *Procholorcoccus* due to its inability to synthesize catalase-peroxidase. Due to presence of other members in the community that act as a sink for H_2O_2 by the action of intracellular catalase-peroxidase, the level of H_2O_2 is maintained at sub-inhibitory level for *Prochlorococcus*.

It has been postulated that over evolutionary time, as a bacterium in a microbial community is provided with an external supply of nutrients, there is a selection for auxotrophic genotypes (Giovannoni *et al.,* 2014, McCutcheon and Moran 2012, Morris *et al.,* 2012). By losing the genes for biosynthesis of the metabolites freely available, the cell saves not only metabolic cost but also gene replication cost. The uptake of amino acids from the environment alone gives a fitness benefit of around 20% to *E. coli* auxotrophs in competition with the wildtype (D'Souza *et al.,* 2014). Hence the uptake of nutrients is a highly beneficial characteristic for individual cells in a community. Can bacterial cells employ different mechanisms to carry out nutrient uptake assuming the fitness benefit of such an interaction? Can the nutrient uptake by such auxotrophs affect the neighboring cells in the community? In this thesis, I present a mechanism of nutrient uptake by auxotrophs from neighboring cells in the environment. Furthermore, I provide a metabolic basis for the functioning of such one-way cross-feeding interactions.

Chapter 6: Aim of the thesis

Microbial interactions have a significant impact on the ecosystem functioning as well as the members of the ecosystem. These interactions between microbes can have both positive and negative effects on the growth of the interacting cells. Metabolite cross-feeding is one such essential interaction that entails the transfer of nutrients from one cell to another. Studying the exchange of metabolites in natural populations is challenging due to fastidious growth requirements of organisms, limited genetic manipulation of genotypes and limited knowledge and control over the genetic background of organisms. Known bacterial model systems on the other hand can be genetically modified to exchange desired metabolites. The nutrient biosynthesis pathways are universal in most bacterial cells hence allowing a close replication of the metabolic exchanges in natural isolates. Taking this into consideration I employed a system comprising of *Escherichia coli* and *Acinetobacter baylyi* cross-feeders to study the transfer of amino acids between cells. A cross-feeder genotype consists of two mutations: (i) loss of amino acid biosynthesis (auxotrophy) and (ii) increased levels of amino acids compared to the wildtype (over-production). The following aspects of this interaction were studied-

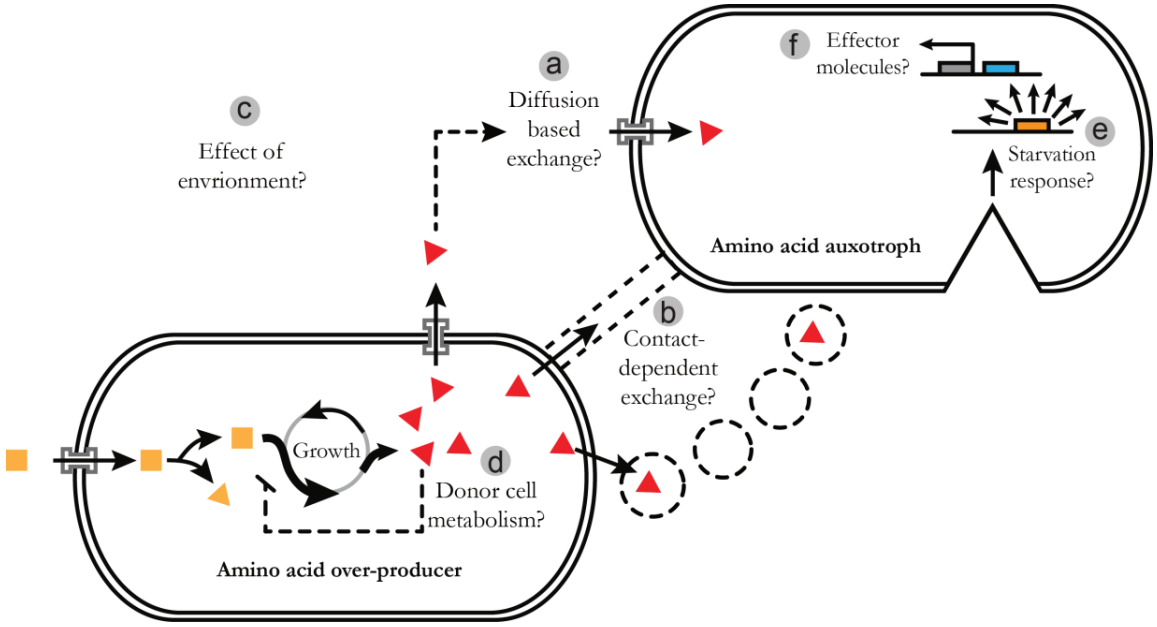

Figure 6: Overview of the main objectives of this study. Identifying the mechanism of amino acid cross-feeding between bacteria, is it (a) diffusion based i.e. through the external environment or is it (b) contact-dependent exchange? (c) What are the nutritional and environmental pre-requisites for amino acid cross-feeding? (d) Elucidating the effect of cross-feeding on the metabolism of the over-producer (donor) in the presence of an auxotroph (recipient). (e) Identifying transcriptional changes in the cross-feeder genotype resulting from amino acid starvation and subsequent cross-feeding. (f) Which genes are differentially expressed during the process of amino acid cross-feeding in *E. coli*?

Mechanism of amino acid exchange

Manuscript I: Contact-dependent exchange of nutrients in bacteria (chapter 7)

Microbes are known to use extracellular appendages for the transfer of genetic material (pili-based conjugation system), virulence-inducing factors (type secretion systems) and toxins for killing (contact-dependent inhibition). There is less known about the use of contact-dependence in nutrient exchange. By genetically modifying the strains to cross-feed, I ask the questions (Fig. 6a-c):

- How the exchange of amino acids takes place between cross-feeding cells?
- If the exchange is contact-dependent then what kind of structures are connecting the cross-feeding partners?
- Is there any effect of the growth conditions on exchange via such structures?

Metabolic basis of amino acid exchange

Manuscript II: Metabolic coupling in bacteria (chapter 8)

Bacterial metabolism is highly regulated and coordinated in the cell. Biochemical reactions that make up the metabolic network are under control of the cytoplasmic level of end products. During cross-feeding interactions there is a transfer of these end-products between two individual cells. Here I was interested to know (Fig. 6d):

- Is the metabolism of a prototrophic cell is affected when in presence of a metabolic sink (auxotroph) in the same environment?
- How does the prototroph adapt to these changes implemented by the one-way interaction?

Genetic basis of amino acid exchange

Manuscript III: Transcriptional insights into bacterial cross-feeding (chapter 9)

The model system depicted amino acid cross-feeding upon a loss-of-function mutation for amino acid biosynthesis in *E. coli*. This mutation renders the strain dependent on an external source of amino acid. However, amino acid starvation is known to induce a variety of stress response elements in bacteria, so I tried to identify the chain of causality from the auxotrophy mutation to nanotube formation by asking (Fig. 6e-f):

- Which genes are differentially regulated in *E. coli* cross-feeding genotypes upon the exchange of amino acids?
- How does the background mutation (auxotrophy or over-production) influence the ability to form nanotubes?
- Are there any supportive structures involved in the process of cross-feeding apart from nanotubes?
- Can a working model be developed to illustrate the process of contact-dependent amino acid cross-feeding?

Chapter 7: Manuscript I

Metabolic cross-feeding via intercellular nanotubes among bacteria

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Summary: This study describes a mechanism of contact-dependent nutrient exchange between two bacterial species, *Escherichia coli* and *Acinetobacter baylyi*. These strains were genetically modified to cross-feed amino acids upon coculture in a shaken liquid medium not supplemented with external amino acids. A single loss-of-function mutation rendering the *E. coli* strain auxotrophic for a specific amino acid was sufficient to induce the formation of nanotubes. A combination of fitness experiments, fluorescent labeling, lipid staining and microscopy was used to identify the structures connecting cross-feeding. Transport of metabolites (amino acid) through nanotubes potentially avoids the loss of this metabolite to the environment and protects the metabolite from degradation or chemical modification.

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Metabolic cross-feeding via intercellular nanotubes among bacteria

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Bacteria frequently exchange metabolites by diffusion through the extracellular environment, yet it remains generally unclear whether bacteria can also use cell–cell connections to directly exchange nutrients. Here we address this question by engineering cross-feeding interactions within and between Acinetobacter baylyi and Escherichia coli, in which two distant bacterial species reciprocally exchange essential amino acids. We establish that in a well-mixed environment E. coli, but likely not A. baylyi, can connect to other bacterial cells via membrane- derived nanotubes and use these to exchange cytoplasmic constituents. Intercellular connections are induced by auxotrophy-causing mutations and cease to establish when amino acids are externally supplied. Electron and fluorescence microscopy reveal a network of nanotubular structures that connects bacterial cells and enables an intercellular transfer of cytoplasmic materials. Together, our results demonstrate that bacteria can use nanotubes to exchange nutrients among connected cells and thus help to distribute metabolic functions within microbial communities.

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Microorganisms are ubiquitous on our planet and their metabolic activities significantly contribute to vital ecosystem processes[1]. In most cases, microbes exist in taxonomically diverse communities, whose structure is determined by a complex interplay between environmental factors and ecological interactions among its community members[2,3]. Strong selection pressures for limiting resources have resulted in the evolution of diverse strategies to survive and reproduce under these conditions and gave rise to the stunning richness of ecological interactions that can be observed within microbial communities[4].

Biotic interactions between two microorganisms can either positively or negatively affect the fitness of the interacting individuals. Negative fitness consequences can result from predation, parasitism or chemical warfare between microorganisms, while cooperative or mutualistic relationships can significantly benefit the strains involved[2]. Many of these interactions rely on either the active or passive release of chemical molecules into the environment[5,6] or, alternatively, a direct contact between bacterial cells[7]. In both cases, the spatial organization of a given microbial community dictates the outcome of these interactions[8]. For example, short distances between surface-attached cells facilitate the exchange of diffusible nutrients[9] and communication signals[10]. However, such a diffusion-based transfer of molecules between two cells bears a number of risks: unintended third parties may eavesdrop on the signal or consume the nutrient. Moreover, the exchanged molecule may degrade or be lost by a too fast diffusion[11].

To circumvent these problems, bacteria have evolved a multitude of different ways to directly deliver molecules to intended recipients. Such mechanisms include, for example, the production of outer membrane vesicles, as they are used by many bacterial species to deliver cargo to other cells[12–16]. Alternatively, bacterial cells may connect via channels[17], nanotubes[18], pili[19] or transiently fuse their outer membrane[20] to transfer cytoplasmic components or outer membrane materials.

These direct cell–cell connections serve primarily three functions: first, genetic material such as plasmids is horizontally transferred between cells[12,21], supplying the recipient with potentially beneficial genetic functions[22,23]. Second, chemical signals that help to coordinate social activities within microbial communities are trafficked between cells[16]. Third, proteins can be transferred between cells[18,20], which are involved in social movement[14] or serve predatory bacteria as interspecific killing factors[15]. Another possibility, which remains virtually unexplored, is the utilization of intercellular connections to transfer nutrients between bacterial cells. While recent data suggest that significant proportions of the cytosol can be exchanged between connected cells[18], clear experimental evidence for a possible nutritional function of the exchanged cytoplasmic constituents is lacking.

Here we address this question by interrogating synthetically engineered cross-feeding interactions within and between the two bacterial species Acinetobacter baylyi and Escherichia coli. We establish that in a well-mixed environment E. coli, but likely not A. baylyi, can connect to other bacterial cells via membrane-derived, tubular structures (hereafter referred to as `nanotubes') and use these to exchange cytoplasmic constituents. Furthermore, we show that cell attachment is demand-driven and contingent on the nutritional status of auxotrophic cells. Together, our results suggest that nanotubes can mediate the exchange of cytoplasmic nutrients among connected bacterial cells and thus help to distribute metabolic functions within microbial communities.

Results

Construction of synthetic cross-feeding interactions. We engineered obligate cross-feeding interactions within and between A. baylyi and E. coli to achieve two main goals. First, genotypes should be unable to produce a certain amino acid and rely on an external supply of this metabolite for growth. Second, amino acid production levels should be sufficiently high to allow growth of a complementary cross-feeding genotype. To achieve this, two pairs of genes were deleted from the wild type (WT) background of both species: deleting the terminal genes of the histidine (His) and tryptophan (Trp) biosynthesis pathways hisD and trpB resulted in two `auxotrophs' of both species unable to grow in the absence of an external supply of either His or Trp (Supplementary Fig. 1). In addition, the regulatory regions of the His and Trp biosynthesis operons hisL (encodes the operon leader peptide) and trpR (encodes the tryptophan repressor protein) were deleted to eliminate negative transcriptional regulation of these two pathways, yielding two amino acid `overproducers' (that is, genotypes impaired in the feedback control of amino acid production levels). Combining two deletion mutations (that is, DtrpB and DhisL; DhisD and DtrpR) in the same genetic background resulted in two `cross-feeders' (that is, genotypes auxotrophic for one amino acid, which constitutively produce increased amounts of the other amino acid) with complementary metabolic requirements and amino acid production characteristics that allowed assembling four intra- and interspecific pairs of cross-feeders (Fig. 1a). Finally, each cross-feeding mutants was labelled with one of two plasmids that constitutively expressed either egfp or mCherry for subsequent differentiation.

The His and Trp production levels of the enhanced green fluorescent protein (EGFP)-labelled variants of all newly constructed mutants and the WTs of both A. baylyi and E. coli were determined by coculturing each genotype together with one of two E. coli mutants auxotrophic for either His (DhisD) or Trp (DtrpB). Since the latter mutants require an external source of the focal amino acid to grow (Supplementary Fig. 1), their growth in coculture is indicative of the amino acid production levels of the respective other strain[24]. Quantifying the amino acid production levels of all tested donors by determining the growth of cocultured auxotrophs (that is, number of colony-forming units (CFUs) formed during 24 h) revealed that overproducers and cross-feeders of both species produced His and Trp levels that significantly exceeded the production levels of both WTs and the corresponding other auxotrophs (Fig. 1b). Moreover, the corresponding overproducers and cross-feeders of both species supported the growth of cocultured auxotrophs to a similar extent (Fig. 1b), indicating that their amino acid production levels did not differ. Thus, the amino acid overproduction mutations (that is, DhisL, DtrpR) significantly increased the His and Trp production levels of the corresponding mutants.

Physical separation of strains prevents cross-feeding. The newly designed cross-feeding genotypes were scrutinized for their ability to support the growth of a complementary partner of the same or the other species. Indeed, all cross-feeding mutants of E. coli grew in coculture with complementary E. coli cells, as well as cross-feeding A. baylyi genotypes (Fig. 2a). Surprisingly, the same cross-feeding mutants of A. baylyi that showed marked growth in coculture with E. coli exhibited virtually no growth when paired up with complementary genotypes of the same species (Fig. 2a). Next, we asked whether the transfer of amino acids between cross-feeders was based on the diffusion via the surrounding medium or whether a physical contact was required for cells to interact. To test this, the same coculture experiment was performed again, but this time bacterial populations were separated

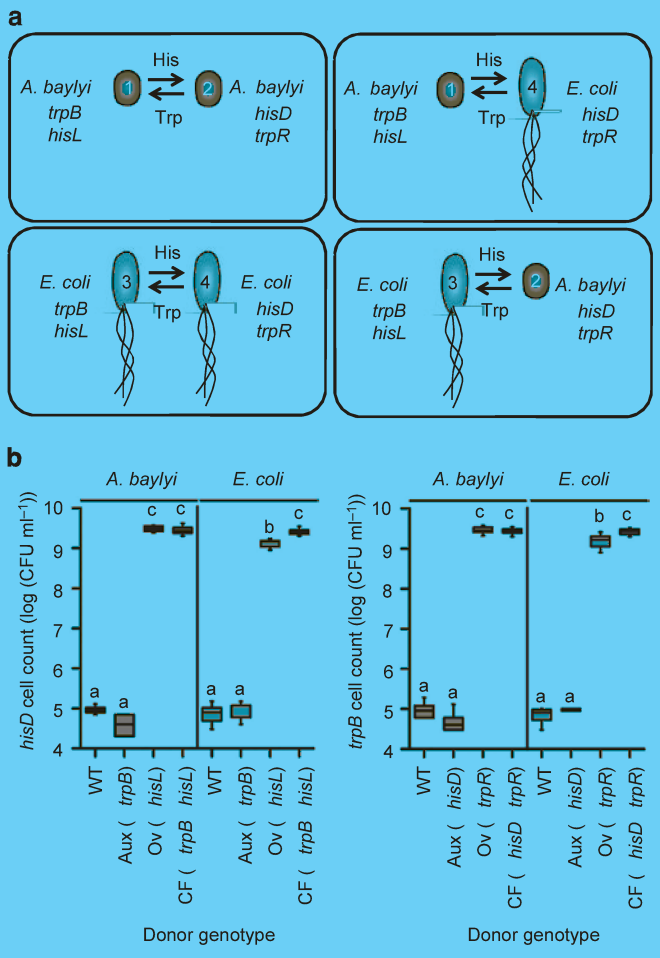

Figure 1 | Design of cross-feeding interactions and amino acid production levels of all genotypes. (a) Overview over all engineered cross-feeding interactions within and between Acinetobacter baylyi and Escherichia coli. Deletion of hisD and trpB rendered the resulting mutants auxotrophic for His and Trp, respectively, while deletion of trpR and hisL increased production levels of Trp and His, respectively. (b) Amino acid production of wild type (WT) and all generated mutant genotypes of A. baylyi (dark-grey boxes) and E. coli (light-grey boxes) as determined by coculturing each donor genotype (x axis) together with one E. coli biosensor (initial ratio: 1:1) that was auxotrophic for either histidine (DhisD, left panel) or tryptophan (DtrpB, right panel). `Cell count' refers to the number of biosensor cells that formed in coculture with a WT, auxotroph (Aux), amino acid overproducer (Ov) or cross-feeding genotype (CF) during 24 h. Values are colony-forming units (CFU) after 24 h minus the initial inoculum size. Different letters above boxes indicate significant differences between donor genotypes of both species (LSD post hoc test: P○0.05, n¼8).

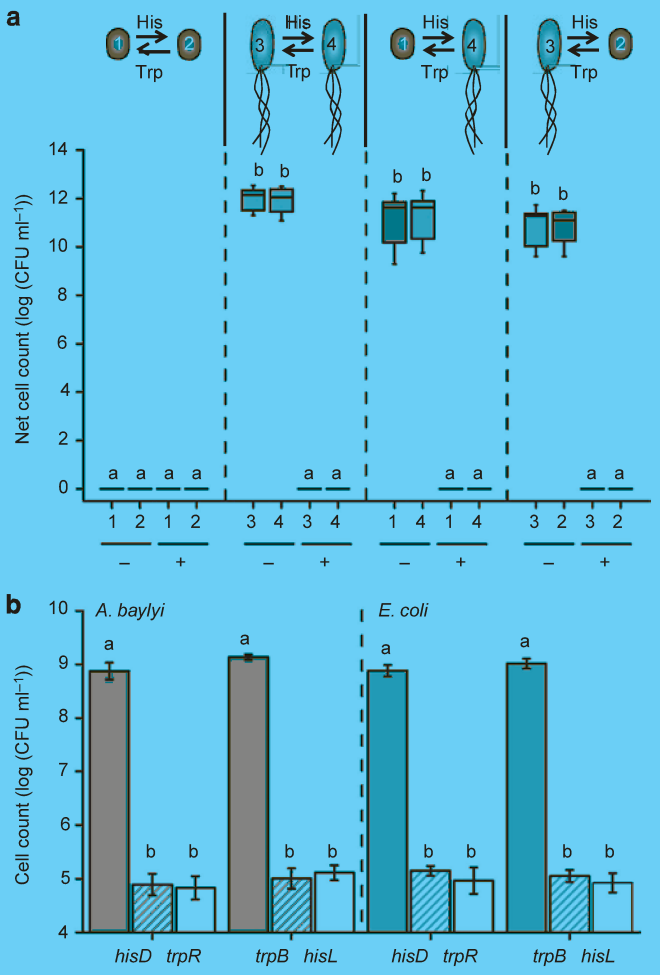

Figure 2 | Cross-feeding is contact-dependent. (a) Coculture experiments between all inter- and intraspecific cross-feeding interactions generated (genotype numbers and pictograms as in Fig. 1a) in the absence (–) or presence (þ) of a membrane filter that separated both populations to prevent a direct cell–cell contact (Supplementary Fig. 2), yet allowed passage of free amino acids (Supplementary Fig. 3). `Net cell count' is the number of colony-forming units (CFU) a genotype reached in coculture during 24 h in minimal medium minus the CFU number reached in monoculture. Cytoplasmic markers (EGFP and mCherry) were swapped between cocultured genotypes. Each combination was replicated four times. (b) Growth of the cross-feeding genotypes at saturating concentrations of the required amino acid (that is, His or Trp, 100 mM, grey bars), a concentration of the required amino acid that significantly exceeded the amounts cross-feeding genotypes would have produced during 24 h of growth (that is, 0.5 mM, striped bars), or the absence of amino acids (white bars). `Cell count' refers to the number of CFUs after 24 h minus the initial inoculum size. Mean values (±95% confidence interval) are shown. (a,b) Different letters indicate significant differences between groups (LSD post hoc test: P○0.05, n¼8). The two species are colour-coded as A. baylyi (dark grey) and E. coli (light grey).

using a filter membrane (Supplementary Fig. 2). For all combinations of cross-feeders tested, virtually no growth was detectable when the two bacterial populations were separated in this way (Fig. 2a).

While significant growth occurred when the two cross-feeding mutants were exposed to saturating concentrations of His and Trp (that is, 100 mM), virtually no growth was detectable when cross-feeders were inoculated into unsupplemented minimal medium or minimal medium supplemented with 0.5 mM of either amino acid (that is, a concentration that exceeded the amount the corresponding intra- or interspecific counterparts would have released during 24 h, two- to fivefold, Supplementary Fig. 3) (Fig. 2b). This finding provided evidence that the amount of amino acids released by these mutants was not sufficient to explain the growth of cross-feeding genotypes in coculture (Fig. 2a). Moreover, an experiment to test whether His and Trp could freely pass through the filter membrane provided no evidence for the membrane acting as a diffusion barrier (Supplementary Fig. 4).

Together, these experiments established that cocultures of cross-feeding mutants among E. coli and between A. baylyi and E. coli showed a marked synergistic growth, which could not be explained by a release of His and Trp by cross-feeders into the

extracellular environment, but instead required the physical presence of both partners in the same environment for cells to interact.

Cross-feeding cells exchange cytoplasmic constituents. An alternative to a diffusion-based transfer of amino acids through the external environment is direct cell–cell interactions that could enable the exchange of cytoplasmic amino acids among cells via, for example, nanotubes or pili. In such a case, other cytoplasmic constituents could be transferred to the respective other cross-feeding mutant along with the focal amino acids. To scrutinize this possibility, pairs of cross-feeding mutants within E. coli or between A. baylyi and E. coli were assembled and the cytoplasm of one partner (that is, the donor) was labelled with a plasmid that conferred resistance to the antibiotic ampicillin and constitutively expressed the fluorescent protein EFGP. In case of a cytoplasmic exchange among cells, both the plasmid and cytoplasmic protein might be transferred from the labelled donor to the previously unlabelled recipient cell. Transfer of plasmids was determined by plating dilutions of cocultures after 0 and 24 h on ampicillin-containing plates that were supplemented with either His or Trp to distinguish individual genotypes. Transfer of EGFP was assessed by quantifying the population-level proportion of cells that fluoresced in green colour at the onset of the experiment and after 24 h of coculture via flow cytometry. This experiment provided no support for a transfer of plasmids between cells under the given experimental conditions, since not a single colony formed on the corresponding antibiotic-containing plates. Although the proportion of plasmid-containing donor cells did not change significantly in any of the tested combinations during 24 h, a significant increase of EGFP-labelled cells during the same time implied that cytoplasmic protein was transferred from donor to recipient cells (Fig. 3a). Moreover, within the same combination of genotypes, EGFP was transferred from A. baylyi to E. coli and vice versa, thus indicating a bidirectional mode of transfer (Fig. 3a).

To determine whether all combinations of cross-feeding mutants exchange cytoplasmic protein in this way, all mutants of both species were labelled with plasmids constitutively expressing either EFGP or mCherry. The emergence of double-labelled cells during coculture—which would be indicative of a transfer of cytoplasm from one single-labelled cell to another one—was monitored by flow cytometry. This experiment demonstrated for all populations involving cross-feeding genotypes of E. coli that, already after 24 h of growth in shaken liquid culture, more than 50% of all cells within these populations were simultaneously labelled with both EGFP and mCherry (Fig. 3b; Supplementary Fig. 5a–h). Extending the time of coculture until 48 h even resulted in more than 60% of all cells being simultaneously labelled with both fluorescent proteins (Supplementary Fig. 5a–h). In contrast, virtually no double-labelled cells (that is, ≤2%) could be detected in cross-feeding consortia consisting exclusively of A. baylyi (Fig. 3b; Supplementary Fig. 5a,b). Repeating the same experiment with a more fine-grained temporal resolution provided evidence for double-labelled cells already 12 and 15 h after the onset of the experiment (Supplementary Fig. 5i–k). A striking link between the increase in productivity of cross-feeding consortia and the formation of double-labelled cells over time bolstered the interpretation that growth of these cultures was indeed contingent on direct cell–cell interactions (Supplementary Fig. 5i–k).

The observation that populations of mCherry-labelled cells consistently declined much faster than cocultured EGFP-labelled cells (Supplementary Fig. 5) could be explained with significantly increased fitness costs incurred by the mCherry-containing

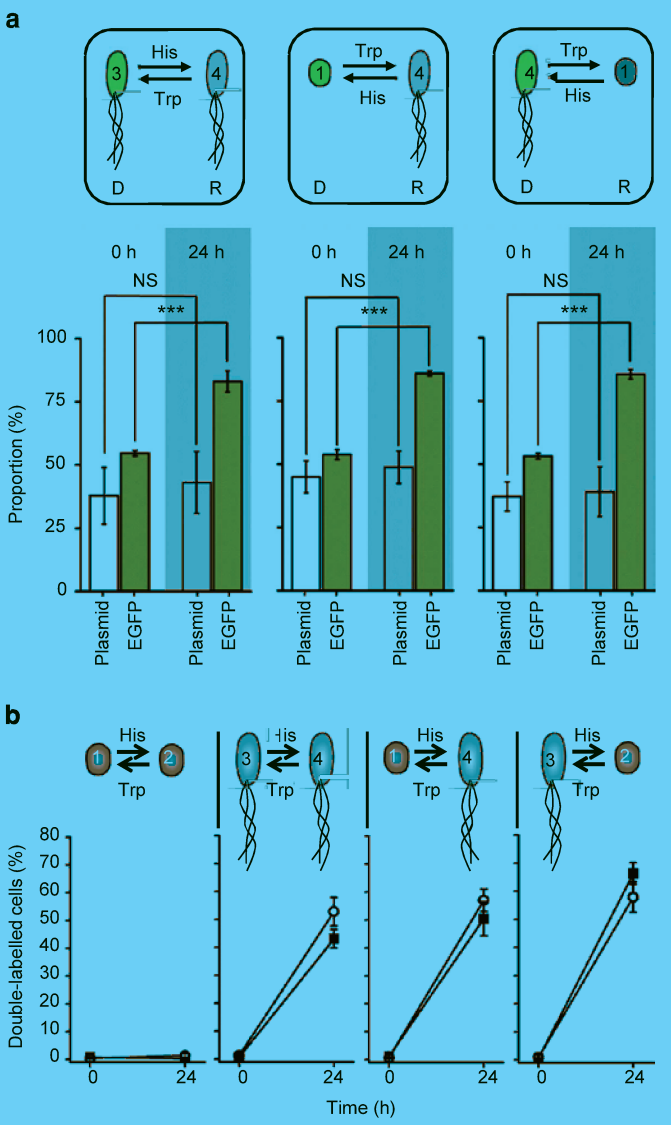

Figure 3 | Cross-feeding cells bidirectionally exchange cytoplasmic protein. (a) Two cross-feeding genotypes of E. coli or A. baylyi and E. coli were cocultured for 24 h. The donor (D) contained an EGFP-expressing plasmid that conferred resistance to the antibiotic ampicillin, while the recipient (R) was unlabelled. Shown is the mean proportion (±95% confidence interval) of all cells containing the labelling plasmid or the EGFP protein at the onset of the experiment (0 h) and after 24 h. Cell numbers were determined by plating on antibiotic-containing plates (plasmid presence) or flow cytometry (EGFP fluorescence emission). The proportion of plasmid-containing cells did not differ between time points (NS ¼ not significant by a paired t-test: $P \geq 0.05$), while the proportion of EGFP-containing cells increased significantly during 24 h (paired t-test: ***$P \leq 0.001$). This experiment was replicated three times. (b) The cytoplasm of cross-feeding genotypes of A. baylyi and E. coli was labelled with plasmids expressing either EGFP or mCherry. The appearance of double-labelled cells (that is, containing both colours) in cocultures indicates a cytoplasmic exchange. Shown is the mean proportion (±95% confidence interval) of double-labelled cells after 0 and 24 h of growth in minimal medium, as determined by flow cytometry. This experiment was replicated eight times. Symbols represent the two possible arrangements of how markers were distributed among cross-feeders: mCherry/EGFP (circle) and EGFP/ mCherry (box). (a,b) Genotype pictograms like in Fig. 1a.

plasmid relative to its EGFP-expressing counterpart (Supplementary Fig. 6). Cultivating monocultures of A. baylyi and E. coli cross-feeders that contained either the mCherry- or the EGFP-expressing plasmid for 48 h and determining emission levels of both red and green fluorescence after 0, 24 and 48 h by flow cytometry corroborated that all cultures analysed emitted light exclusively at the expected wavelength over the entire experimental period (Supplementary Fig. 7). Thus, this experiment ruled out that the previously observed emergence of double-labelled cells was due to the emission of both colours from single-labelled cells.

However, a cell that is scored as emitting both green and red fluorescence in the flow cytometer may also result from the physical attachment of two differentially labelled cells, rather than an exchange of cytoplasmic protein. However, three lines of evidence support the interpretation that indeed single cells contained both fluorescent proteins: first, dissociating possible cell clumps by vortexing did not significantly reduce the number of double-labelled cells (Supplementary Fig. 8a). Even treatment with a commercially available cell dissociation solution reduced the number of double-labelled cells by 19 and 11% only in the within-E. coli cocultures, while it did not affect the number of double-labelled cells in any of the interspecific cocultures (Supplementary Fig. 8a). Second, the size distribution of double-labelled cells determined as the forward scatter (FSC) in the flow cytometer[25] was congruent with the size distribution of EGFP-labelled WT cells of E. coli. Third, analysing cocultures of pairwise cross-feeding consortia within E. coli or between A. baylyi and E. coli under the fluorescence microscope revealed individual cells that fluoresced in both red and green colour (Fig. 4c).

Taken together, these results show that cross-feeding cells can bidirectionally exchange cytoplasmic constituents such as protein, yet they provided no evidence for a transfer of plasmid DNA (that is, pJBA24) between cells.

Cytoplasmic exchange depends on nutritional status of cells. Since both cross-feeding genotypes vitally required one amino acid to grow, we hypothesized that externally adding the focal amino acid to the growth environment should abolish the experimentally imposed obligate requirement for cross-feeding. As a consequence, these conditions should also eliminate the exchange of cytoplasmic markers such as EGFP and mCherry among cross-feeders. An experiment to test this hypothesis clearly validated that the transfer of cytoplasmic constituents was governed by the physiological demand for amino acid cross-feeding: after 24 h, cocultures of cross-feeding consortia that consisted of either two E. coli mutants or A. baylyi and E. coli exhibited a population-level ratio of double-labelled cells of around 40% when grown in unsupplemented minimal medium. In contrast, when the growth medium was supplemented with His and Trp, the proportion of double-labelled cells reached less than 1.4% of the total population (Fig. 4a), thus indicating that the exchange of cytoplasmic constituents was driven by the nutritional status of an E. coli cell. Similarly, no double-labelled cells could be detected by fluorescence microscopy when cocultures of cross-feeding genotypes were supplemented with His and Trp (Fig. 4c).

Finally, coculture experiments between all possible combinations of differentially labelled WTs, auxotrophs, overproducers, and cross-feeders of both species revealed two conditions for a cytoplasmic exchange to be detectable by flow cytometry (Fig. 4b). First, one of the two partners needed to carry a mutation causing amino acid overproduction. Second, the respective other cells needed to be an E. coli cell auxotrophic for the corresponding amino acid. Altogether, this set of experiments revealed that the species-specific exchange of cytoplasmic constituents between two bacterial cells was driven by the physiological demand for a given amino acid, as well as the presence of other cells satisfying this need.

Cytoplasmic exchange requires the mixing of cocultures. All experiments so far have been conducted under shaken environmental conditions. A static incubation of cocultures, however, could enhance the exchange of cytoplasmic constituents due to facilitated cell–cell interactions under non-disturbed conditions. This hypothesis was tested by incubating cross-feeding consortia consisting of one (that is, E. coli) or both species under static or continuously shaken environmental conditions, and quantifying the proportion of double-labelled cells after 0 and 24 h by flow cytometry. In contrast to expectations, this experiment clarified that the shaking of cultures was essential for a cytoplasmic exchange to take place (Supplementary Fig. 9). Under shaking conditions, around 50% of cells were double-labelled after 24 h of growth, while almost no double-labelled cells emerged when cocultures were incubated under static conditions (Supplementary Fig. 9a,c,e). The lacking cytoplasmic exchange under static incubation conditions strikingly coincided with a severely reduced growth of these cultures (Supplementary Fig. 9b,d,f) relative to cocultures that were incubated under shaken conditions, thus corroborating that an exchange of cytoplasmic material was essential for growth of these obligate cross-feeders. In contrast to our expectations, this experiment indicated that the shaking of cultures promoted the exchange of cytoplasmic constituents.

Cells connect via membrane-derived nanotubes. What is the structural basis for the observed transfer of cytoplasmic elements among cross-feeding cells? To answer this question, scanning electron micrographs of cross-feeding consortia consisting of either E. coli or A. baylyi and E. coli were recorded. In the presence of amino acids (that is, His and Trp, 100 mM each), cocultures of cross-feeding genotypes grew as individual cells with no discernible sign of a physical attachment of two or more cells (Fig. 4c). However, without an external supply of His and Trp, cells of both types of cocultures consistently formed tubular structures that connected cells (Figs 4c and 5). The average diameter of these nanotubes was 80±10 nm, and the distance covered between two cells ranged between 0.05 and 14 mm.

To identify whether these intercellular connections consist of membrane-derived lipids, 9-h-old interspecific cocultures of the cross-feeding mutants A. baylyi DhisDDtrpR and E. coli DtrpBDhisL were labelled with the lipophilic dye DiO that intercalates in lipid membranes[20]. Subsequent in vivo fluorescent imaging of the otherwise mCherry-labelled cells should pinpoint the potential lipid-based nature of extracellular appendages by their green fluorescence. In line with these expectations, the lipophilic dye stained threads that connected multiple cells, indicating they consist of membrane-derived lipids (Fig. 6).

Finally, imaging consortia of cross-feeding mutants of A. baylyi (DhisDDtrpR containing pJBA24-mCherry) and E. coli (DtrpBDhisL labelled with pJBA24-egfp) by fluorescence microscopy clearly showed that nanotubes fluoresced in green colour (Fig. 7a,b), implying they are hollow and contained EGFP. Next, time-lapse fluorescence microscopy experiments were set up to visualize transfer of cytoplasmic material using the same interspecific pair of cross-feeding mutants. However, nanotubes did not establish when cross-feeding genotypes were cocultured on agarose pads. Instead, when cells were allowed to form nanotubes in a shaken, liquid culture and subsequently

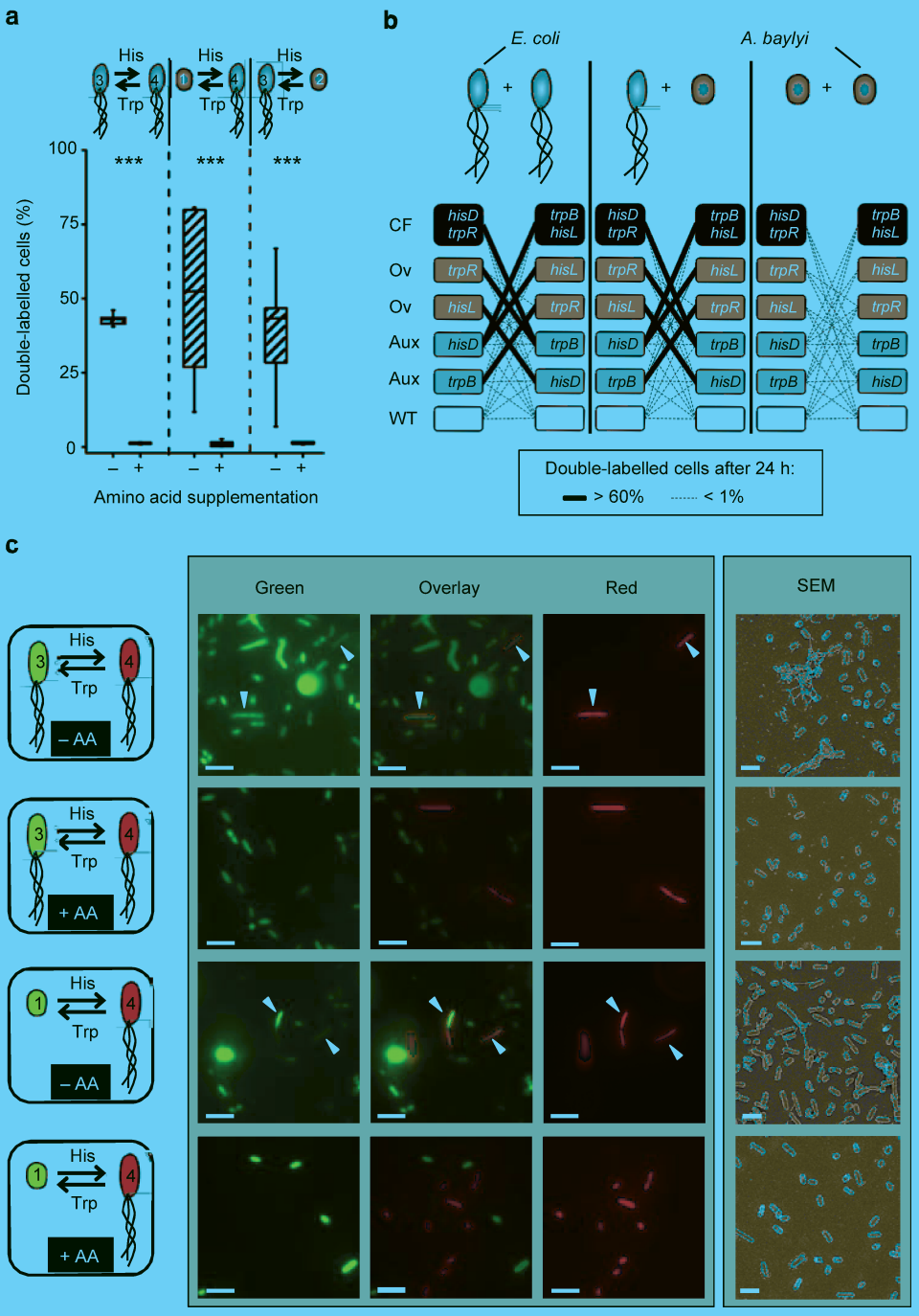

Figure 4 | Transfer of cytoplasmic markers depends on amino acid availability. (a) Proportion of double-labelled cells as determined by flow cytometry in cocultures of cross-feeding genotypes after 24 h of growth in the absence (–) or presence (þ) of the required amino acids His and Trp (100 mM each). Paired t-test: ***P o 0.001. This experiment was replicated eight times. (b) Proportion of double-labelled cells formed in pairwise coculture experiments of differentially labelled wild type (WT), auxotrophs (Aux), amino acid overproducers (Ov), or cross-feeding genotypes (CF). Number of double-labelled cells after 24 h was determined by flow cytometry. This experiment was replicated four times. (c) Four representative pairs of cross-feeding interactions within E. coli (rows 1 and 2) or between A. baylyi and E. coli (rows 3 and 4) were cocultured in the absence (rows 1 and 3) or presence (rows 2 and 4) of the two required amino acids (AAs) His and Trp (100 mM each). Fluorescence pictures show cells labelled with EGFP (green), mCherry (red) or both markers simultaneously (overlay). Scale bars, 5 mm. White triangles highlight cells where transfer occurred. The last column shows scanning electron micrographic (SEM) images of the corresponding cocultures. Scale bars, 2 mm.

transferred to agarose pads, we observed a striking example for a cytoplasmic transfer via nanotubes: a dividing mCherry-labelled cell (most likely A. baylyi) that was connected to several EGFP-labelled cells (most likely E. coli) via green-fluorescing nanotubes displayed a continuous, yet moderate increase in its green fluorescence levels over 32 min (Fig. 7b,c; Supplementary Movie 1). Then, within 2 min, the intensity of green fluorescence jumped from 113±11 to 455±34 arbitrary fluorescence units (Fig. 7c). This stark increase in green fluorescence during such a short time is unlikely to be caused by an increased EGFP expression level, thus further supporting that protein but not plasmids are transferred between cells. Control measurements from the same

images of five randomly chosen mCherry- and EGFP-labelled cells that only showed fluorescence in their respective channel and which were not visibly connected to any other cell (Fig. 7d,e) ruled out that the previously observed increase in green fluorescence was due to stochastic fluctuations. These findings strongly suggest the involvement of nanotubes in mediating the observed intercellular transfer of cytoplasmic constituents.

Altogether, these analyses established that cross-feeding cells exchanged cytoplasmic constituents via an intercellular network of nanotubular structures that consisted of membrane-derived lipids.

Discussion

Cooperative metabolic interactions are very common among both Archaea and Bacteria[6,26] and these `syntrophic' interactions are in many cases vitally important for the fitness of the microbes involved. However, since the exchanged commodities are often costly to produce, a diffusion-based transfer between cells via the extracellular environment entails risks: the metabolite may be lost or be consumed by an unintended third party. This problem even aggravates in agitated, aqueous ecosystems, where released compounds diffuse at high rates, thus hampering the build-up of sufficiently high local concentrations.

Our results demonstrate that E. coli can solve this problem by employing cell–cell connections for a targeted transfer of cytoplasmic constituents. Utilizing engineered obligate cross-feeding interactions, we show that (i) E. coli, but likely not A. baylyi, can exchange nutrients and proteins in a contact-dependent manner with cells of the same or a different species (A. baylyi), (ii) the observed exchange was bidirectional and required a mixing of cocultures, (iii) lipid-based nanotubes were the structural basis for the observed cytoplasmic exchange, and (iv) the establishment of intercellular bridges and the exchange of cytoplasmic materials was strongly dependent on the nutritional status of a given cell. As such, our study provides first experimental evidence for an intercellular transfer of nutrients via nanotubes.

The observation that E. coli established tubular connections with other bacterial cells is strikingly reminiscent of Myxococcus xanthus[15,27] cells, which have been shown to form a discrete, three-dimensional network of membrane tubes that interconnects cells. Cells of this social bacterium use membrane extensions to mediate the intercellular transfer of outer membrane proteins and lipids[14], yet not cytoplasmic contents[27]. Given the recent evidence that Bacillus subtilis cells can exchange cytoplasmic elements such as protein and plasmid DNA via tubular connections[18], it is tempting to speculate that this type of contact-dependent metabolite exchange may be more common in bacteria than previously thought.

Many bacteria[28,29], including E. coli[12], are known to produce membrane vesicles that are involved in an intercellular transport of chemical signals[16], lipids[30], protein[31], and DNA[12], as well as in promoting adherence to other bacterial or eukaryotic host cells[32,33]. In most of these cases, membrane vesicles are believed to pinch off the producing cell and traverse the external environment as cell-independent units that eventually fuse with potential recipients[16]. Also in our experiments we observed vesicular structures that appeared attached to bacterial cells or seemingly free-floating in the cell external environment (Fig. 7a,b). While the results of the population separation experiment (Fig. 2a) and the fluorescence microscopic analyses are inconsistent with the idea that cytoplasmic elements were trafficked with free membrane vesicles, their functional role in

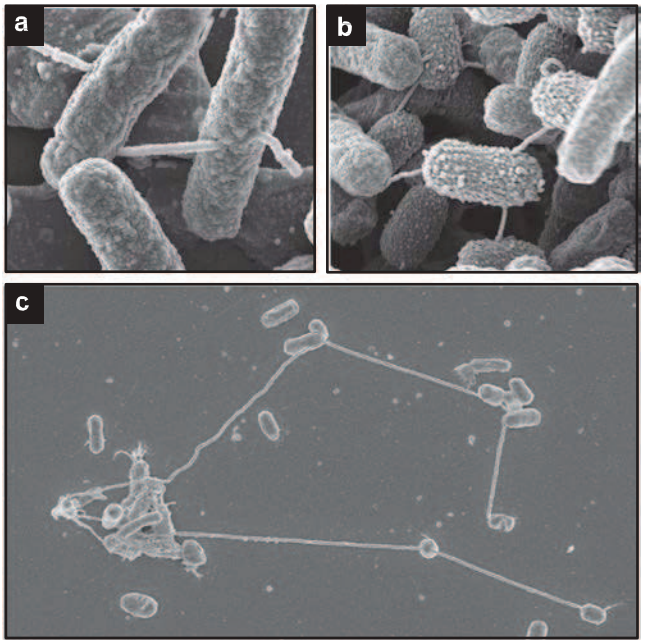

Figure 5 | Nanotubular structures connect cross-feeding cells. Intercellular connections form between two cross-feeding genotypes of E. coli, as well as between A. baylyi and E. coli. Shown are scanning electron micrographic images of (a) an E. coli DtrpBDhisL and E. coli DhisDDtrpR coculture after 24 h, as well as (b,c) an A. baylyi DhisDDtrpR and E. coli DtrpBDhisL coculture after (b) 48 h and (c) 24 h of growth in unsupplemented minimal medium. Scale bars, 0.2 mm (a,b), 2 mm (c).

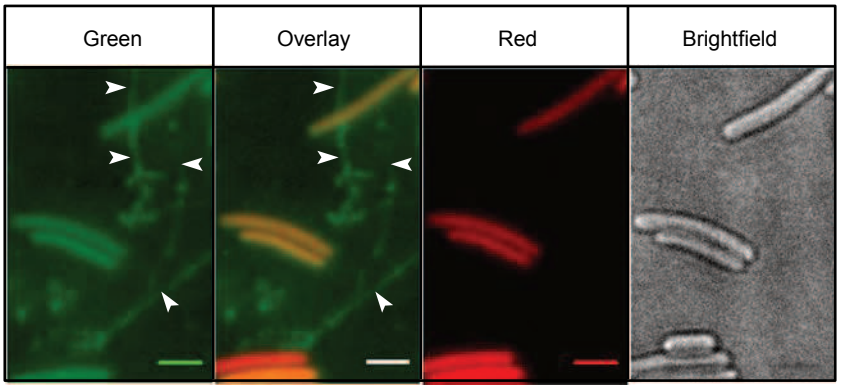

Figure 6 | Nanotubes consist of membrane-derived lipids. Cocultures of cross-feeding genotypes of A. baylyi DhisDDtrpR and E. coli DtrpBDhisL (both mCherry labelled) after 9 h of growth were stained with a lipophilic dye (DiO) that intercalates in membranes. Lipid membranes fluoresce in green colour. Pointers indicate nanotubes that connect cells. Scale bars, 2 mm.

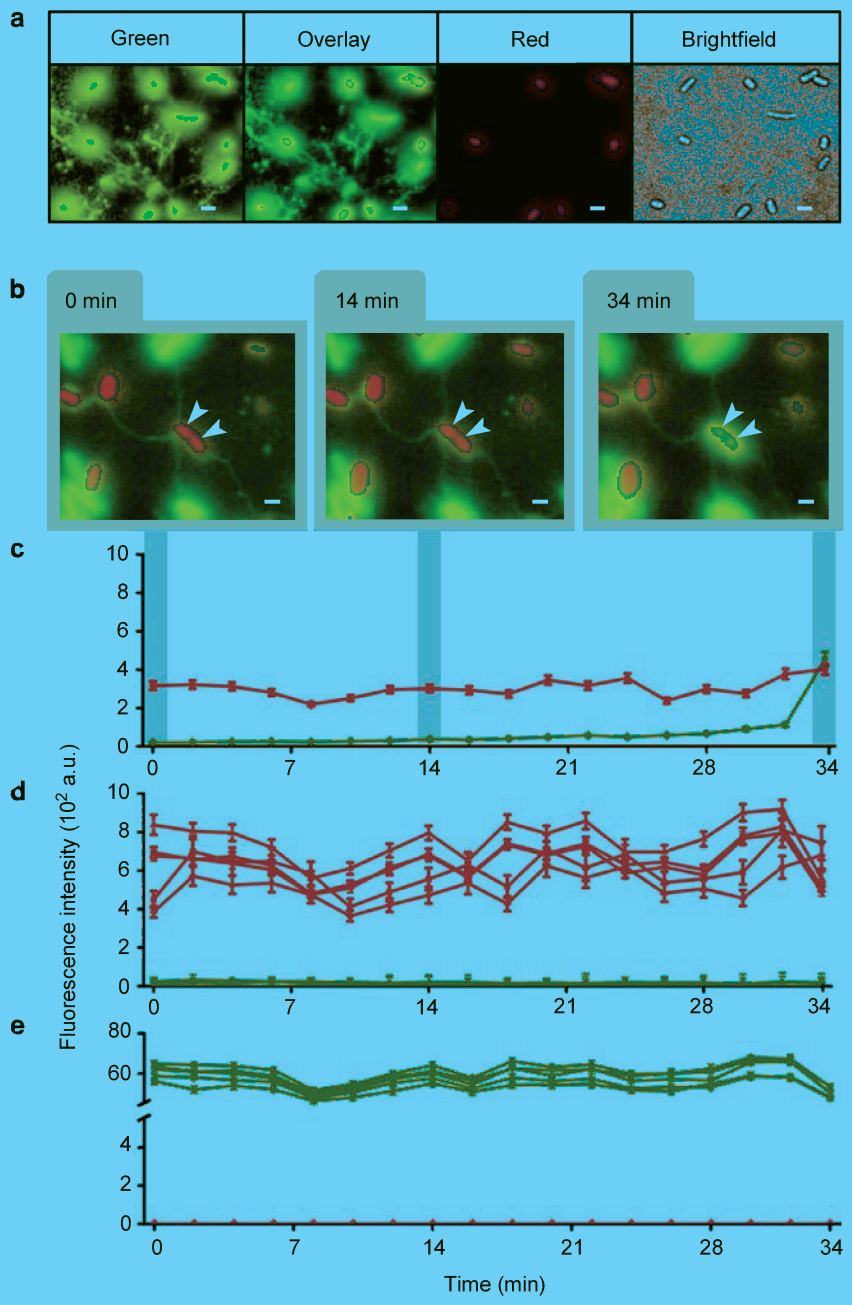

Figure 7 | Nanotubes transport cytoplasmic constituents. Cocultures of cross-feeding genotypes of A. baylyi DhisDDtrpR (mCherry labelled) and E. coli DtrpBDhisL (EGFP labelled) after 9 h of growth. (a) Fluorescence and brightfield microscopic images show a network of nanotubes and vesicles that contain EGFP. Scale bars, 4 mm. (b) Fluorescence time-lapse microscopy reveals an increase in green fluorescence in a dividing mCherry-labelled cell (pointers) over time. The focal cells are attached to an EGFP-containing nanotube that projects to several EGFP-containing cells. Scale bars, 2 mm. (c–e) Change in red (red lines) and green (green lines) fluorescence intensity (10^2 arbitrary units (a.u.)) over time. Values for each time point were derived by analysing cells on the same microscopic field. Points are means (±95% confidence interval) of 30 longitudinal measurements per cell with background fluorescence levels subtracted. Background fluorescence varied marginally over time (95% confidence intervals of green fluorescence: ±2.9 a.u., red fluorescence: ±1.8 a.u.). Shown is the change in fluorescence levels of (c) the focal cells highlighted in b, (d) five randomly chosen mCherry-containing cells, and (e) five randomly chosen EGFP-containing cells. The transfer event shown in b and c is the same as that depicted in Supplementary Movie 1 (unprocessed images are available at the Dryad Digital Repository, http://doi.org/10.5061/dryad.ds68k).

our experiments remains elusive. Currently, we are working on identifying the structural basis of nanotube formation and elucidating the potential involvement of membrane vesicles in this process. The results of this analysis will be reported elsewhere.

What triggered the formation of nanotubes in our experiments? Deleting key steps of amino acid biosynthetic pathways likely rendered the resulting E. coli mutants starving for His and Trp[34]. This experimentally induced nutrient limitation represents a strong stimulus[35] that could have triggered the observed response. Accordingly, both the transfer of cytoplasmic markers between cells (Fig. 4a) and the formation of nanotubes (Fig. 4c) could be effectively prevented by supplementing the growth medium with the two focal amino acids. These findings imply the existence of a regulatory mechanism, which, depending on the degree of nutrient starvation, activates the nanotube-mediated

cell attachment in E. coli to remedy shortcomings for the required metabolites.

In our study, cross-feeding E. coli cells could both serve as donor and recipient of amino acids (Fig. 2a) and protein (Figs 3 and 4), suggesting the exchange of cytoplasmic constituents was bidirectional on a population level. A bidirectional exchange of cytoplasmic elements has been previously documented in B. subtilis cells that exchanged plasmids and cytoplasmic protein via nanotubes[18], as well as in archaea[36,37] that use cytoplasmic bridges to reciprocally exchange non-conjugative plasmids between cells. Strikingly, the labelling plasmid used in this study (pJBA24) was not transferred between cells, which could imply the plasmid was bound to the host's chromosome and/or its inner membrane[38]. Future work should investigate whether other plasmids that differ in, for example, their copy number or mode of replication/segregation can be exchanged in this way. Finally, the degree of specificity with which cytoplasmic elements can be exchanged between cells remains unknown. In particular, an uncontrolled passage of regulatory or catalytic elements through nanotubes may jeopardize homeostasis and development of connected cells. Thus, it will be interesting to explore whether nanotube-forming bacteria utilize diffusion barriers[39] or specialized secretion systems[40] to selectively control nanotube traffic.

In this study, a passive movement was required for cell–cell connections to successfully establish (Supplementary Fig. 9). A possibility to account for this observation is that outgrowing nanotubes might have impaired the active swimming motility of cells. Given the length nanotubular structures can reach (that is, up to 14 mm, Figs 5c and 7b), it seems likely that these appendages impede swimming. As a consequence, physical mixing of cells was required to increase the chance of a given recipient to encounter and attach to suitable donor cells. This is in contrast to previous reports on B. subtilis[18] or M. xanthus[27,41] in which cell–cell interactions required a solid surface. In our experiments, nanotubes did not establish on (agarose) surfaces. Even when cells were allowed to pre-form nanotubes in liquid cultures and then were transferred to an agarose pad, nanotubes were only transiently visible and then appeared to destabilize over time.

To successfully attach to other bacterial cells in an agitated, liquid environment, E. coli needed not only to be able to recognize suitable cells, but also required an efficient mechanism to capture detected cells. In our experiments, amino acids released from cross-feeding genotypes could have served as a chemical cue to recognize suitable host cells[42–44]. However, it remains unclear whether E. coli utilizes chemosensory mechanisms to specifically identify target cells or whether the binding of nanotubes is rather promiscuous. Our finding that E. coli readily accepted A. baylyi as a suitable host suggests metabolic suitability rather than kin discrimination mechanisms[45] governed the choice of E. coli. Identifying the molecular mechanisms that regulate the attachment of two bacterial cells as well as degree of specificity that underlies this process are exciting areas of research that should be addressed in the future.

Starving cells that were part of the intercellular network `imported' certain metabolites, while other cells functioned as a source for the required compounds. Depending on the net benefit incurred to the cells involved, the spectrum of possible ecological interactions that could result from this attachment process ranges from truly parasitic, where mainly the receiver benefits, to mutualistic interactions, in which both parties benefit[46]. Although in this study the ecological interactions were synthetically contrived, the observation that E. coli but not A. baylyi was capable of satisfying its metabolic requirements by connecting itself to other bacterial cells was not experimentally arranged. The discovery that the loss of conditionally essential biosynthetic genes induced the formation of nanotubes (Fig. 4) represents a previously unknown strategy of E. coli that may help starving cells to survive in their natural environment.

Given the widespread occurrence of pili, nanotubes, or other mechanisms bacteria utilize to exchange molecules, the intentional or enforced establishment of intercellular connections to engage in cooperative and parasitic nutritional interactions may represent a common ecological strategy pursued by bacteria. One of the best-studied examples of a metabolic parasitism involves two species of archaea: Nanoarchaeum equitans grow attached to the surface of Ignicococcus hospitalis[47]. Probably as a consequence of this metabolic parasitism, N. equitans has a drastically reduced genome size of only 0.5 megabases and thus depends entirely on its host for provisioning essential metabolites such as lipids, nucleotides, amino acids, and cofactors[48]. Interestingly, membranous structures connect host and symbiont and N. equitans obtains its amino acids from I. hospitalis[49], which strikingly parallels the observations of our study. Also the recent observation that a shortage of nutrients seems to trigger an exchange of cytoplasmic constituents between cocultured cells of Desulfovibrio vulgaris and Clostridium acetobutylicum[50] corroborates the interpretation that bacteria may commonly establish direct cell-cell connections to counter nutritional stress. Several other examples have been documented, in which bacteria and/or archaea engage in obligate, metabolic interactions[51,52]— virtually all of which rely on close, physical associations between interacting partners.

A direct intercellular transfer via nanotubes likely minimizes the loss of a given compound by diffusion, thereby reducing the physiological costs of producing the metabolite. Thus, nanotube-mediated cross-feeding is likely most relevant in nutrient-limiting or aqueous environments, whereas a diffusion of metabolites via the cell external environment should be more prevalent in nutrient-rich habitats, such as milk[53], or when the exchanged metabolite is released in sufficiently high concentrations (for example, as a metabolic by-product[54]). Furthermore, also for cells that are located within a close physical proximity such as a bacterial biofilm, it may be more efficient to transfer metabolites by diffusion[9] than to activate a contact-dependent transfer mechanism. However, it remains to be determined how intercellular networks affect the growth and metabolism of the cells involved, as well as which ecological factors favour their establishment.

Theory predicts for a population of cells that perform two vital functions that, phenotypic specialists should emerge when their cumulative costs are less than the cost for one cell to perform both tasks[55]. Indeed, analysing cocultures of two E. coli genotypes that both required a different amino acid to grow, yet produced increased amounts of others, revealed a significant fitness advantage of cooperative cross-feeding relative to prototrophic WT cells[56]. Especially, the tremendous fitness advantage auxotrophic bacteria gain when the required metabolite is supplied externally[56–58] should exert a strong selection pressure that favours cross-feeding of essential metabolites among bacterial cells. The enormous variation in gene content that is commonly found among different genomes of E. coli[59,60], together with the observation that often seemingly essential biosynthetic functions are lost[56] may reflect the ability of E. coli to compensate its metabolic deficiencies by connecting to other cells.

Our discovery that bacteria utilize intercellular connections to exchange nutrients and thus complement each other's metabolic requirements has significant implications for microbial ecology and physiology. The possibility that by connecting via nanotubes two or more bacterial cells can significantly extend their biochemical repertoire without the need for genetic change, suggests bacteria may function as multicellular, interconnected entities rather than as individual, physiologically autonomous units.

Methods

Strains and plasmids. Genetic targets, which would lead to metabolic auxotrophies for His and Trp (that is, hisD and trpB) or an overproduction of His and Trp (that is, hisL and trpR) upon deletion from the genomes of Acinetobacter baylyi ADP1 and Escherichia coli BW25113 were identified using the KEGG pathway database[61].

Acinetobacter baylyi ADP1 deletion mutants were constructed as described[62]. In brief, linear constructs of the kanamycin cassette with 5′-overhangs homologous to the insertion site were produced by PCR. For this, DNA of the plasmid pKD4 (ref. 63) was used as a template to amplify the kanamycin resistance cassette (Supplementary Table 1). Upstream and downstream regions homologous to hisD, trpB, hisL, and trpR were amplified using primers with a 5′-extension that was complementary to the primers used to amplify the kanamycin cassette (Supplementary Table 1). The three resulting products were combined by PCR to obtain the kanamycin cassette fused to the upstream and downstream homologous overhangs. Natural competence of A. baylyi was utilized to transform the linear fragments into the WT. Transformation was done by diluting 20 ml of a 16-h-old culture grown in LB medium. This diluted culture was incubated at 30 °C with shaking. Fifty ml PCR mix containing the deletion cassette was added to this culture and incubated at 30 °C with shaking for 2 h. Finally, the culture volume was concentrated to 100 ml, plated on LB agar plates containing kanamycin, and incubated at 30 °C for colonies to appear. To construct double-deletion mutants, the kanamycin resistance cassette was removed from the receiver's genome. For this, upstream and downstream regions homologous to hisD, trpB, hisL, and trpR were amplified using primers with a 5′-extension that were complementary to each other (Supplementary Table 1).

E. coli BW25113 (ref. 64) was used as WT, into which deletion alleles from existing single-gene deletion mutants[64] were introduced by P1 transduction[65]. Double-deletion mutants were constructed using auxotrophic mutants as receiver and amino acid overproducing mutants as donor strains. For this, the kanamycin resistance cassette was removed from the receiver's genome as described[63].

The cytoplasm of all mutant and WT strains were individually labelled with either pJBA24-egfp[24] or pJBA24-mCherry—two plasmids that constitutively express the ampicillin resistance gene bla, as well as a green (EGFP) or red (mCherry) fluorescent protein, respectively. pJBA24-mCherry was constructed by PCR amplifying mCherry using pFPV-mCherry[66] as a template and oligonucleotide primers mCherry-SphI (5′-ATATAGCATGCTGAGCAAGGGCGAG-3′) and mCherry-HindIII (5′-CTCTCAAGCTTACTTGTACAGCTCGTCCATG-3′) (restriction sites are underlined). The resulting amplicon was digested with SphI and HindIII, and the mCherry-containing fragment ligated between an artificial ribosomal-binding site II (RBSII) and transcriptional terminators T_0 and T_1 of pJBA24 (ref. 67). Finally, all strains were transformed using either plasmid. All strains and plasmids used are listed in Supplementary Table 2.

Culture conditions and general procedures. In all experiments, cells were grown in minimal medium for Azospirillium brasilense (MMAB)[68] without biotin, using fructose instead of malate as a carbon source. The required amino acids (that is, His and Trp) were supplemented singly or together if necessary (100 mM each). Replicate precultures were started with individual colonies picked from freshly streaked LB agar plates that have been incubated for 24 h. The next morning, precultures were diluted to an optical density $(OD)_{600\ nm}$ of 0.1 and subsequently 10 ml (≅10^5 CFUs) were used to inoculate 1 ml MMAB medium. Amino acids in the abovementioned concentration were added to the MMAB medium as specified. All cultures were incubated at 30 °C for 24 h under shaking conditions (230 r.p.m,) in 96-deep-well plates (max. volume: 2 ml, Thermo Scientific Nunc) or (100 r.p.m.) in `Nurmikko cells' (see `Population separation experiment' below). Both the EGFP- and the mCherry-labelled variants of the focal genotypes were used in all experiments, unless specified otherwise. Antibiotics were used at the following concentrations: kanamycin 50 mg ml^{-1} and ampicillin 100 mg ml^{-1}.

Supplementation experiment. To identify whether auxotrophs and cross-feeders of both species required amino acids to grow, the corresponding strains were cultivated in 1 ml of unsupplemented or amino acid-containing MMAB medium (one amino acid). Cultures were plated on LB agar plates at 0 h and after 24 h, and the number of CFUs quantified. This experiment was replicated eight times.

Amino acid quantification using biosensors. The two amino acid auxotrophic E. coli strains DhisD and DtrpB were used as biosensors[24] to determine the production levels of His and Trp in growing cultures of WT, auxotrophs, overproducers, and cross-feeders. For this, precultures of all donor genotypes were inoculated (1:1 ratio) together with one of the two auxotrophs in 1 ml of MMAB medium. After 24 h, the population size of auxotrophs was determined by plating cocultures on LB agar plates that did or did not contain kanamycin. This experiment was replicated eight times per biosensor–donor combination.

Amino acid quantification by liquid chromatography. The amount of His and Trp the two cross-feeding genotypes of both A. baylyi and E. coli released during 24 h of growth in the external environment was quantified by liquid chromatography (LC). For this, eight replicate populations of all EGFP-labelled strains were grown separately in 1 ml MMAB medium containing either His or Trp (100 mM). After incubation, cultures were centrifuged (3,800g for 20 min) and the supernatant immediately used for amino acid measurements.

Histidine analysis was performed by reversed-phase high-performance LC on an Agilent 1100 HPLC system (Agilent Technologies, Böblingen, Germany) on a XDB-C18 (50 × 4.6 mm, 1.8 mm, Agilent Technologies) column with o-phtaldialdehyde-mercaptoethanol (OPA) pre-column derivatization. Each sample was pre-mixed with sodium borate buffer (0.5 M, pH 11) to a final volume of 100 ml. Derivatization was performed automatically by an Agilent 1100 autosampler (Agilent Technologies) that added 30 ml of a mixture of OPA (85 mM) and b-mercaptoethanol (130 mM) to the sample, incubated it for 2 min at room temperature and loaded 30 ml onto the column. The mobile phase consisted of a mixture of solvent A (10 mM ammonium acetate) and solvent B (acetonitrile). In the beginning, 10% of solution B was used, which rose to 20% in the course of 10 min. The flow rate was set to 0.8 ml min^{-1}. After 10 min, the mobile phase was switched to 100% solvent B for 4 min. After that the column was reconstituted for 3 min with 10% of solvent B before the next analysis cycle started. The amino acid-OPA derivatives were quantified using a fluorescence detector (Ex: 340 nm, Em: 445 nm). The calibration was done in the same way using a histidine standard in concentrations ranging from 0.005 to 0.05 mM.

For tryptophan analysis, supernatant samples were diluted in a ratio of 1:10 (v:v) in water containing the ^{13}C, ^{15}N-labelled amino acid mix (Isotec, Miamisburg, OH, USA) and directly analysed by LC/MS/MS. The analysis method was modified from a protocol described by Jander et al.[69]. Chromatography was performed on an Agilent 1200 HPLC system (Agilent Technologies). Separation was achieved on a Zorbax Eclipse XDB-C18 column (50 × 4.6 mm, 1.8 mm, Agilent Technologies). 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}. The column temperature was maintained at 25 °C. The LC 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 ionspray voltage was maintained at 5,500 eV. 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 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) was used for data acquisition and processing. All samples were spiked with ^{13}C, ^{15}N-labelled amino acids (algal amino acids ^{13}C, ^{15}N, Isotec) at a concentration of 10 mg of the mix per ml. Trp was quantified using ^{13}C, ^{15}N-Phe as internal standard applying a response factor of 0.42.

Population separation experiment. To determine whether physically separating cross-feeding genotypes prevents the reciprocal exchange of amino acids, a device was designed and constructed (Supplementary Fig. 2) that allowed to separate two populations of bacterial cells with a membrane filter (0.2 mm, polyethersulfone, Pall GmbH, Germany). We named the device `Nurmikko cell' after Veikko Nurmikko, a Finnish biochemist who developed a similar apparatus in 1957 (ref. 70). Pairs of cross-feeders were inoculated (1:1 ratio) in separate growth chambers of a Nurmikko cell (total volume: 8 ml), which were or were not separated by a filter membrane. Each combination (that is, pair of cross-feeder with or without filter) was replicated four times. The entire assembly was incubated at 30 °C for 24 h under shaking conditions (100 r.p.m.). The density of each cross-feeding partner was determined at the onset of the experiment and after 24 h by plating on MMAB plates containing selective amino acids (His or Trp).

To test whether the membrane filter can hinder the diffusion of amino acids between two growth chambers of a Nurmikko cell (Supplementary Fig. 2), each labelled variant of the individual cross-feeding strains was inoculated in one side of a Nurmikko cell. Afterwards, 80 ml of a 10-mM amino acid solution the genotype required for growth (that is, His or Trp) was supplemented either into the same growth chamber or, alternatively, across the filter membrane (final amino acid concentration: 100 mM). Cultures were incubated as before and plated on LB agar plates to estimate CFU counts at 0 h and after 24 h. This experiment was replicated four times.

A control experiment was performed to test whether the growth of unseparated cross-feeders could be explained by a release of amino acids into the external growth environment. For this, the amount of His and Trp released by the GFP-labelled variants of the cross-feeding mutants of both species during 24 h of growth was determined by LC. Then, cross-feeding genotypes were cultivated for 24 h in saturating concentrations of either His or Trp (that is, 100 mM), a concentration that significantly exceeded the amounts cross-feeding genotypes would have produced during 24 h of growth (that is, 0.5 mM), or unsupplemented MMAB medium. After 0 and 24 h, cultures were plated on unsupplemented and kanamycin-containing LB agar plates and the number of CFUs was determined. This experiment was replicated eight times.

Plasmid transfer experiment. To determine whether plasmids are transferred between cells, pairs of cross-feeding genotypes within E. coli and two combinations of A. baylyi and E. coli were co-inoculated (1:1 ratio) in MMAB medium. However,

only one of the two cross-feeding genotypes was labelled with the pJBA24-egfp plasmid, which also expressed ampicillin resistance (that is, bla gene). The population density of each cross-feeder was determined after 0 and 24 h by plating dilution series of vortexed cocultures on MMAB agar plates that contained either His or Trp, and the frequency of plasmid carriage was quantified by supplementing the amino acid-containing agar plates with ampicillin or no antibiotic as control. The proportion of EGFP-labelled cells at 0 h and after 24 h in each of the cocultures was determined by flow cytometry. This experiment was replicated three times.

Flow cytometry. The proportion of mCherry-, EGFP- and double-labelled cells (that is, cells containing both EGFP and mCherry) within a given coculture was determined by flow cytometry. To this end, cells were excited at 488 nm by a blue solid-state laser at 20 mW and at 561 nm by a yellow solid-state laser at 100 mW using a Partec CyFlow Space flow cytometer (Partec GmbH, Germany). Green and red fluorescence was detected at 536 nm (FL1) and 610 nm (FL3), respectively. Non-fluorescent E. coli WT culture was used as control to set the gates for EGFP- and mCherry-positive cells. Data acquisition and analysis was done using the FlowMax software (Partec GmbH).

Flow cytometric analysis of cytoplasmic protein transfer. Pairs of cross-feeding genotypes, whose cytoplasms were differentially labelled with a plasmid containing either egfp or mCherry were co-inoculated (1:1 ratio) in 1 ml MMAB medium. After 24 h, cocultures were diluted to $OD_{600\ nm}$ 0.01 and 20 ml of this culture transferred to 1 ml of fresh MMAB medium. Cocultures were sampled after 0, 12, 24 and 48 h and the number of single- and double-labelled cells was determined by flow cytometry. This experiment was replicated eight times. The same experiment was repeated with all three combinations of cross-feeders within E. coli and between A. baylyi and E. coli, yet this time cocultures were sampled at a much finer temporal resolution, namely after 0, 3, 6, 9, 12, 14, 15, 16, 17, 18, 19, 20, 22, 24 and 48 h, and the number of single- and double-labelled cells was determined as before. This experiment was replicated eight times.

Two control experiments were performed to verify whether in the flow cytometrical measurements, double-labelled cells result from the sticking together of two individually labelled cells. First, the proportion of double-labelled cells was determined at 0 and at 24 h after applying no treatment, vortexing for 1 min or treating cells with a commercially available cell dissociation solution (FACSmax, Genlantis, USA). This experiment was replicated eight times. Second, the FSC of all double cells within a given coculture, as well as of E. coli WT labelled with pJBA24-egfp was determined after 24 h. In addition, the FSC of 1- and 2-mm-diameter microspheres (Molecular probes Inc., USA) was quantified. These measurements were replicated six times.

The effect of amino acid availability on the exchange of cytoplasmic constituents between cross-feeding genotypes was determined for all pairs of double-deletion mutants, except the intraspecific comparison within A. baylyi. All cross-feeders were individually precultured and after 24 h diluted to an $OD_{600\ nm}$ of 0.01. Ten ml of these dilutions were used to co-inoculate two cross-feeding genotypes (1:1 ratio) in 1 ml of MMAB medium. After 24 h, the proportion of double-labelled cells in all cocultures was analysed by flow cytometry. This experiment was repeated four times.

The role of the amino acid auxotrophy- or overproduction-causing mutations for inducing the nanotube-mediated exchange of cytoplasmic constituents was investigated by coculturing all possible pairs of EGFP- and mCherry-labelled variants of WTs, auxotrophs, overproducers and cross-feeders of both species, as described before. After 24 h, the percentage of double-labelled cells was quantified by flow cytometry. This experiment was replicated four times.

Autofluorescence of EGFP- and mCherry-expressing cells. To confirm that EGFP- and mCherry-labelled cells fluoresce only at the expected wavelength, all cross-feeding genotypes expressing EGFP and mCherry were inoculated in MMAB medium supplemented with ampicillin, as well as the required amino acid (His and Trp). After 0, 24 and 48 h of incubation, the emission levels of red and green fluorescence was determined by flow cytometry in all eight replicate cultures.

Fitness of EGFP- and mCherry-expressing cells. Competitive fitness experiments between the two variants of the same cross-feeding genotype labelled with either the pJBA24-egfp or pJBA24-mCherry plasmid were co-inoculated in equal densities ($\approx 10^5$ CFUs ml^{-1} each) in MMAB medium containing the required amino acid (that is, His or Trp), which was supplemented with ampicillin. After 0 and 24 h, CFU numbers were determined by plating on LB plates with ampicillin. EGFP- and mCherry-expressing cells were distinguished based on the colour colonies developed on LB agar plates (mCherry: pink; EGFP: green). Competitive fitness of the differentially labelled cross-feeding genotypes was determined by calculating the Malthusian parameter (M) of both genotypes: $M = (\ln (N_f/N_i)/24)$, where N_i is initial number of CFUs at 0 h and N_f is the final CFU count after 24 h[71]. Relative fitness was calculated as the ratio of Malthusian parameters. This experiment was replicated four times.

Cytoplasmic exchange in shaken and static conditions. To test whether static culture conditions enhance the cytoplasmic constituents, pairs of cross-feeding strains, whose cytoplasms were differentially labelled with EGFP and mCherry, were co-inoculated (1:1 ratio) in 1 ml MMAB medium. These cocultures were either incubated at 30 °C under shaken conditions (230 r.p.m.) or in unshaken, static conditions. Cocultures were sampled after 0 and 24 h and the numbers of single- and double-labelled cells were determined by flow cytometry, and the number of CFUs quantified by plating on LB agar plates. This experiment was replicated three times.

Fluorescence microscopy. The emission of red and green fluorescence from individual cells was confirmed by fluorescence microscopy. For this, all possible pairs of EGFP- or mCherry-labelled cross-feeders were co-inoculated (1:1 ratio) in 1 ml of unsupplemented or amino acid-supplemented MMAB medium (that is, both His and Trp). After 24 h, samples were drawn and a drop was fixed on a microscopy slide. An Axio Imager Z1 Zeiss microscope (Carl Zeiss AG, Jena, Germany) was used to observe bacterial cells. Images were analysed using the software AxioVision LE Rel. 4.4 (Carl Zeiss AG).

Fluorescence time-lapse microscopy. Cells were grown in liquid medium as above and thereby allowed to induce nanotube formation. Subsequently, cells were spread on a gel pad 1.5% Ultra Pure Agarose (Invitrogen, Darmstadt, Germany). Images were taken on an inverted epi-fluorescence microscope (Delta Vision Elite Imaging System, GE Healthcare) using a × 100, 1.4 numerical apperture UPlanSApo objective (Olympus) and a PCS Edge sCMOS camera. Focus was maintained using the Ultimate Focus system. Samples were imaged every 2 min using brightfield and fluorescence illumination, respectively, by applying the following filter and exposure conditions: GFP (475/28 nm, 525/50 nm) for 25 ms at 5% intensity, mCherry (575/25 nm 632/60 nm) for 15 ms at 10% and POL (5 ms, 32%). Under these exposure conditions, single-labelled cells did not show fluorescence in the respective other channel. For display, image stacks were split into three channels (red, green, and grey), converted into single images and merged for a specific time point using the Fiji software[72]. To visualize nanotubes, brightness and contrast settings were adjusted and a local contrast enhancement (CLAHE) followed by smoothening was applied in Fiji[72] to obtain the final output (Fig. 6a). Fluorescence intensities of a given cell were determined by first splitting the image into the different channels (green and red). Next, fluorescent images were individually merged with a brightfield microscopic image to determine the cell outline. A line was then mapped across the cell and a plot profile of 30 grey values was generated. Subsequently, the line was shifted to a region with no cells in a radius of 20 mm from the focal cell to determine the background fluorescence. For calculation of final values, the average background fluorescence was subtracted from the fluorescence intensities (grey values) of the cells. Fluorescence intensities were determined for each of the 17 frames (interval: 2 min).

Lipid dye experiment. To determine whether nanotubes were lipid-based, 5 ml of Vybrant DiO Cell-Labelling Solution (Invitrogen) were added to 1 ml of a 9-h-old coculture of the interspecific cross-feeding consortium, of which both strains were labelled with the mCherry-expressing plasmid. After that, cells were incubated for 30 min in the dark at 30 °C and subsequently imaged on agar pads using inverted epi-fluorescence microscopy as described above. Visualization of membrane structures was improved by applying a nonlinear histogram adjustment to fluorescence images using Fiji software[72].

Scanning electron microscopy. Cross-feeding strains were co-inoculated (1:1 ratio) in 1 ml of unsupplemented or amino acid-supplemented MMAB medium (that is, both His and Trp), and after 24 h of growth, sedimented on poly-L-lysine-coated (Sigma-Aldrich) glass coverslips. Samples were fixed for 1 h with 2.5% glutaraldehyde in sodium cacodylate buffer (0.1 M, pH 7.0) and dehydrated with ethanol in serially increased concentration, followed by critical point drying in a Leica EM CPD300 Automated Critical Point Dryer (Leica, Germany). Then, samples were sputter-coated with gold (layer 25 nm) in a BAL-TEC SCD005 Sputter Coater (BAL-TEC, Liechtenstein) and analysed at different magnifications with a LEO 1530 Gemini field emission scanning electron microscope (Carl Zeiss) at 5 kV acceleration voltage and a working distance of 5 mm using an in-lense secondary electron detector.

Statistical analysis. Growth of a given (co-) culture during 24 h was expressed as `cell count', which refers to the number of CFUs reached after 24 h minus the CFU number at 0 h. `Net cell count' is the cell count a given strain reached in coculture minus its cell count in monoculture. Statistical differences between two experimental groups were identified using paired t-tests. Wilcoxon signed rank tests were performed when variances were inhomogeneous. Univariate analyses of variance followed by a LSD post hoc test were used to compare more than two experimental groups. Statistical differences in the proportion of EGFP-labelled cells at 0 and 24 h were identified using a replicated G-test of goodness-of-fit. One-sample t-tests were performed to test whether the competitive fitness of differentially labelled cross-feeding genotypes deviated significantly from 1 (that is, no fitness difference).

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Author contributions

S.P., S.S. and C.K. conceived and designed the study. S.P., S.S. and L.F. performed all experiments. S.P., S.S., L.F. and C.K. analysed and interpreted the results. F.B. constructed pJBA24-mCherry. C.C. and M.W. helped with recording electron micrographs. I.B.B. helped with single-cell imaging and analysing microscopic data. C.K. wrote the manuscript with contributions from all authors.

Additional information

Accession codes: The unprocessed raw image files used to compile Supplementary Movie 1 and generate Fig. 7b,c are available at the Dryad Digital Repository (http://doi.org/10.5061/dryad.ds68k).

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Chapter 8: Manuscript II

Metabolic coupling in bacteria

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Summary: Here the question of how cellular metabolism deals with the process of metabolite exchange between cross-feeding cells is answered. Single gene deletion mutants of *E. coli* were used that render them auxotrophic for amino acid (recipient) or producers of amino acid (donor). A co-culture of the recipient and donor illustrated a contact-dependent exchange of amino acid between the two partners. Using a combination of, (i) co-culture experiments, (ii) cell internal amino acid sensors and (iii) transcriptional activity sensors, changes in the cytoplasm of both partners were quantified over the time. The results from this study provide a biochemical explanation for the establishment of cross-feeding consortia in nature.

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.

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

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

MATERIALS AND METHODS

Strains and plasmids used in the study

Escherichia coli BW25113 was used as wild type, from which mutants that overproduce amino acids *(Δmdh, ΔnuoN, ΔhisL,* and *ΔtrpR)* and mutants that are auxotrophic for histidine (*ΔhisD*), lysine (*ΔlysR*), or tryptophan (*ΔtrpB)* 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 [18]) 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 supplemental experimental procedures for plasmid construction and characterization of reporter constructs).

Culturing methods and general procedures

Minimal media for *Azospirillum brasiliense* (MMAB) [19] 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) [20] 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, *Δmdh*, *ΔnuoN*, *ΔhisL,* and *ΔtrpR*) was individually paired with each recipient (i.e. *ΔhisD*, *ΔlysR,* and *ΔtrpB*) 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 combination was replicated 4-times for both experimental conditions (i.e. with and without filter).

Flow cytometric analysis of cytoplasmic protein transfer

A previously established protocol was applied to identify a transfer of cytoplasmic material from donor to recipient genotypes [16]. For this, pairs of donor and recipient cells with differentially labeled cytoplasms (i.e. containing EGFP or mCherry) were co-inoculated into 1 ml MMAB. At the beginning of the experiment (0 h) and after 24 h of growth, the sample was analyzed in a Partec CyFlow Space flow cytometer (Partec, Germany). In the flow cytometer, cells were excited at 488 nm with a blue solid-state laser (20 mV) and at 561 nm with a yellow solid-state laser (100 mV). Green (*egfp*) and red (*mCherry*) fluorescence emission was detected at 536 nm and 610 nm, respectively. *E. coli* WT devoid of any plasmid was used as a non-fluorescent control. The number of single- and double-labeled cells in a population was quantified at both time points. Data analysis and acquisition was done using the FlowMax software (Partec GmbH, Germany). The experiment was conducted by coculturing eGFP-labelled donor with mCherry-labelled recipient gentoypes and *vice versa* in all possible combinations (i.e. each donor paired with each recipient, except in case of *ΔhisL* and *ΔtrpR*, which were only paired with *ΔhisD* and *ΔtrpB,* respectively) for 24 h. Each combination was replicated 4-times.

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.

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 (*ΔlysR*) 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 *ΔlysR* recipient.

RESULTS

Construction and characterisation of uni-directional cross-feeding interactions

To establish unidirectional cross-feeding interactions within *Escherichia coli*, five different genotypes served as amino acid donors: Two single gene deletion mutants (*Δmdh* and *ΔnuoN*) that produce increased amounts of several different amino acids [15], two deletion mutants that produce increased amounts of either histidine or tryptophan (*ΔhisL* and *ΔtrpR*) [21], as well as non-manipulated *E. coli* WT cells. Three genotypes served as recipients, which were auxotrophic for the amino acids histidine (*ΔhisD*), lysine (*ΔlysR*), and tryptophan (*ΔtrpB*) (Figure 1, Supplementary table 1) and thus essentially required an external source of these metabolites to grow [22].

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 *ΔnuoN* 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 *Δmdh* 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 *ΔtrpR* mutant were significantly elevated over WT-levels (Mann Whitney U-test: $P<0.05$, $n=4$, Supplementary figure 1). In contrast, *ΔhisL* 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 (Figure 2A-C). Auxotrophic recipients grew significantly better when cocultured with amino acid overproducers (*Δmdh*, *ΔnuoN*, *ΔhisL*, and *ΔtrpR*) 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 (Figure 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 (*Δmdh*, *ΔnuoN*, *ΔhisL*, and *ΔtrpR*) 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: $P<0.05$, $n=4$, Figure 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 (Figure 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 (Figure 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 (Figure 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 [23-25]. 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 [26, 27]. 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 (Figure 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* [28]. Introducing the plasmid-borne reporter construct (hereafter: *Lys-riboswitch*,

Supplementary figure 2) into the lysine auxotroph *ΔlysR* 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 (*ΔlysR*) and growing the resulting strain in lysine-supplemented media revealed consistently elevated levels of cytoplasmic lysine throughout the experiment (Figure 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, Figure 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, Figure 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 (Figure 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, *Δmdh*, and *ΔnuoN*. Each of these donor genotypes were then grown in monoculture as well as in coculture with the lysine-auxotrophic strain *ΔlysR*. 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 *ΔnuoN* 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 *Δmdh* 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 *Δmdh* and the *ΔnuoN* 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 (Figure 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: $P<0.03$, $n=4$, Figure 4C). After that lysine levels dropped significantly before increasing back to monoculture levels (Figure 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 *Δmdh* and *ΔnuoN* 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 (Figure 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 (Figure 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 [29, 30]. 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 [27], 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 [31] or by direct binding of the amino acid to the operon [32].

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 [33]. For analysing the focal cross-feeding interactions, the fusion constructs for *hisL* and *trpL* were selected, which sense the cytoplasmic concentration of histidine [34] and tryptophan [32, 35], respectively. Correlating GFP emission levels with the cytoplasmic concentration of the corresponding amino acid as quantified chemically via LC/MS/MS revealed a significantly negative relationship for both histidine ($r=-0.407$, $P<0.001$, Supplementary figure 3) and tryptophan ($r=-0.237$, $P=0.038$, Supplementary figure 3), confirming

the link between transcription of metabolic genes and the cytoplasmic concentration of the corresponding amino acids.

These promoter-GFP-fusion constructs were introduced into donor cells (i.e. WT, *Δmdh*, *ΔhisL*, and *ΔtrpR*), which were then cultivated for 24 hours in the absence or presence of the *ΔhisD* or *ΔtrpB* auxotrophic recipient cells. In line with expectations, donor strains WT, *ΔhisL*, and *ΔtrpR* displayed a starkly increased transcription of the respective biosynthetic operon in the presence of auxotrophic recipients as compared to donors growing in monoculture (FDR-corrected paired t-tests: $P<0.05$, $n=4$, Figure 5). Together, these results demonstrate that the presence of auxotrophic recipients significantly increased the amino acid production of donor cells. By withdrawing amino acids from the cytoplasm of donor cells, auxotrophic recipients prompted donor cells to readjust their amino acid levels by up-regulating the transcription of the corresponding amino acid biosynthesis genes.

DISCUSSION

Our study demonstrates for the first time that the deletion of a single metabolic gene from a bacterial genome can be sufficient to couple the metabolism of two previously independent bacterial cells. Auxotrophic cells that had lost the ability to autonomously produce a certain amino acid established intercellular nanotubes to derive the amino 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 [36]. An excess or deficit of metabolites within cells can disturb the cell-internal equilibrium and thus cause stress [37]. 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 [38, 39]. 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 [40, 41]. 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, 42, 43]. In many cases, this physical contact is followed by an exchange of cytoplasmic contents between interacting cells [16, 42, 44]. Structurally similar connections between cells are known to be involved in short- and long-distance communication in many multicellular organisms [45, 46]. 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 [47]. 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 [40] 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 [48]. 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.

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AUTHOR CONTRIBUTIONS

CK and SS 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.

Supplementary information accompanies this paper on The ISME Journal website (http://www.nature.com/ismej)

. FIGURES

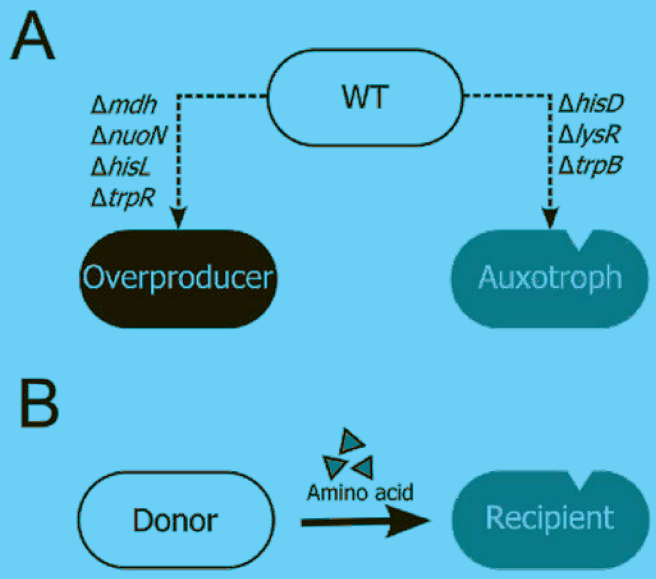

Figure 1. Experimental system used. (A) Design of genotypes. Single genes were deleted from *E. coli* BW25113 wild type (WT) to generate mutants that produce increased amounts of amino acids (overproducer, black) as well as mutants that essentially require a certain amino acid to grow (auxotroph, grey). (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.

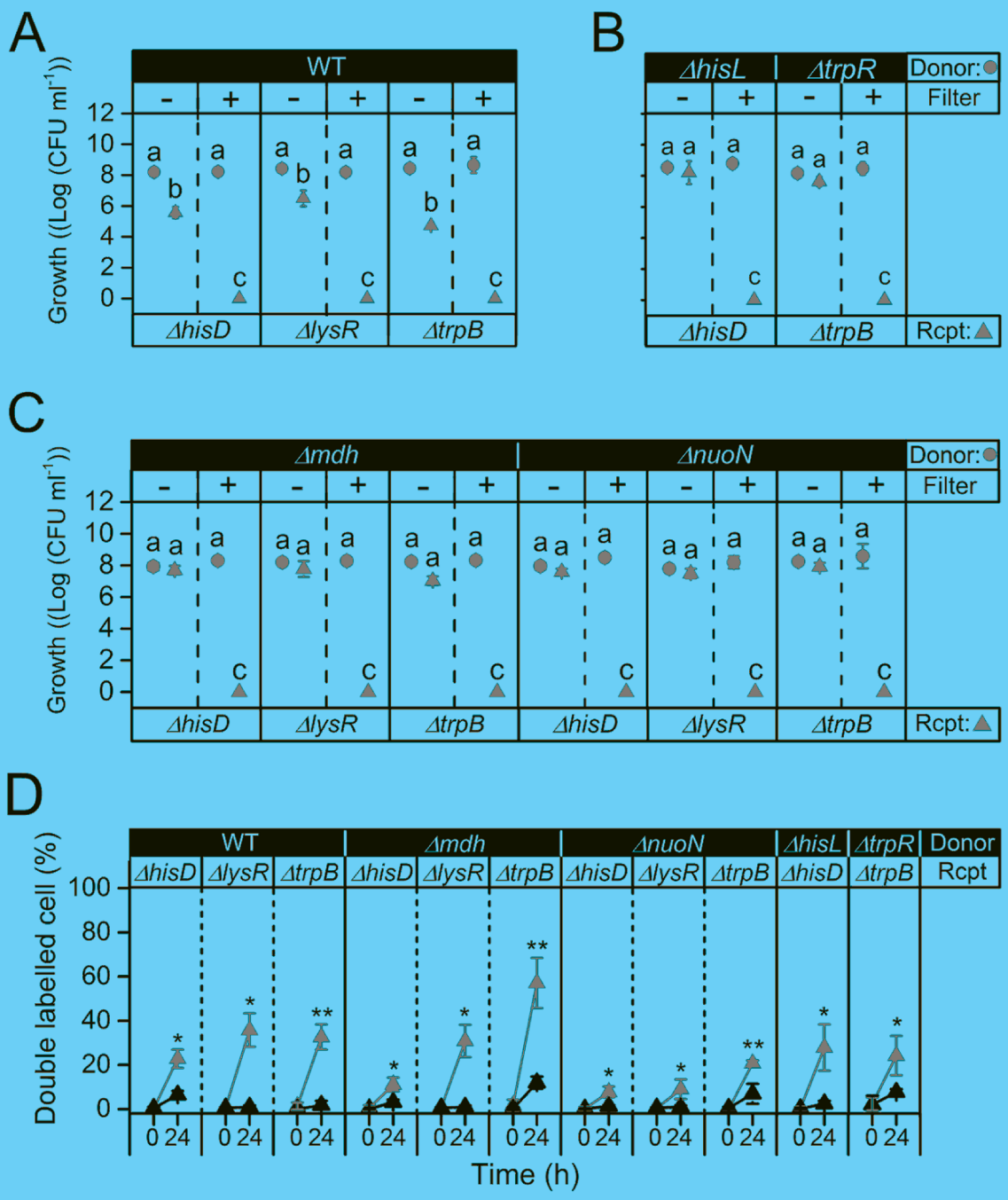

Figure 2. Contact-dependent exchange of cytoplasmic amino acids. (**A-C**) Amino acid exchange is contact-dependent. Amino acid donors (circles) were cocultured with auxotrophic recipients (Rcpt, triangles) either together in the same compartment (- Filter) or separated by a filter membrane (+ Filter) 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.

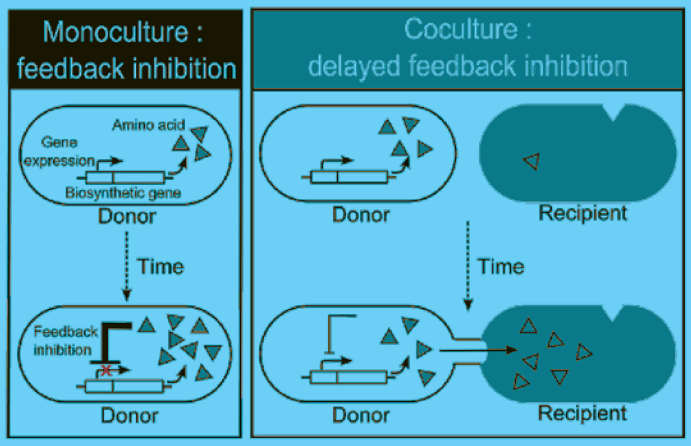

Figure 3. **Delayed feedback inhibition hypothesis.** In monoculture, amino acid concentrations in the cytoplasm of donor cells build up over time. When a certain concentration threshold is reached, these metabolites inhibit their own production by supressing the expression of the corresponding amino 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.

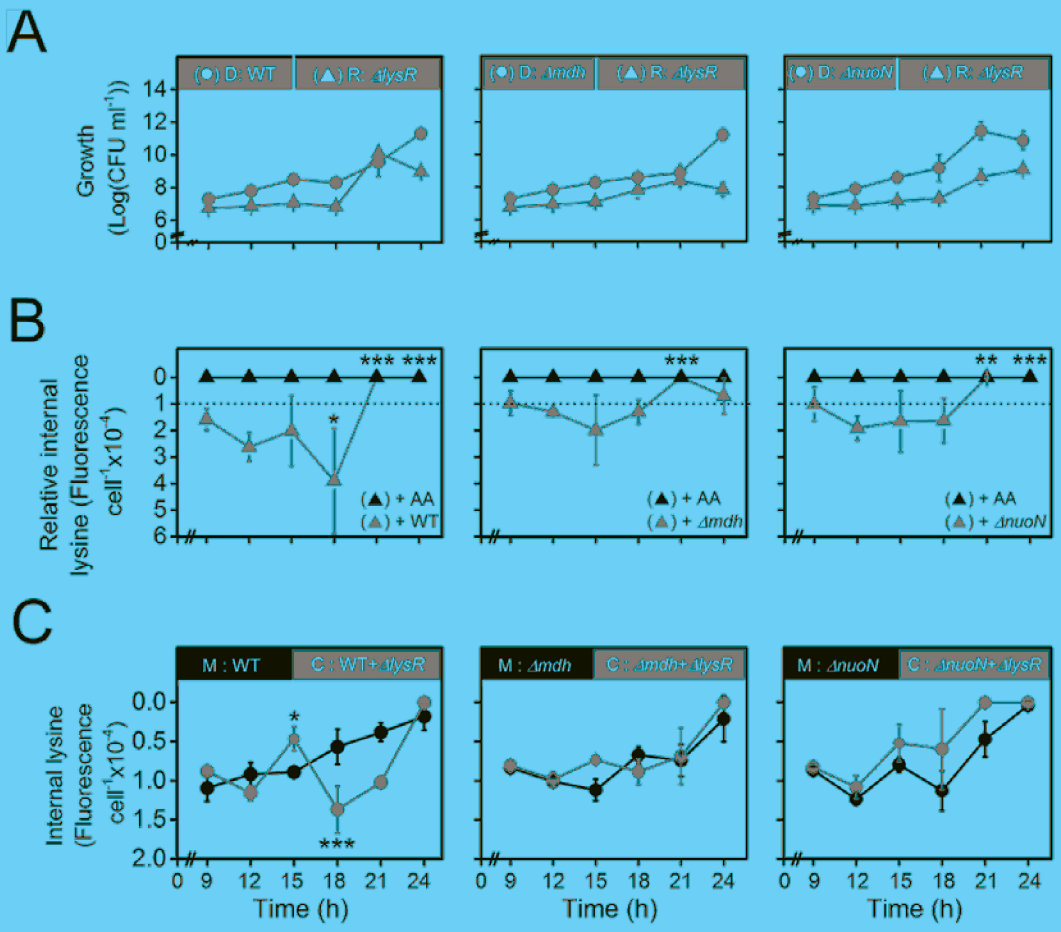

Figure 4. The presence of auxotrophs increases cytoplasmic amino acid levels in donor cells. (**A**) Growth of each partner in cocultures of donor (D, circles) and recipient (R, triangles) populations was determined as the number of colony-forming units (CFUs) ml^{-1} over 24 h. (**B, C**) Cytoplasmic 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 *Δmdh* and *ΔnuoN*, 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).

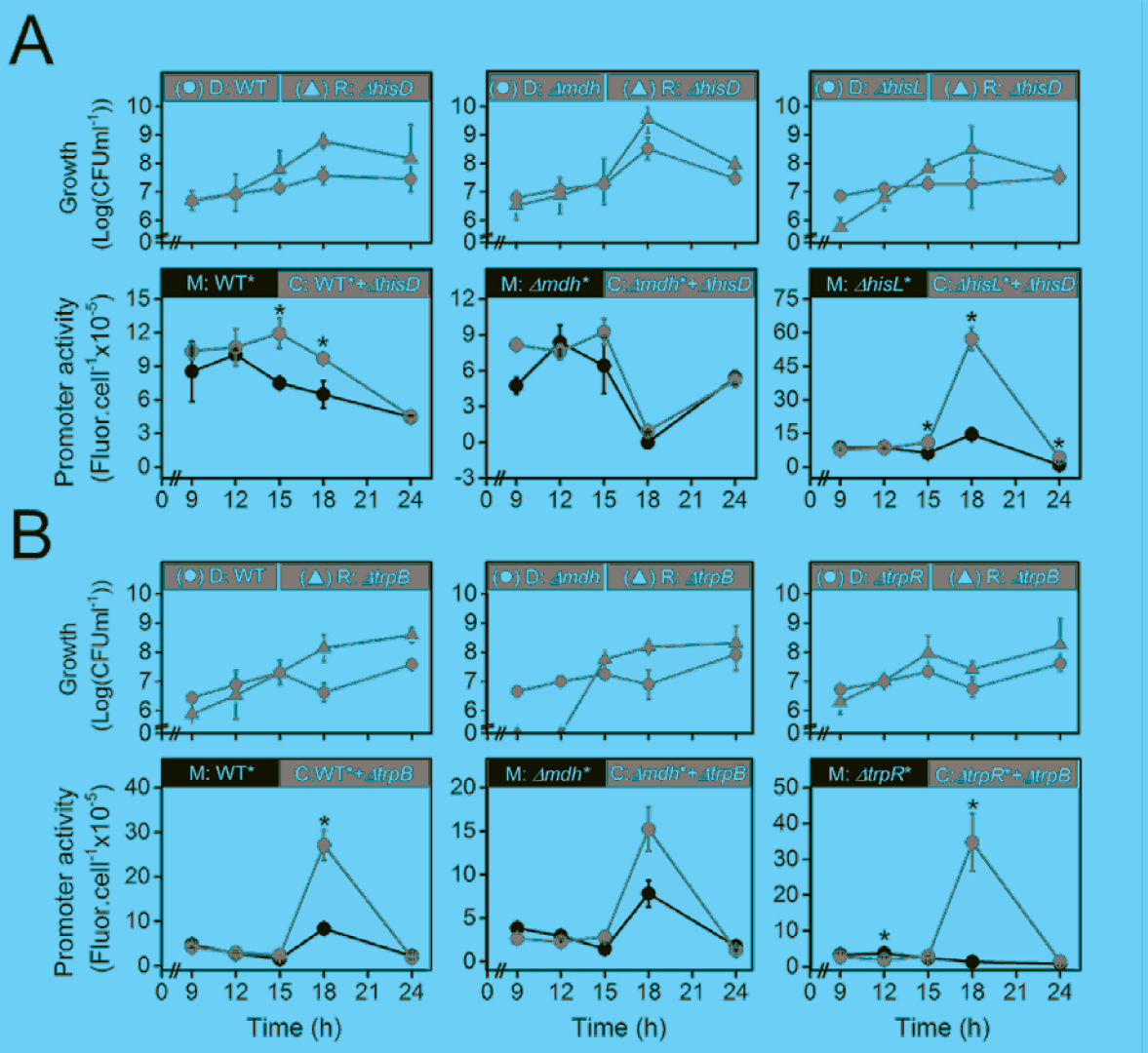

Figure 5. The presence of auxotrophs increases transcription of biosynthetic genes in the donor. (**A, B**) Top panels depict growth of donor (D, circle) and recipient (R, triangle) in coculture over time quantified as the number of colony-forming units (CFU) per ml. Bottom panels show promoter activity 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 (*ΔhisD*) or (**B**) the tryptophan auxotrophic recipient (*ΔtrpB*). In all cases, mean (±95% confidence interval) are shown.

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Chapter 9: Manuscript III

Transcriptional insights from metabolite cross-feeding bacteria

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Under preparation

Summary: The transfer of nutrients between cells through nanotubes requires the activity of several gene products and the coordination of different pathways. This study shines light on the transcriptional changes arising in *E. coli* cross-feeding genotypes resulting from, (i) amino acid starvation and (ii) the exchange of cytoplasmic contents through nanotubes. A combination of gene expression analysis and targeted gene deletions in different genotypic backgrounds followed by growth quantification was used. The results of this study indicate the involvement of c-di-GMP signaling and membrane vesicles in the process of cross-feeding in *E. coli*.

Transcriptional insights from metabolite cross-feeding bacteria

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ABSTRACT

Inter-cellular connections are widespread in nature. Through structures such as gap junctions, type secretion systems, membrane vesicles and pili, cells in a given environment exchange information in the form of chemical compounds. Contact-dependent interactions in microbial communities have been predominantly studied for the exchange of toxins, virulence factors or genetic elements. Recently, a phenomenon of contact-dependent exchange of essential nutrients between bacteria was observed. A synthetically engineered cross-feeding system of *Escherichia coli* and *Acinetobacter baylyi*, depicted the use of nanotubes to transfer cytoplasmic contents between complementary cross-feeding genotypes. Gene expression data revealed differential regulation of genes involved in cellular adhesion, chemotaxis, motility and membrane vesicle formation. The effect of individual gene targets was studied in a simplified system comprising of an auxotrophic recipient and over-producing donor genotype. In this unidirectional cross-feeding pair a potential role of c-di-GMP signaling in the aggregation of cells for enhancing the process of cross-feeding was identified. Furthermore, increased vesicle production by the auxotrophic recipient resulted in significantly higher amino acid uptake by the recipient. Interestingly, the increased uptake of amino acid by the auxotroph through vesicles was found to be independent of the donor genotype. This study implies the far-reaching effects of a single loss-of-function mutation in bacteria that live in communities with potential donor cells which exists often in naturally occurring populations.

INTRODUCTION

Cross-feeding in microbial communities, is the phenomenon in which one cell supports the growth of another cell. Growth support is provided to the recipient cell through by-products or end-products of a metabolic pathway, co-factors, low molecular weight compounds or ions [1, 2]. Species that depict cross-feeding have been shown to grow better when in co-culture with the interacting partner than in monoculture by division of metabolic labor [3, 4]. The interacting partner (recipient) behaves as a metabolic sink that takes up by-products resulting from the metabolic activity of the focal cell for growth. By constantly removing these by-products or ions, the concentration of the by-product is maintained at sub-inhibitory levels [5]. The focal cell is hence uninhibited by an accumulation of the by-product or end-product can grow better. Being part of a cross-feeding consortium also appears to be a beneficial and preferred lifestyle for many microorganisms in their natural habitat [2, 6-9].

Exchange of metabolites within cross-feeding consortia can take place in a variety of ways. The metabolites that are actively transported or undergo passive diffusion across the cell membrane are subsequently available for uptake in the environment [10]. This type of cross-feeding is considered to be diffusion-based cross-feeding between two partners in the same environment. Alternatively direct cell contact established between interacting partners also serves as a

transport channel. Organisms are often seen in close associations when grown in coculture indicating a role of cell adhesion in the process of metabolite exchange [11]. Experimental evolution of auxotrophic *Escherichia coli* genotypes depicted the formation of clusters during growth in liquid media [12]. Establishing connections between interacting partners is a way of positive assortment of reciprocally beneficial phenotypes, through the establishment of a feed-forward loop [13-15]. A contact-dependent mechanism for cross-feeding of nutrients could thus help in long term maintenance of the interaction as compared to a diffusion-based exchange.

Contact-dependent mechanisms for exchange of molecules have been employed by eukaryotic systems as well [16-18]. Eukaryotic multicellular organisms use inter-cellular channels like gap junctions for exchanging low-molecular weight compounds between adjacent cells [19, 20] whereas exosomes, plasmodesmata or tunneling nanotubes transfer larger components like organelles over long distances [21, 22]. Contact-dependent exchange can also be observed in symbiotic associations involving eukaryotic hosts and microorganisms. The plant rhizosphere, consisting of plant growth promoting rhizobacteria (PGPR), depicts colonization of the plant root by the PGPRs [23]. Upon colonization of the plant cell by the rhizobacterium, there is a transport of sucrose from plant phloem to the rhizobacterium. A close association between the plant cell and symbiont allows efficient transport of fixed nitrogen (in the form of ammonium) back to the plant cell. Contact-dependent exchange is hence common in the ecosystem.

Recently a contact-dependent exchange of amino acids was observed in cross-feeding genotypes of *Escherichia coli* and *Acinetobacter baylyi* [24]. Wildtype cells of each species had been genetically modified by introducing mutations that imparted: (i) dependence on external supply of one amino acid (auxotrophy) and (ii) increased production of a second amino acid (over-production). A combination of these two mutations in one background resulted in a cross-feeding genotype (Fig. 1). By pairing a given cross-feeder with another cross-feeder, harboring mutations in complementary amino acid biosynthetic pathways, the cross-feeders were capable of synergistic growth. The cross-feeders employed nanotubes for the exchange of cytoplasmic content when grown in shaken liquid medium devoid of an external supply of amino acids. The loss-of-function mutation resulting in auxotrophy in *E .coli* was found to be a prerequisite for nanotube formation. However changes in the transcriptome resulting from the auxotrophy were unclear making it challenging to provide a model for the process of amino acid cross-feeding via nanotubes. Furthermore, little was revealed about the structure and composition of the nanotubes through staining and microscopy. Membrane lipids were detected in the nanotubes upon staining with the Vybrant DiO solution. The biosynthetic processes leading to the inclusion of these membrane lipids in the nanotubes was unknown.

This study provides a holistic view of the changes in gene expression and consequently a determination of the pathways involved in the process of nanotube-mediated cross-feeding in *E.coli*. Gene expression analysis of cross-feeding genotypes in different conditions was carried out as a first step to identify changes in transcription during amino acid cross-feeding. Differential expression was observed for genes involved in: (i) lipid biosynthesis, (ii) biofilm formation, (iii)

toxin production, (iv) vesicle formation and (v) amino acid biosynthesis. Characterization of candidate genes was performed through deleting the gene in an auxotrophic genotype of *E. coli* resulting in a double deletion mutant. Several double deletion mutants depicted reduced growth in coculture with an over-producer genotype. Surprisingly, two of the candidate genes, namely *yhjH* and *degP*, upon deletion in the auxotrophic background resulted in increased growth of the auxotroph. Increased growth in co-culture of the *yhjH* double deletion mutant, combined with gene expression data indicates a role of c-di-GMP signaling in the process of cross-feeding. Furthermore by introducing the *degP* deletion in different genetic backgrounds (auxotroph and over-producer) of *E. coli*, the involvement of membrane vesicles in cross-feeding was identified. Taken together, our results show a coordination of different cellular processes during amino acid cross-feeding in *E. coli*. The fact that these processes are triggered upon nutrient starvation highlights the possibility of such interactions taking place between bacteria in nutrient-poor environments.

MATERIALS & METHODS

Strain construction

Cross-feeding genotypes of *Escherichia coli* were used for transcriptomic analysis from previous studies [24]. In short, *E. coli* BW25113 was used as the wildtype (WT), into which deletion alleles of amino acid biosynthesis genes were sequentially introduced via P1 phage transduction [25]. The auxotrophic mutation (*ΔhisD* or *ΔtrpB*) was first introduced into the WT resulting in the auxotrophic genotypes. These auxotrophic genotypes then served as the background strain for introducing the overproduction mutation (*ΔtrpR* or *ΔhisL*). Before introduction of the second mutation (overproduction) the kanamycin resistance cassette was removed from the auxotrophic receiver strain using the pCP20 plasmid with the method described elsewhere (Datsenko and Wanner, 2000). All generated strains have been represented in figure 1.

Candidate genes obtained from the analysis of the gene expression data were tested for involvement in growth through cross-feeding by deleting the genes in different backgrounds. These double deletion mutants for the selected genes were also constructed using P1 phage transduction. The auxotrophic *E. coli* (*ΔhisD* or *ΔtrpB*) were used as recipients and the Keio collection strains were the donors. The confirmation of double mutants was done through growth measurements in presence and absence of amino acid (for the auxotrophy) and PCR (for the second mutation). Two sets of primers were used for PCR confirmation as described in Baba *et al.*, 2006 forward and reverse primers of the target gene as well as K1 and K2 primers for the kanamycin resistance cassette. The K1 and K2 primers bind to the center of the sequence in the kanamycin resistance cassette. In order to confirm the deletion the forward primer was paired the K1 primer whereas the reverse primer was paired with the K2 primer to obtain two separate PCR fragments. Primer sequence are provided in supplementary table 1.

Growth media and culture conditions

All cultures were grown in minimal media (MMAB) without biotin, with fructose (5 g L^{-1}) as a carbon source. Media supplemented with amino acid (histidine or tryptophan) at a final concentration of 100 µm was used for the growth of auxotrophic genotypes in monoculture. All genotypes were first precultured by inoculating a single colony in 1 ml MMAB for 18 h, followed by adjusting the optical density (OD_{600}) to 0.1. The final cultures were prepared by inoculating 10 µL of the above adjusted preculture into 1 mL of MMAB liquid, in case of cocultures 5 µL of each partner was inoculated into 1 mL MMAB liquid. This dilution of the cultures results in a starting population size of $1*10^5$ cells ml^{-1}. All cultures were incubated at 30°C in shaking conditions (220 rpm). A total cell count was obtained through spread-plate technique on solid media (MMAB agar). The solid medium was modified by addition of either kanamycin (50 µg ml^{-1}) or amino acid (100 µM) as required for double deletion genotypes and auxotrophic genotypes. The cultures were appropriately diluted prior to spreading at 0 h and 24 h. A difference between the cell count at 0 h and 24 h was used to quantify the growth of a given genotype.

Experimental set-up for the microarray analysis

Gene expression profiling was done for six different conditions (Fig. 2) using the Agilent one color *E. coli* Gene Expression Microarray, 8x15K, which contains probes for the following strains: K12-MG1655, O157:H7 EDL933, O157:H7 VT2-Sakai, CFT073. Monocultures of cross-feeders (*ΔhisD ΔtrpR* and *ΔtrpBΔhisL*), were first grown in MMAB media supplemented with the respective amino acid (100 µM) for 18 hours in order to obtain a cell density of 0.2 OD_{600}. These monocultures were then centrifuged and washed twice with non-supplemented liquid MMAB. After washing the cultures were split into two conditions: (i) liquid MMAB supplemented with amino acid (histidine sample label H and tryptophan sample label T) and (ii) non-supplemented MMAB (samples labeled as Hs and Ts). These cultures were further incubated for 20 hours before harvesting for RNA extraction. In case of the coculture of cross-feeders, the individual strains were pre-cultured as described earlier. The cultures were then inoculated in a 1:1 ratio in two conditions: (i) MMAB media supplemented with amino acid (histidine and tryptophan, both at 100 µM, sample label CC) and (ii) non-supplemented MMAB (sample label CCs). The cocultures CC and CCs were harvested after 20 hours for RNA extraction. The total culture volume for each replicate of the described samples (H, T, Hs, Ts, CC, and CCs) was 80 mL and grown under 220 rpm shaking conditions at 30°C. All samples were in replicates of four.

RNA extraction, labeling, and hybridization

Each of the above six cultures (H, T, Hs, Ts, CC, and CCs) was centrifuged to obtain cell biomass for RNA extraction using the innuPREP RNA mini kit (Analytik Jena, Germany). The cells were lysed using lysozyme followed by centrifugation and filtration steps as mentioned in the protocol provided by the supplier to separate the total RNA. The purified RNA was quantified and tested

for purity using the 2100 Bioanalyzer (Agilent Technologies, CA, USA). The 2100 Bioanalyzer provides a RNA integrity number (RIN) to depict the quality of RNA using an algorithm that integrates the RNA electropherogram characteristics with the ribosomal subunits. Only the samples showing a RIN > 8.0 were selected for labeling. This purified RNA was labeled and analyzed with the One-color microarray-based gene expression analysis kit (Agilent Technologies, CA, USA). This method employs the low input quick amp labeling protocol. All required reagents were ordered and used as per kit provider protocols. Briefly, the RNA in the samples was converted to cDNA followed by conversion to cRNA and subsequent labeling with the Cy3 dye. This labeled cRNA was purified using the RNeasy minikit by Qiagen (Qiagen GmbH, Hilden, Germany) before application onto the microarray chip for hybridization. The labeled samples were tested for quality by calculating the amount of Cy3 label present per ng of RNA, following which only those labeled samples with a value greater than 5.0 pmol ng^{-1} were selected for application on the microarray chip. After 17 hours of hybridization at 65 degrees celcius, the chip was washed and scanned using an Agilent SureScan microarray scanner (Agilent Technologies, CA, USA).

Bioinformatics analysis

Scanning of the microarray chip resulted in a list of fluorescence values for each probe (15,208 probes in total) with the fluorescence intensity indicating the quantity of mRNA that bound to a given probe. A list was obtained for each of the 24 samples (6 conditions and 4 replicates). These lists of the fluorescence intensity values were initially tested for quality control and further analyzed using the GeneSpring software (provided by Agilent Genomics). The median normalized samples were first categorized by the following baseline selection: (i) media conditions (with or without amino acid), (ii) culture conditions (monoculture or coculture), (iii) type of amino acid auxotrophy (histidine or tryptophan), (iv) replicate number (1, 2, 3, or 4). Pairwise comparisons between the 6 different samples (H, T, Hs, Ts, CC and CCs) were carried out to identify differentially expressed (DE) genes detected in at least 3 of the 4 replicate samples. This set of DE genes was filtered by expression using a cut-off of 100 for the upper values and 20 for the lower values to remove background noise. This filtered set of DE genes was then checked with other pairs for significant overlap. The comparisons are illustrated in figure 3.

The above processing and filtration steps resulted in a set of 325 DE genes which were subjected to further analysis to identify gene ontology (using the PANTHER v.8.0 software) [26]. To identify the metabolic pathways affected, the KEGG Mapper (v.2.8) [27] was used whereas for the obtaining protein association networks the STRING software (v.10.0) [28] was used (supplementary figure 1). Finally to determine the gene regulatory networks affected by the differential expression of the 325 genes enlisted, the Regulondb (v.9.0) [29] was used.

Fitness experiments

The involvement of a gene in metabolite cross-feeding was tested by deleting the gene of interest in an auxotrophic recipient and measuring the effect of deletion on growth of the auxotroph. The deletion was introduced into the auxotrophic recipient (*ΔhisD* or *ΔtrpB*) or the donor (WT, *ΔtrpR* or *ΔhisL*) using P1 transduction as mentioned before. The double mutants were then paired with the respective recipient or donor as required and cocultured in a 1:1 ratio in liquid MMAB. The initial cell count at 0 hour was calculated by spread plate technique on selective plates (described in `growth and culture conditions'). After 24 hours of incubation the final count was determined again by serial dilution and plating. The initial cell count (CFU mL^{-1}) was subtracted from the final cell count (CFU mL^{-1}) to obtain the growth.

Vesicle fraction extraction

The double-deletion genotype *ΔhisDΔdegP* were used for obtaining a cell-free vesicle rich fraction. The strains were grown as monocultures in presence of histidine for 18 to 20 hours till an OD_{600} of 0.2 was obtained. These cultures were then washed twice with liquid MMAB and finally suspended in fresh MMAB medium without histidine. The cultures were further incubated in these amino acid starved conditions for 12 hours to induce vesicle production. Amino acid starvation in *E. coli* WT cells is known to induce hypervesiculation [30, 31]. Cells were centrifuged at 10,000 r.p.m. for 10 minutes to obtain a firm pellet. The supernatant was filtered (0.45 µM, polyethersulfone, Pall GmbH, Germany) to remove unsettled cells and large cell debris. This filtrate was used as the growth medium for cocultures of recipients (*ΔhisD*, and *ΔhisDΔnlpA*) with donor cells (WT and *ΔhisL*).

Scanning electron microscopy

Cross-feeding genotypes were co-cultured in 1 ml of liquid MMAB without amino acid supplementation for 24 h. 1 ml of culture was then fixed using a 2.5% glutaraldehyde solution prepared in a sodium cacodylate buffer (0.1 M, pH 7.0) for 1 h at room temperature. The same procedure for fixation was carried out for cocultures of auxotroph and over-producer genotypes. All fixed samples were allowed to sediment onto poly-L-lysine coated glass coverslips (Sigma-Aldrich) for an additional 1 h time period. The glass coverslips were sputter coated with gold layer (25 nm) in a BAL-TEC SCD005 Sputter Coater (BAL-TEC, Lichtenstein). The gold coated samples were visualized using a LEO 1530 Gemini field emission scanning electron microscope (Carl Zeiss, Jena) at 5 kV acceleration voltage and a working distance of 5 mm using an in-lens secondary electron detector.

Statistical analysis

All datasets containing growth of the different genotypes were tested for normal distribution using the Kolmogorov-Smirnov test (normal distribution considered if $P>0.05$). Homogeneity of variances was determined using Levene's test (variances considered homogenous if $P>0.05$).

The growth of double deletion mutants was compared to the single deletion strain (auxotroph or over-producer genotype) for significant difference using an independent sample t-test.

RESULTS & DISCUSSION

Microarray set-up for transcriptional analysis of *E. coli* cross-feeders

The aim of this study was to identify specific genes involved in the formation of nanotubes as well as in the process of metabolite cross-feeding. To this end, a transcriptional profiling of *E. coli* cross-feeding strains was done since this species depicted intra- and inter-species cross-feeding of amino acids. The cross-feeding genotypes (Fig. 1) were grown in conditions that induce uptake of nutrients from the partner (cross-feeding) in comparison to those in which the nutrients are taken up from the environment (Fig. 2). An external supply of amino acids was previously shown to decouple the otherwise obligate cross-feeding interaction. Hence, s complementary set of *E. coli* cross-feeding genotypes, *ΔhisDΔtrpR* with *ΔtrpBΔhisL*, was cocultured in media with amino acid (CC) and without amino acid (CCs) supplementation. Apart from the two conditions (CC and CCs) additional treatments were carried out to remove background noise and to check for inherent changes in gene expression when an auxotrophic *E. coli* is exposed to starvation. To achieve this, monocultures of the cross-feeders in conditions with histidine (H) or tryptophan (T) and without histidine (Hs) or tryptophan (Ts) were used as control (Fig. 2).

A large number (approximately 2000) of differentially expressed genes (DEGs) was obtained when the coculture conditions (CC vs CCs) were subjected to a pairwise analysis. This dataset of DEGs was processed through stepwise comparisons to acquire an enriched dataset of genes solely involved in amino acid cross-feeding (Fig. 3). Firstly, to filter out the effects of amino acid starvation the DEGs in the monoculture pairs, (H vs Hs and T vs Ts), were identified (Fig. 3A). These genes were tested for overlap with the DEGs in the coculture conditions (CC vs CCs). The genes that were common between the above monoculture (H vs Hs and T vs Ts) and coculture (CC vs CCs) comparisons would be those involved in amino acid stress irrespective of the presence of another genotype. Subsequently these genes were omitted from the set of DEGs in the coculture pair (CC vs CCs), resulting in a set of 350 genes (Fig. 3A). Apart from the effect of the amino acid scarcity, the effects due to the presence of another genotype were also filtered out (Fig. 3B). In order to do this the combined set of genes expressed in the coculture conditions (CC + CCs) was compared individually to those in monoculture with amino acid (H and T). The genes differently regulated in monocultures in presence of amino acids are in nutrient rich conditions where cytoplasmic exchange was not observed. Thus this set of genes was subtracted from the combined set ((CC + CCs) – H and (CC + CCs) – T). Furthermore, this refined set (CC + CCs) was compared individually to the genes expressed in monocultures without amino acid, Hs and Ts. This comparison yielded those genes that are differently regulated for cross-feeding of individual amino acids (histidine or tryptophan) as well as for cross-feeding in general. This

second approach of filtration for the presence of another genotype resulted in a list of 50 genes (Fig. 3B).

Upregulation of cell adhesion and c-di-GMP production

Previous studies on cross-feeding in *E. coli* provided three main structural insights [24], first that nanotubes connect cells. Secondly, nanotubes are composed of membrane lipids and lastly nanotubes were of varying lengths. Furthermore, *E. coli* depicted the formation of nanotubes in coculture with *E. coli* cross-feeding genotypes as well as *A. baylyi* cross-feeding genotypes. *A. baylyi* on the other hand was not able to exchange cytoplasmic content when paired with the other *A. baylyi* cross-feeding. These characteristics of *E. coli* and the nanotubes, hinted towards the involvement of specific functions in *E. coli*. As a result, the above enriched dataset was analyzed for genes involved in the following four categories: (i) cellular adhesion, (ii) chemotaxis, (iii) motility, (iv) vesicle formation and (v) synthesis of the membrane lipids and fatty acids (Fig. 4, supplementary figure 1). Candidate genes in these categories were deleted in the auxotrophic recipients, *ΔhisD* and *ΔtrpB*. These double deletion mutants were then tested for the ability to cross-feed amino acids by pairing with the complementary over-producer, *ΔhisL* or *ΔtrpR* (Fig. 4).

Five gene deletions led to a reduction in fitness in both the auxotrophic backgrounds when in co-culture with the over-producer genotype. Noteworthy among these were genes involved in cellular adhesion like *csgE*, *ppdA* and *ydeQ*. *csgE* is a regulator for the formation of curli fibers on the cell membrane [32], *ppdA* is a peptidase that is hypothesized to play a role in biofilm formation [33] and *ydeQ* is a fimbrial-like adhesion protein present on the cell surface [34]. Individual gene deletions of *csgE* and *ydeQ* are known to cause a decrease in curli formation and hence stickiness in *E. coli* WT. Cellular adhesion is highly beneficial in syntrophic consortia to form clusters of interacting cells [11, 14, 35]. By forming clusters the interacting partners are stable in a spatially unstructured environment and in close proximity which enhances molecule exchange. Moreover, fimbriae in *E. coli* are known for connecting cells at short distances like those seen in a biofilm based lifestyle. Similarly when an auxotrophic recipient is present with potential donor cells in a shaken liquid environment, cell adhesion could be beneficial, first for establishing a connection with a neighboring cell and second to maintain this connection.

Genes involved in chemotaxis and motility like *flgM*, *tap* and *ycgR*, upon deletion in the auxotroph resulted in a decreased fitness. Interestingly, the deletion of motor protein genes, *motA* and *motB*, did not have significant effects on the fitness, however the deletion of a molecular brake, *ycgR*, decreased fitness of the double deletion auxotroph compared to a single deletion auxotroph (Fig. 4). *ycgR* is known to play a role in reducing flagellar motor speeds upon c-di-GMP binding in the stationary phase [36]. An absence of *ycgR* has a two-fold effect: (i) higher motility in stationary phase and (ii) free c-di-GMP in the cytoplasm. Motility is beneficial for recipient cells to access the available space in search of a donor cell. Secondly c-di-GMP, a second messenger that binds to a various effector molecules, is known to promote sessile behavior and biofilm formation [37].

Recently c-di-GMP was also shown to play a role in intercellular communication in *Myxococcus xanthus* and *Burkholderia cepacia* for coordinated multicellular behavior [38].

C-di-GMP signaling enhances growth in co-culture

Transcriptomic analysis of *E.coli* cross-feeding genotypes indicated three DEGs that regulate the levels of c-di-GMP in the cell. First, the gene *yhjH*, responsible for cleavage and subsequent deactivation of c-di-GMP was downregulated in the cross-feeding coculture. Second, a diguanylate cyclase (*ydaM*/*dgcM*) which is responsible for the production of c-di-GMP was upregulated in the cross-feeding coculture. Third, the molecular brake, *ycgR*, which binds to c-di-GMP to reduce motility, was found to be downregulated in the cross-feeding coculture. The cumulative effect of changes in the gene expression of these three candidate genes would result in increased intracellular levels of c-di-GMP. An increase in c-di-GMP in *E. coli* is known to induce production of amyloid curli fibres by activating the transcription factor, *csgD*. Furthermore c-di-GMP also mediates the synthesis of the exopolysaccharide poly-β-1,6-*N*-acetyl-glucosamine (PGA) which trigger biofilm formation [37].

We hypothesized that increased intracellular levels of c-di-GMP would lead to increased cell adhesion between auxotrophic recipient and over-producing donor genotypes when in co-culture. Hence the introduction of a mutation that results in increased c-di-GMP production in either background (recipient or donor), should lead to increased cross-feeding and subsequently increased growth. To this end, the *yhjH* gene was deleted in both genotypes and tested for the effect of this deletion on growth. *ΔyhjH* when introduced into the recipient background, *ΔhisD*, and paired with a donor, *ΔhisL*, depicted a significant increase in growth (independent t-test, *** $P<0.001$, $n=4$). The recipient double-deletion mutant, *ΔhisDΔyhjH*, was able to cross-feed better in comparison to the single-deletion mutant recipient, *ΔhisD* (Fig. 5A). This increased growth of *ΔhisDΔyhjH* did not affect the growth of the donor negatively. Upon introduction of the *ΔyhjH* deletion in the donor genotype, *ΔhisLΔyhjH*, there was no change in the growth of the recipient, *ΔhisD* (Fig. 5A). Hence the presence of the *ΔyhjH* mutation only in the auxotrophic background has a benefit indicating potential epistasis between the auxotrophic mutation and *ΔyhjH*. Neither double-deletion mutants (*ΔhisDΔyhjH* or *ΔhisLΔyhjH*) depicted a reduced growth in coculture with amino acid indicating no basal effect on growth due to the mutation. Additionally the donor double mutant in comparison to the donor showed similar growth thus ruling out the possibility of epistatic effects of the over-production mutation and the *ΔyhjH* mutation.

A recent study of nanotubes produced by *B. subtilis* revealed the involvement of *ymdB*, a c-di-GMP phosphodiesterase and also a homologue of *yhjH* [39]. Interestingly here a *B. subtilis* donor produces *ymdB* to assist the exchange of the plasmid to a recipient cell. In contrast to this we see that the absence of *yhjH* leads to higher growth in *E. coli* auxotrophs. It is hence the cellular response mediated by effectors of c-di-GMP that play a role rather than the yhjH protein itself. A deletion of *yhjH* results in higher c-di-GMP levels, which induces the formation of curli fibres as

well as PGA molecules, both of which lead to an aggregative lifestyle. SEM imaging of the *ΔhisDΔyhjH* double mutant confirmed the presence of increased curli expression (Fig. 5B).

Presence of vesicles enhances cross-feeding

Scanning electron microscopy (SEM) of cross-feeding genotypes revealed the presence of membrane-bound spherical vesicles in the surrounding (Fig. 6A). In some cases these vesicles seemed tethered to the nanotubes bridging multiple cells together. Additionally SEM imaging of an auxotrophic recipient co-cultured with over-producer donor genotypes also depicted vesicles (Fig. 6B). Vesicle chain-like structures were also observed associated to cells in the auxotroph-over-producer coculture (Fig. 6C). In line with expectations, the vesicles were not observed when the donor-recipient pair was co-cultured in the presence of amino acid (Fig. 6D) indicating the role of vesicles in cross-feeding of amino acids.

Transcriptional analysis hinted towards the differential expression of genes regulating vesicle formation. An upregulation of the gene *nlpA*, an inner membrane lipoprotein anchoring point for new vesicles, was observed in cross-feeding cocultures. Moreover the downregulation of *degP*, a serine protease present in the periplasmic space was also observed in the same cocultures. A deletion of *degP* in *E. coli* K12 leads to accumulation of misfolded or aggregated proteins in the periplasm to which the cell counteracts by hyper-vesiculating (increased vesicle production than WT) [40]. Thus the combined effect of an upregulation of *nlpA* and downregulation of *degP* would lead to higher vesicle production in the cell. First, the effect of deleting these genes (*degP* and *nlpA*) in an auxotrophic background was determined. The deletion of *nlpA* in the *ΔhisD* auxotroph resulted in a decreased growth compared to the single-deletion auxotroph, when cocultured with the *ΔhisL* over-producer (Fig. 4). Subsequently, the deletion of *degP* in the auxotroph, *ΔhisD,* led to an increase in growth (Fig. 4).

There are two possible origins for the production of vesicles, (i) vesicles are produced by the donor genotype or (ii) vesicles are produced by the auxotrophic recipient. Proteomic analysis of outer-membrane vesicles (OMVs) produced by *E. coli* depicts the presence of proteins for amino acid transport [41]. Hence the OMVs produced by an over-producing donor cell would contain the focal amino acid. Alternatively, the OMVs produced by an auxotrophic recipient cell would not transport the focal amino acid into the vesicle. An increased production of OMVs in the donor background should hence result in increased growth of the auxotroph. This hypothesis was tested by introducing the deletion resulting in hypervesiculation, *degP*, in the auxotrophic recipient as well as the over-producing donor. The recipient double mutant (*ΔhisDΔdegP*) showed higher growth when compared to a single mutant recipient (*ΔhisD*) (independent samples t-test, * $P<0.05$, n=4, Fig. 7A). In this coculture of the double-deletion auxotroph (*ΔhisDΔdegP*) and the over-producing donor (*ΔhisL*), the donor also depicted slightly higher growth. No difference was observed in recipient (*ΔhisD*) growth when the *ΔdegP* deletion was introduced the donor genotype (*ΔhisLΔdegP*) (Fig. 7A); contrary to the expectation that hypervesiculation in the donor genotype should result in increased growth of the auxotroph.

Nutrient starvation in bacteria is known to induce the formation of OMVs [42, 43]. Auxotrophic recipient cells when cocultured with donor cells, in the initial growth phase, experience nutrient stress in the form of amino acid starvation. When a *degP* deletion is now introduced in an amino acid starved auxotroph, this double-deletion mutant (*ΔhisDΔdegP*) will produce a large quantity of OMVs. The over-producer genotype on the other hand does not face nutrient stress nor any other environmental stress (pH, temperature, antibiotics) which is also known to induce vesicle formation [44, 45]. Since all experiments were performed at 30°C, it is possible that the double-deletion in the donor background, *ΔhisLΔdegP,* did not result in an increased level of OMVs.

Vesicles increase growth of auxotrophic recipient in coculture independent of donor genotype

An increase in growth of the auxotroph due to an over-production of OMVs indicates the use of vesicles as something other than just transport of cytoplasmic content. A vesicle produce by the auxotroph that is devoid of the focal amino acid could act as building blocks for inter-cellular connections. Vesicles have been shown to fuse and form chain-like structures in *Myxococcus xanthus* for coordinating growth and exchanging signals between cells [46]. Similarly vesicles produced by an auxotroph could fuse together to initiate inter-cellular connections. To answer this question the vesicle fraction was separated from the auxotrophic double-deletion genotype (*ΔhisDΔdegP)*. Monoculture of the recipient double-deletion mutant, *ΔhisDΔdegP,* was grown and starved to induce higher vesicle production. An increased vesicle density was necessary to facilitate vesicle separation during ultra-centrifugation. The vesicle fraction from these cultures (*ΔhisDΔdegP*) was added to auxotrophic recipient (*ΔhisD*) as well as another recipient double-deletion mutant (*ΔhisDΔnlpA*) and cocultured with the over-producer genotype (*ΔhisL*) (Fig. 7B). The *nlpA* deletion in *E. coli* as discussed before induces a hypo-vesiculating phenotype and a double mutant of *nlpA* in the auxotrophic background depicted reduced fitness (Fig. 4). Here we wanted to test if the addition of the vesicle fraction from the *ΔhisDΔdegP* monoculture can facilitate cross-feeding in both, the single-deletion auxotroph, *ΔhisD,* and the double-deletion auxotroph, *ΔhisDΔnlpA*. Interestingly the *ΔhisDΔnlpA* recipient performed better when supplemented with the vesicle fraction as compared to growth in the absence of the vesicle fraction (independent samples t-test, * $P<0.05$, n=4, Fig. 7B). The growth of the recipient double mutant was not significantly different from that of the recipient single-deletion mutant. This vesicle fraction also resulted in increased growth of the recipient (*ΔhisD*) when it was cocultured together with *E. coli* WT as a donor ((independent samples t-test, ** $P<0.01$, n=4, Fig. 7B). The WT strain does not produced increased quantities of amino acids as compared to an over-producer genotype owing to the presence of feedback regulation for amino acid biosynthesis [47]. In cocultures, this WT as a donor hence does not support auxotroph growth as much as an over-producer genotype. The growth advantage imparted to the auxotroph by the vesicle fraction seems to be independent of the donor genotype present in the environment.

Although the exact chemical composition of the vesicle fraction is yet unclear, the following characteristics hold true, (i) the vesicle fraction does not contain the focal amino acid (histidine)

required for growth and (ii) the fraction may contain additional growth inducing factors. Monocultures of the double-deletion auxotroph, *ΔhisDΔdegP,* were grown in liquid MMAB devoid of histidine, for 12 hours prior to harvesting for vesicle fraction collection. This starvation was performed to increase production of OMVs. Any histidine, potentially carried over from the previous medium, would be utilized during the starvation phase. There is however a possibility of other molecules being present in the vesicle fraction that were released as a response to amino acid starvation by the double-deletion auxotroph. Transcriptomic data obtained from cross-feeding cocultures indicated the upregulation of *hokC* and *hokD* genes which are toxins involved in cell death [48, 49]. Amino acid starvation of the double-deletion auxotroph, *ΔhisDΔdegP,* might have resulted in the production of these toxins which get released into the medium upon cell lysis. Toxins present in the medium would pass through the membrane during filtration and lead to cell death in the coculture of recipient and donor genotypes. Since donor genotypes are present in a higher number owing to an autonomous metabolic state, the toxin-mediated killing would result in higher donor cell death. Although growth measurements of the donor genotype in coculture does not show a significant decrease in comparison to the donor in monoculture, the possibility of toxin-mediated cell death needs to be addressed in the future.

CONCLUSION

The results of this study provide multiple insights into the process of amino acid cross-feeding in bacteria. Transcriptomics revealed the cellular responses of one cell to amino acid induced starvation and subsequent acquisition of amino acids from another cell in the environment. A concerted effect of differential regulation of genes induced pathways that specifically resulted in increased cell adhesion and vesicle formation. Hence a single loss-of-function mutation (auxotrophy), triggered significant changes in the transcription of genes that further led to enhanced growth in coculture. Similarly auxotrophic genotypes found in natural habitats or in association with a host could also employ these transcriptional changes to obtain nutrients for growth.

FIGURES

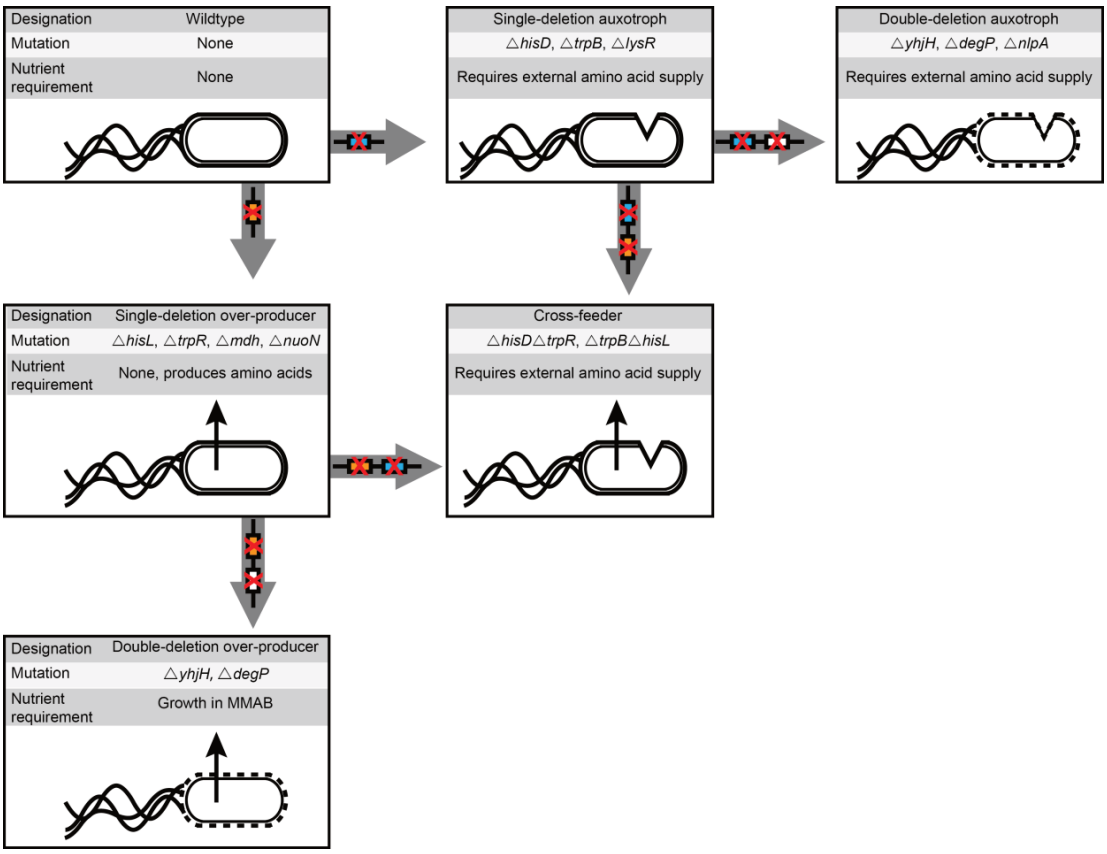

Figure 1: Overview of genotypes used in this study. *E. coli* BW25113 was the wildtype strain from which all other genotypes were derived using P1 transduction for targeted gene deletion. Mutant genotypes were selected by the presence of the kanamycin resistance cassette. Strain designation, the gene deleted (mutation) and nutrient requirement (phenotype with respect to amino acid requirement) have been mentioned for each genotype. Grey arrows with representative genes inside indicate the number of deletions required for the derived genotype. Single-deletion mutants are obtained through a one-step gene inactivation whereas double-deletion mutants required an additional mutation. Before the introduction of the second mutation in double-deletion mutants, the kanamycin cassette was removed using the pCP20 plasmid based method.

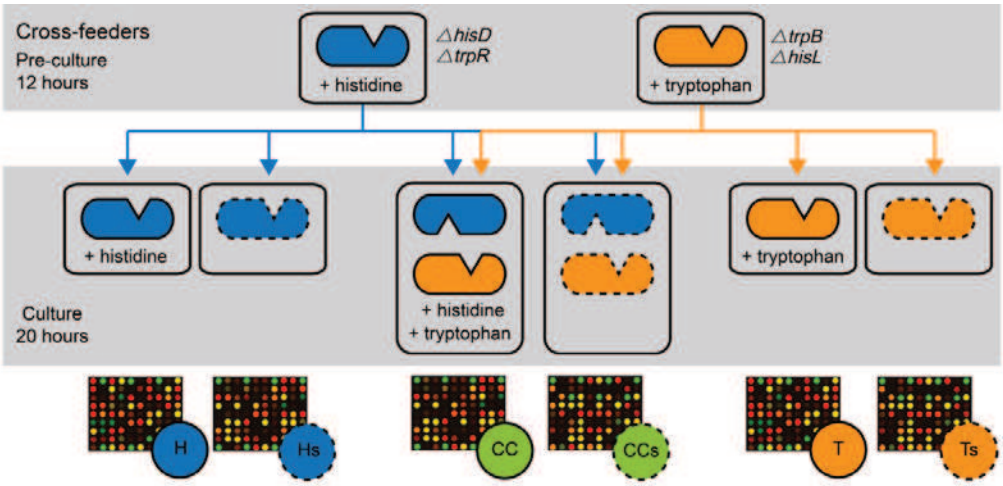

Figure 2: Strains and culture conditions for transcriptional analysis of cross-feeding genotypes. In order to determine the set of gene differentially regulated during the process of amino acid cross-feeding in *E. coli*, the following experimental set up was used. Cross-feeding genotypes (i.e. *ΔhisDΔtrpR* (blue) and *ΔtrpBΔhisL* (orange)), were subjected to six different conditions as follows, individual cross-feeders were grown as monocultures in presence of amino acid, (i) histidine (H) and (ii) tryptophan (T), as well as exposed to, (iii) histidine starvation (Hs) and (iv) tryptophan starvation (Ts). The same cross-feeding genotypes were subjected to coculture conditions (green), (v) in the presence of both amino acids (CC) and, (vi) in the absence of amino acids (CCs). The monocultures and cocultures were incubated for 20 hours at 30 °C followed by RNA extraction, purification and Cy3 labeling (see materials & methods)

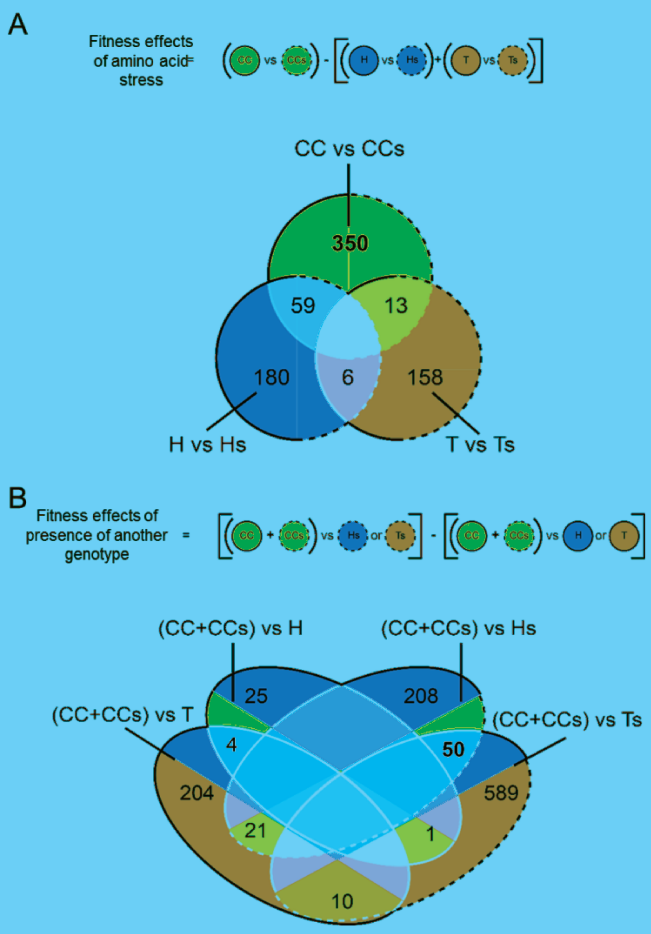

Figure 3: Comparisons applied to obtain significant, differentially expressed genes (DEGs). Scanning of a microarray chip containing labeled RNA provides the expression level of each gene that binds to the probe preloaded on the chip. In order to identify relevant genes out of the 15,208 genes whose probes are present in a microarray for an *E. coli* K12 genome, pairwise comparisons were carried out (for details see materials & methods). A pairwise comparison, for instance CC vs CCs, results a refined list of only those genes up or down-regulated (>2 fold) in one condition (CCs) relative to the other (CC). **(A)** In order to filter out the effects of aminno acid starvation, the set of differentially regulated genes in monocultures grown in presence and absence of histidine (blue) and tryptophan (orange) was subtracted from the list of DEGs in coculture comparisons (CC vs CCs, green). This resulted in a set of 350 genes (in bold) showing differential regulation in cocultures of cross-feeding genotypes. **(B)** To identify the genes differently regulated when a partner strain is present in the environment, multiple comparisons were carried out. The combined set of genes from the cocultures with and without amino acid supplementation (CC + CCs), was compared to different monoculture conditions. The DEGs from the monocultures in the presence of histidine (H) and tryptophan (T) were then subtracted from those in the absence of histidine (Hs) and tryptophan (Ts). A final set of 50 genes (in bold) was obtained that were common for the absence of both amino acids.

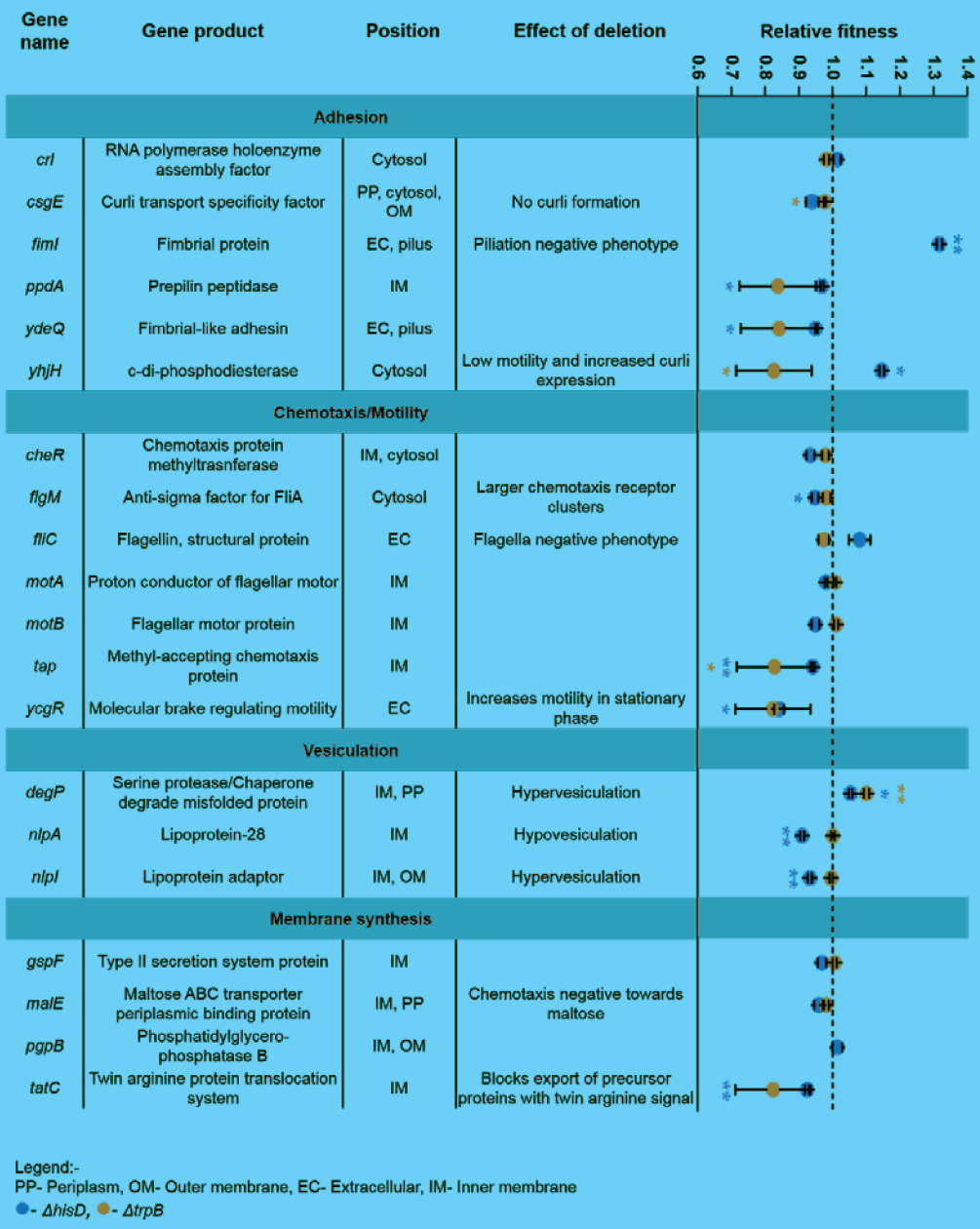

Figure 4: Relative growth of double-deletion auxotrophic genotypes. Genes from the analyzed dataset of differentially regulated genes were categorized into the following groups, (i) cell adhesion, (ii) chemotaxis or motility, (iii) vesicle formation, and (iv) membrane protein synthesis. Table indicates the gene name, gene product, position of the gene product in the cell and known effect of the gene deletion on *E. coli* K12 wildtype. Each individual gene was introduced into the auxotrophic background, *ΔhisD* (blue) and *ΔtrpB* (yellow). These double-deletion genotypes were then tested for growth in coculture with the complementary over-producer (*ΔhisL* or *ΔtrpR)*. The growth of double-deletion genotypes quantified relative to the single-deletion auxotroph, indicated the positive or negative effect of the gene on cross-feeding of amino acids (independent t-test, * P>0.05, ** P>0.01, n=4). The dashed line indicates the fitness of single-deletion auxotroph, *ΔhisD* or *ΔtrpB*.

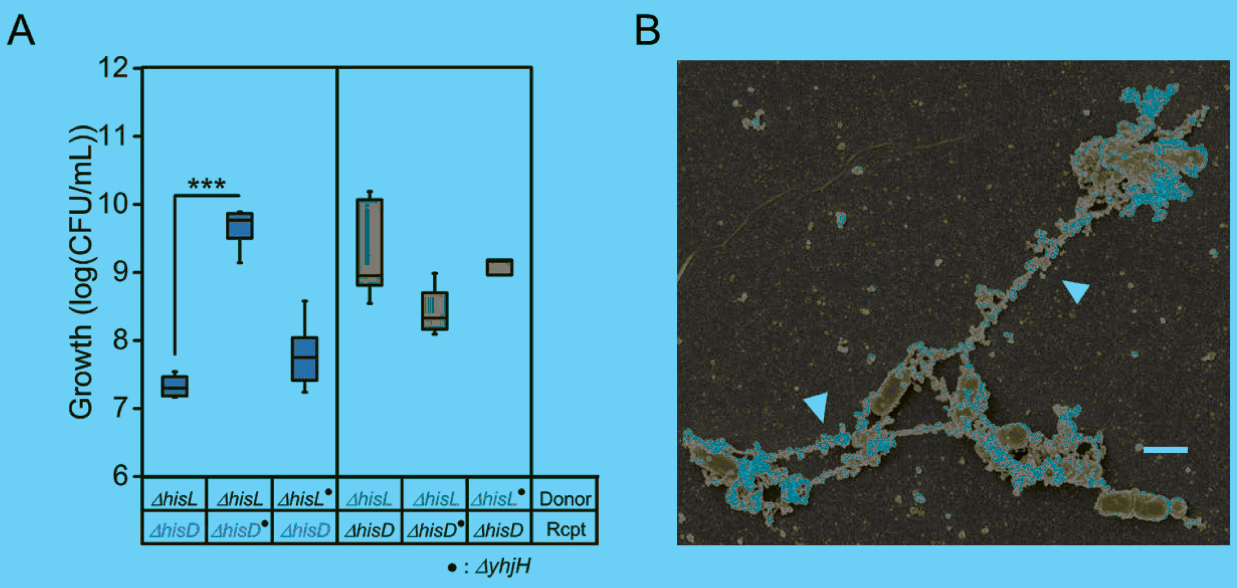

Figure 5: **Increased c-di-GMP levels in the auxotrophic recipient enhances growth**. **(A)** The auxotrophic recipient, *ΔhisD* (blue), and a double-deletion mutant, *ΔhisDΔyhjH* (blue, indicated by ●), were grown in coculture with *ΔhisL* (grey) as the donor. All recipient genotypes indicated as "Rcpt" and donor genotypes as "Donor". A significant increase in growth was observed for the *ΔhisDΔyhjH* genotype as compared to the *ΔhisD* genotype (independent t-test, *** P<0.001, n=4). The same deletion when introduced in the *ΔhisL* background (*ΔhisLΔyhjH*, grey, indicated by ●) did not significantly affect the growth of the recipient nor that of the donor when in coculture. **(B)** A deletion of *yhjH*, a c-di-GMP phosphodiesterase, results in increased intracellular levels of c-di-GMP which enhance curli-based adhesion to cells. Scanning electron micrographs of the double-deletion genotype, *ΔhisDΔyhjH*, in coculture with the single-deletion over-producer genotype (*ΔhisL*), indicates increased curli fibres and cell adhesion. Scale bar represents 1 μm.

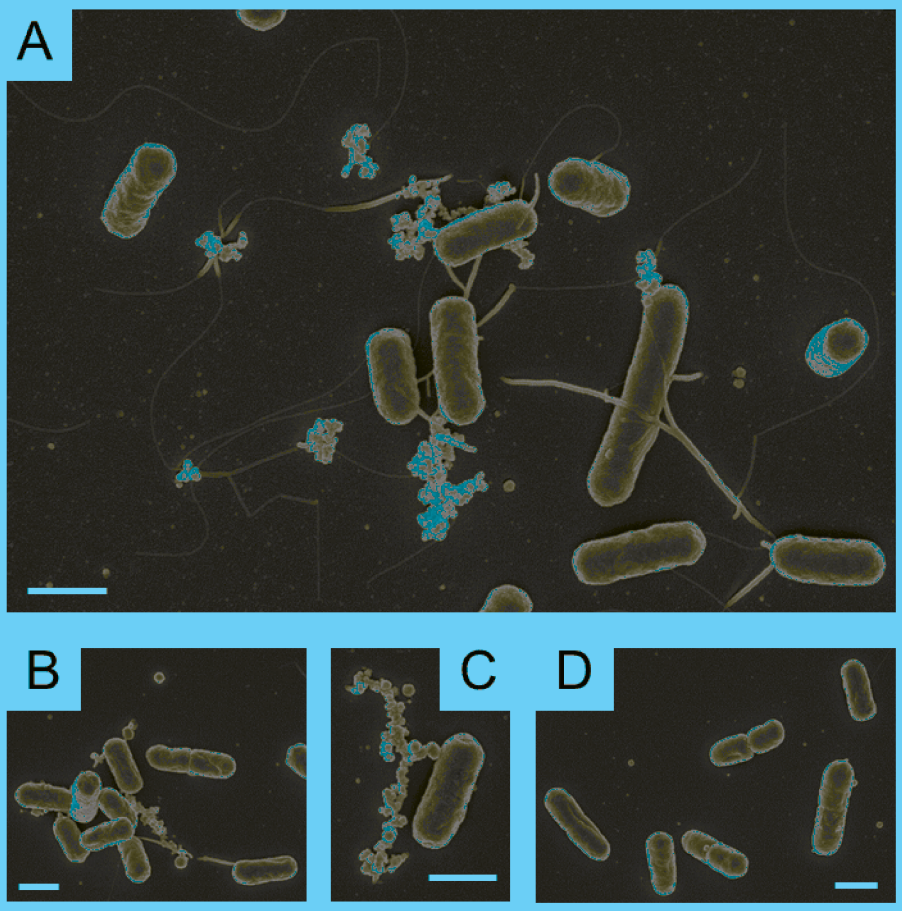

Figure 6: **Vesicles observed during amino acid cross-feeding**. Scanning electron micrographs of different cocultures with or without amino acid supplementation indicate a role of vesicles in amino acid cross-feeding. **(A)** Cross-feeding genotypes (i.e. *ΔhisDΔtrpR* and *ΔtrpBΔhisL*) were cocultured without amino acid supplementation and imaged after 24 hours. Vesicles in the form of aggregates were observed in association with cells as well as separated in the medium. **(B)** Similar vesicles were observed when the single-deletion auxotroph (*ΔhisD)* was cocultured with a single-deletion over-producer genotype (*ΔhisL)*. **(C)** Vesicles observed in the form of fused chain-like structures attached to a cell in cocultures of single-deletion auxotroph (*ΔhisD)* and single-deletion over-producer (*ΔhisL)* genotypes. **(D)** A coculture of single-deletion auxotroph (*ΔhisD)* and single-deletion over-producer (*ΔhisL)* genotypes, in presence of histidine (100 μM) depicts an absence of vesicles as well as nanotubes. Scale bar represents 1 μm.

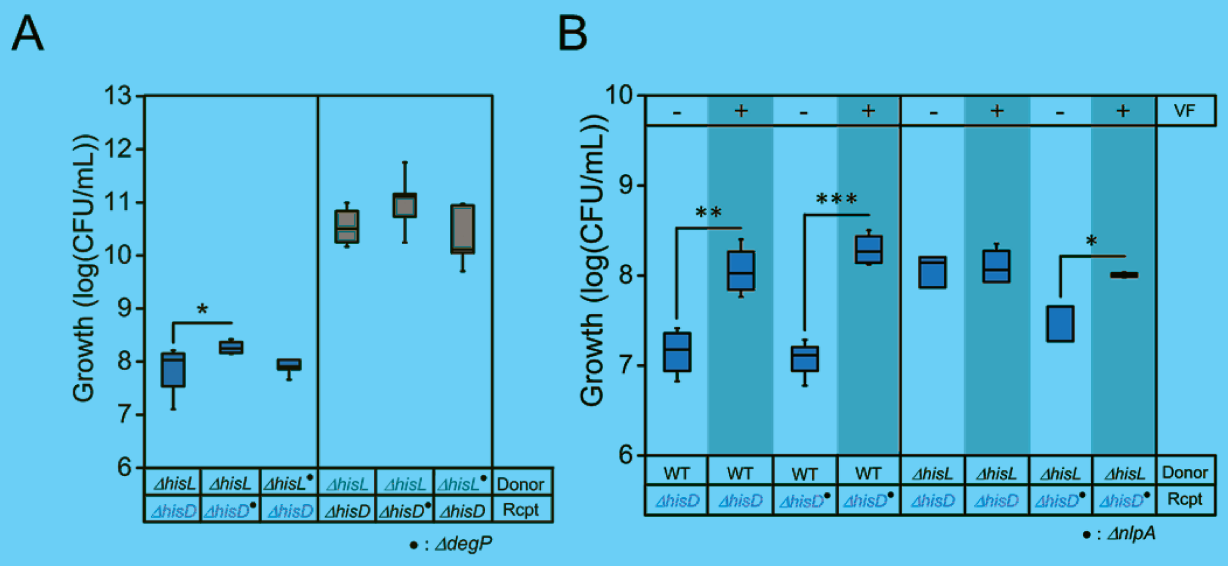

Figure 7: **Vesicles produced by the auxotrophic recipient increases growth independent of donor genotype**. **(A)** In order to identify the origin of vesicles in a cross-feeding pair, the auxotrophic recipient, *ΔhisD* (blue), and a double-deletion genotype, *ΔhisDΔdegP* (blue, indicated by ●), were grown in coculture with single-deletion over-producer, *ΔhisL* (grey), as the donor. A *degP* deletion in *E.coli* WT K12 strain induces vesicle formation. A significant increase in growth was observed for the *ΔhisDΔdegP* recipient double-deletion genotype as compared to the *ΔhisD* recipient (independent samples t-test, * P<0.05, n=4). The same deletion when introduced in the *ΔhisL* background (*ΔhisLΔdegP,* grey, indicated by ●) did not alter the growth of either partners in coculture . **(B)** The cell-free fraction of a recipient double-deletion genotype (*ΔhisDΔdegP*) known to over-produce vesicles upon amino acid starvation was obtained (see materials & methods). This cell-fee fraction, denoted as vesicle fraction (VF), when added to the coculture of single-deletion auxotroph (*ΔhisD*) and WT, significantly increased the growth of the recipient *ΔhisD* (blue) (independent samples t-test, ** P<0.01, n=4). A recipient double-deletion genotype *ΔhisDΔnlpA* (blue, indicated by ●) when cocultured with the WT, showed a significant increase in growth (independent samples t-test, * P<0.05, n=4). Interestingly when this recipient double-deletion genotype (*ΔhisDΔnlpA*) was cocultured in the VF with *ΔhisL* as the donor, the growth of *ΔhisDΔnlpA* was equivalent to that of the sigle-deletion genotype (*ΔhisD)* with *ΔhisL* as a donor.

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Chapter 10: General discussion

Microbial communities are highly diverse and display a range of interactions. These interactions are often broadly classified as, (i) parasitic, where one organism attains growth benefit at the cost of another organism, or (ii) commensal, wherein one organism attains growth benefit without positively or negatively affecting the other organism or, (iii) mutualistic, where both organisms benefit from the interaction. Each interaction hence leads to a different output with respect to the growth of individuals involved. Despite these differences in the outcomes, microbial interactions do show similarity in one aspect, the medium of communication. Majority of these interactions take place through metabolic exchange between individuals (Kouzuma *et al.,* 2015, Kuramitsu *et al.,* 2007, Morris *et al.,* 2013, Phelan *et al.,* 2012), thus highlighting the importance of bacterial metabolism in the functioning of microbial interactions and consequently the community (Johnson *et al.,* 2012, Mori *et al.,* 2016). A study analyzing 1,297 natural communities obtained from varied habitats (soil, water and human gut) for co-occurrence of species depicted a large number of metabolic dependencies. Applying genome-scale metabolic modeling further revealed metabolic cross-feeding of amino acids and sugars as the mechanistic basis for the observed dependencies (Chaffron *et al.,* 2010, Zelezniak *et al.,* 2015). A classification of microbial interactions in the context of metabolism and nutrition results in the following groups depicting, (i) no interaction (Fig. 7a), wherein both organisms utilize different resources from the environment, or (ii) competition (Fig. 7b), where both organisms metabolize a common substrate, or (iii) cross-feeding (Fig. 7c), where the metabolite produced by one organism serves as a substrate for the other organism, this metabolite may be a by-product of biosynthesis or exclusively produced for the other organism.

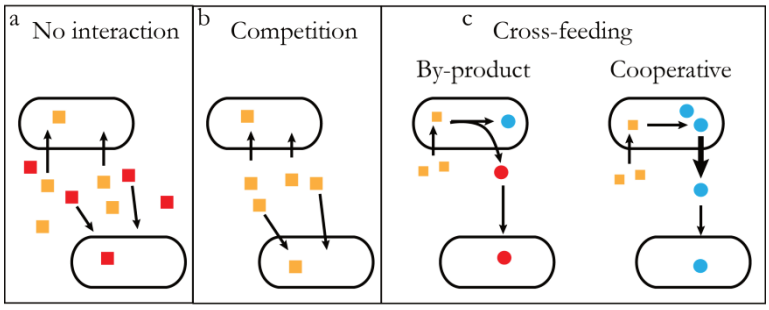

Figure 7: Classification of microbial interactions based on metabolic complementation. (a) Organisms may specialize on different substrate, both available in the environment. (b) Organisms may compete for the same substrate for growth resulting in depletion if the resource is limited. (c) Organisms may utilize products of another cell's metabolic activity, these can be by-products (no additional cost to producer) or cooperative (organism invests additional cost to provide the metabolite for another cell.

The cross-feeding of amino acids between bacteria was studied to understand the mechanistic and metabolic, basis for amino acid cross-feeding. Studying a cross-feeding consortium from natural habitats, although ecologically relevant, poses various problems during experimentation. Problems like fastidious growth requirements, insufficient characterization of organisms, unknown genotypes and exchange of multiple products, make natural consortia unappealing for answering mechanistic questions. Synthetic ecology provides tools to engineer an interaction in model systems that are genetically tractable making them a popular model for microbial community

studies (Dolinšek *et al.,* 2016, Harcombe 2010, Mee and Wang 2012, Mee *et al.,* 2014, Shou *et al.,* 2007, Wintermute and Silver 2010).

A synthetically engineered cross-feeding system in *Escherichia coli* and *Acinetobacter baylyi* was used to study the mechanism of amino acid exchange (chapter 7). *E. coli* was found to produce membrane-derived nanotubes upon introduction of a mutation that renders it auxotrophic for an amino acid. Cross-feeding genotypes were able to exchange cytoplasmic content only when either partner was *E. coli* and no amino acid was supplied externally. The absence of amino acid seems to trigger several cellular responses in the cross-feeding genotype as revealed by transcriptomics (chapter 9). By simplifying the cross-feeding system to a unidirectional transfer of amino acids, I identified individual genes that assist amino acid cross-feeding mediated growth. Specifically, cell adhesion and membrane vesicles were found to increase the growth of an auxotrophic genotype when cocultured with an over-producer. Amino acids form a significant part of bacterial metabolism and are tightly regulated. This regulation of amino acid biosynthesis was studied in the context of cross-feeding by observing changes in the levels of internal amino acid as well as the transcriptional activity of their biosynthetic genes (chapter 8). Here I saw the emergence of metabolic coupling between cells in a source-sink-like manner. The transport of amino acids from the donor (WT or overproducer) to the recipient (auxotroph) led to a delay in feedback inhibition of amino acid biosynthesis in the donor.

10.1 Components of a microbial network

Cross-feeding interactions between individual microorganisms can have profound effects on the community and the inhabited ecosystem (Falkowski *et al.,* 2008, Gil-Turnes *et al.,* 1989, Guarner and Malagelada 2003, Phelan *et al.,* 2012, Schultz and Brady 2008). With the help of a synthetically engineered system in *E. coli,* key aspects of a cross-feeding interaction that can affect microbial community dynamics have been identified.

10.1.1 The interacting partners: donors and recipients

The model system consists of cross-feeding genotypes that contain an auxotrophy-causing mutation and an overproduction mutation that results in amino acid exchange. Auxotrophic genotypes are dependent on an external source of nutrients due to loss of the biosynthetic gene. A prototrophic cell, on the other hand devotes a significant portion of cellular precursors, energy (ATP) and reducing agents (NAD, NADP) for amino acid biosynthesis. If these amino acids are now available either through the environment or from a neighboring cell then the cell saves this cost of biosynthesis (D'Souza *et al.,* 2014). Auxotrophic cells would hence have a growth advantage over prototrophic cells in an environment with amino acid supply. An environmentally-induced auxotrophic lifestyle is seen often in host-microbe symbiosis wherein endosymbionts are nutritionally dependent on the host (Douglas 2016, McCutcheon and von Dohlen 2011, Mori *et al.,* 2016, Van Leuven *et al.,* 2014). For instance

the pea aphid, *Acyrthosiphon pisum*, harbours an endosymbiont, *Buchner asp.* APS, within specialized cells called bacteriocytes (Shigenobu *et al.,* 2000). A mutual dependence and complementation of metabolic pathways for amino acid biosynthesis has been observed in this host-symbiont pair. Many endosymbionts are known to undergo genome streamlining (a loss of genes that are functionally redundant) during their association with the host (McCutcheon and Moran 2012). Free-living bacteria like *Lactobacillus bulgaricus*, also exhibit genome reduction and auxotrophy due to constant exposure to nutrient-rich environment (yoghurt) (van de Guchte *et al.,* 2006). Apart from environmental sources, nutrients may also be made available by neighboring cells as a consequence of cell membrane permeability and extracellular substrate degradation (Garcia *et al.,* 2015, Morris *et al.,* 2012, Stams *et al.,* 2006, Vollbrecht *et al.,* 1979).

The other partner in the cross-feeding interaction is a donor strain which provides metabolites to the auxotrophic recipient. A donor cell could produce extracellular enzymes that degrade complex polymers like cellulose, the degradation products (monosaccharaides, oligosaccharides) of which are then available for neighboring cells (Koschwanez *et al.,* 2011). Additionally, primary and secondary metabolites are often released into the environment during bacterial growth owing to the high permeability of the cell membrane (Konings *et al.,* 1992, Nikaido 2003). Cells are known to have differential permeability for molecules such as hydrogen peroxide, iron sequestering compounds, gaseous intermediates and organic wastes (Belenguer *et al.,* 2006, Boone *et al.,* 1989, Costa *et al.,* 2006). The transport of such molecules in the surrounding of an autonomous cell can benefit the growth of any auxotrophs in the surrounding. Similar to the results presented in this thesis (chapter 8), a wildtype autonomously replicating cell may also act as a donor for metabolites in nature.

10.1.2 External conditions inducing contact-dependent cross-feeding

Soil, oceans, glaciers, lakes and animals gut, are some of the major natural habitats of bacteria (Curtis and Sloan 2004). The growth of bacteria in most of these habitats is found to be surface-attached in the form of biofilms (Dunne 2002, Mazumdar *et al.,* 2013, Rao *et al.,* 2005, Ren *et al.,* 2015). Biofilms in these natural habitats are characterized by fluid flow on the external surface as well as through micro-channels within the biofilm (Azam and Malfatti 2007, Hall-Stoodley *et al.,* 2004). This flow leads to the gradual removal of products released by resident cells, necessitating mechanisms of exchange that circumvent the problem of dilution (Drescher *et al.,* 2014). Soil structure has been shown to have an effect on the type bacteria found in the rhizosphere (Berg and Smalla 2009, Lauber *et al.,* 2008). Structure, in this case, is attributed by components like quartz, clay, sand and/or organic matter. Depending on the coarseness of the soil, water channels are formed within these aggregates. Microorganisms often colonize at the interface of water and organic matter or clay. The flow of water through the soil aggregate results in dilution or significant loss of a released metabolite. By testing the process of amino acid cross-feeding in a shaken liquid environment, a solution emerges. These results (chapter 7) provide a mechanistic explanation to how these effects resulting in loss of the metabolite can be avoided. Using nanotubes, a

nutritionally-starved cell (auxotroph) is able to connect to a neighboring cell and obtain nutrients without exposing the nutrients to the environment.

10.1.3 Cellular conditions inducing contact-dependent cross-feeding

Loss-of-biosynthetic genes in a prototrophic cell results in metabolic dependency as discussed before (Section 4.1.1). In the absence of externally supplied metabolites, a starvation response is triggered in the cell (Betts *et al.,* 2002, Givskov *et al.,* 1994, Kjelleberg 2013). Amino acid starvation in *E. coli* specifically induces guanosine 5',3' bispyrophosphate (ppGpp) mediated cessation of mRNA transcription (Srivatsan and Wang 2008, Traxler *et al.,* 2008). A reduced transcription is important for conserving available amino acid till nutrient supply is no longer limiting. Apart from the production of ppGpp during stress response, the cell also increases production of bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP). C-di-GMP is known to induce genes involved in biofilm formation in *E.coli* (Hengge 2009, Landini 2009). Genes like *csgD*, *csgB*, *csgA* and *csgC,* increase cellular adhesion through the expression of curli fibers on the cell membrane (Chapman *et al.,* 2002). Transcriptomics analysis of cross-feeding genotypes reveals similar changes, (i) upregulation of a diguanylate cyclase (*dgcM*) and (ii) downregulation of cyclic-di-GMP phosphodiesterase (*yhjH*) (chapter 9). Both these changes in gene expression lead to higher intra-cellular c-di-GMP levels which can explain the physical connectivity required during cross-feeding.

A starvation of nutrients is also known to induce the formation of outer membrane vesicles in some bacterial species. In *Myxococcus xanthus* for example, starvation induces the formation of fruiting bodies for which some inter-cellular coordination is of essence. In order to coordinate between neighboring cells, membrane vesicles produced by the cells form chain-like structures (Remis *et al.,* 2014). These vesicle chains, composed of lipids and carbohydrate moieties, exchange signaling proteins, CglB and Tgl, required for motility. A similar production of vesicles by auxotrophic genotypes of *E. coli* was observed (chapter 9). The vesicles in case of *E. coli* were not shown to form fused vesicle chains, however they do enhance growth and hence play a role in amino acid exchange.

10.1.4 Recalibration of the metabolism of cross-feeding cells

Metabolic coupling of an auxotrophic recipient and a donor cell when they exchange cytoplasmic amino acids is shown (chapter 8). Coupling of amino acid biosynthesis is shown to be a result of an intrinsic regulatory function of amino acid biosynthesis, end-product mediated feedback inhibition. A delay in feedback inhibition of amino acid biosynthetic gene, caused by the recipient leads to increased amino acid production by the donor. Similar coupling-based interactions have been observed in other biological systems that consist of physically connected organisms. For instance an extreme case of metabolic coupling is seen between a eukaryotic cell and its organelles, mitochondria or chloroplast. Hypothesized to have evolved from a symbiotic association (Kiers and West 2015, Kooijman and Hengeveld 2005, López-García and Moreira 1999), a long co-evolutionary history has resulted in

complete inter-dependence of interacting units. However, the initial phase of this symbiosis is yet unclear. End-product mediated feedback regulation is a universal control system for biosynthetic pathways (Umbarger 1978). Hence the ancestors or precursor cells of mitochondria and chloroplasts also harbored similar regulatory mechanisms. A delayed feedback inhibition can help explain the initial steps of a symbiotic relationship. Alternatively, it can explain the start of parasitic interaction like that seen between the archaeal symbiont, *Nanoarchaeum equitans*, and its host, *Ignicoccus* sp. (Waters *et al.,* 2003). *N. equitans* is found to derive lipids, amino acids and nucleotides from *Ignicoccus* sp. through direct cell-cell connections which potentially link the cytoplasm of either species.

The results from this thesis indicate an increased transcriptional activity of the amino acid biosynthetic genes as a functional basis for higher amino acid production in the donor. This response in transcriptional activity was a consequence of the auxotroph behaving like a metabolic sink. Metabolic pathways like amino acid biosynthesis can be regulated in two ways to result in increased production, (i) higher activity of the enzyme catalyzing the reaction or (ii) increasing level of the enzyme (Neidhardt *et al.,* 1990). Modification of the activity of allosteric enzymes (which catalyze all biosynthesis pathways) (Monod *et al.,* 1963), requires binding of an allosteric effector like cytidine triphosphate (CTP) for an enzyme like aspartate transcarbamylase (Bethell *et al.,* 1968). Each enzyme in a given pathway is regulated by such effectors which may be similar or different (Cunin *et al.,* 1986, Sahm *et al.,* 1995, Sanwal 1970), all of which may not be available in the cell during the interaction. Increasing the level of enzymes on the other hand takes place through increased transcription. Bacterial cells have an operon- based biosynthesis gene cluster which is regulated mainly through the activity at the promoter region (Neidhardt *et al.,* 1990). An increased rate of transcription at the promoter thus results in sufficient enzymes to drive biosynthesis.

10.2 A model for contact-dependent cross-feeding

The results from this thesis, collectively considered, provide a framework of how contact-dependent cross-feeding of amino acids takes place in bacteria. There are three possibilities for establishing tubular connections between an auxotrophic recipient and a donor cell which are presented.

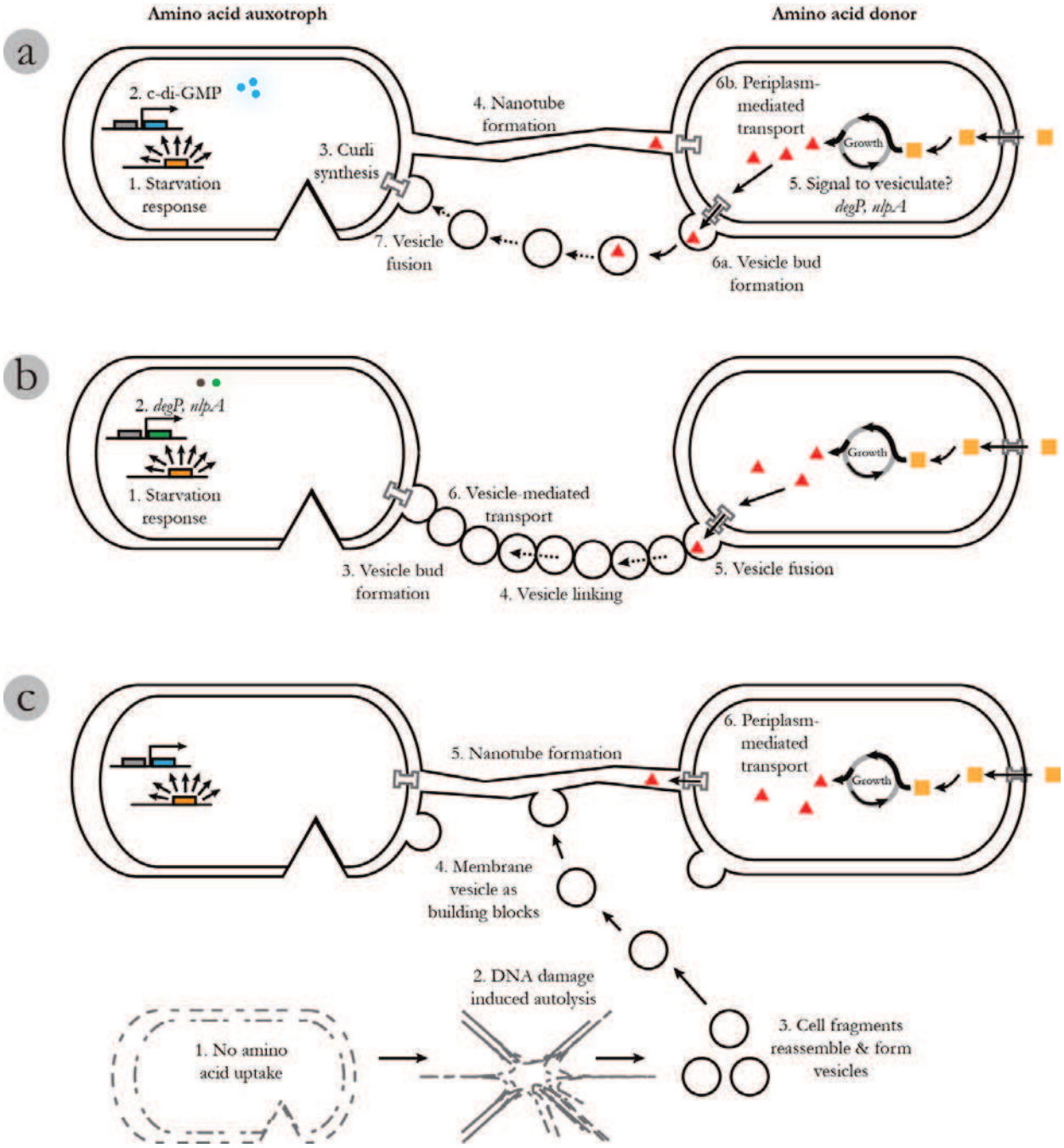

Figure 8: Overview of models for contact-dependent amino acid transfer from donor to auxotroph. The numbers within each horizontal panel indicates the hypothetical sequence of events. (a) Nanotubes connect cell, vesicles transport cytoplasmic content. (b) Vesicles bud out of the cell during formation and remain linked or form chain-like structures that connect auxotroph to donor. (c) Autolysis of other auxotrophic cells results in fragmentation of cell material which may reassemble to form vesicles. These vesicles and membrane fragments are used to build nanotubes between cells. Transport of amino acids in all cases is represented through transporters in the periplasm.

10.2.1 Nanotubes connect cells, vesicles transport cytoplasmic content

Amino acid starvation due to loss of the biosynthetic gene in an auxotroph triggers cellular processes that lead to the accumulation of second messenger (c-di-GMP). Three genes found to be differentially regulated (*yhjH*, *ycg*R and *dgcM*), cumulatively lead to c-di-GMP production as per the transcriptomic analysis (chapter 9). C-di-GMP in turn induces two pathways for biofilm matrix production namely, (i) curli fibres (through the *csgD* global transcription factor) and (ii) poly-β-1,6-N-acetyl-glucosamine (PGA) exopolysaccharide (through the PgaC and PgaD inner membrane proteins). Curli fibres are filamentous appendages known to assist the attachment of *E. coli* to inert surfaces (Chapman *et al.,* 2002, Van Houdt and Michiels 2005). PGA, an exopolysaccharide, on the other hand is known to promote intercellular adhesion (Van Houdt and Michiels 2005, Wang *et al.,* 2004). Hence a combination of both structures could be used to build nanotubes over a long distance (Fig. 8a).

After establishing a connection there are two ways in which cytoplasmic exchange can take place, (i) through the periplasmic space or (ii) through outer membrane vesicles. The periplasmic space is an aqueous but densely packed region present between the outer and inner membrane of gram negative bacteria and consists of proteins involved in cell nutrition (Neidhardt *et al.,* 1990). The inner membrane consists of transporters for efflux of sugars and amino acids into this periplasmic space before further release out of the cell (Ehrmann 2007, Silhavy *et al.,* 2010). Amino acids that are pumped into this periplasm would get exchanged between connected partners (here from donor to recipient) through the periplasm. The requirement and continued uptake of the amino acid by the recipient would result in increased export of amino acid through the periplasm as a result of metabolic coupling (chapter 8). Alternatively the transport of cytoplasmic content could take place through membrane vesicles which have been observed in cross-feeding consortia (chapter 9). The biogenesis of membrane vesicles starts with bulging of the outer membrane followed by fission of the grown vesicle bud (Fig. 8a) or continued attachment of vesicle bud to the cell (Fig. 8b) (Gorby *et al.,* 2008, Kulp and Kuehn 2010, McBroom *et al.,* 2006). Cytoplasmic contents like genetic material and amino acids present in such vesicles get transported directly into the recipient cell through vesicle attachment and fusion. Real-time microscopy of fluorescently labeled cross-feeding genotypes indicates such an attachment and fusion event leading to the transport of cytoplasmic GFP (chapter 7). There might be a change in the mechanism used depending on the size of the molecule exchanged considering that free amino acids are much smaller than GFP molecules and also have dedicated transporters in the cell membrane.

Another possibility is that once the nanotube is formed, vesicles are transported within this tube from one cell to another. This however would require that the vesicles are made from the inner membrane of *E. coli* which is a highly complex structure. It is also composed of a phospholipid bilayer and associated proteins like the outer membrane. However the inner membrane being the final layer encompassing cytoplasmic material, maintains cell shape and homeostasis. The formation of inner membrane vesicle requires an invagination of the membrane to form intracellular vesicles (Eriksson *et al.,* 2009). If they are then secreted into

the environment then an additional layer of the outer membrane will result in an outer-inner membrane vesicle as observed in some pathogenic Gram negative bacteria like *Pseudomonas aeruginosa* (Pérez-Cruz *et al.,* 2015). The molecular details of how outer-inner membrane vesicles are formed is however unknown.

10.2.2 Vesicles link to form nanotubes

Candidate genes regulating vesicle biogenesis in *E. coli* were found to be differentially expressed during amino acid cross-feeding namely, *degP*, *nlpA* and *nlpI* (chapter 9). Furthermore the vesicle fraction of a hypervesiculating auxotrophic genotype enhanced growth of other auxotrophs when cocultured with a donor genotype. A disentanglement of the phenotype (increased vesicles) from the genotype (*ΔhisDΔdegP*) opens a new possibility for metabolite exchange in the cross-feeding system (Fig. 8b). Outer membrane vesicles produced as a response to auxotrophy are essentially a lipid bilayer encasing cytoplasmic material. A linking of newly formed vesicles or alternatively post-budding adhesion of vesicles (to each other) would lead to potential inter-cellular connections. Such a chain-like form of vesicles has been observed for intercellular communication in *Myxococcus xanthus* (Remis *et al.,* 2014) as well as in the form of membrane protrusions for electron exchange (Pirbadian *et al.,* 2014). The structural details of how vesicle connections are maintained for *M. xanthus* are unknown however, during nanowire formation *S. oniedensis* forms outer membrane protrusions. These protrusions swell up to increase surface area for the adherence of electron pumps followed by a collapse of the membrane at specific regions said to be influenced by electron density.

10.2.3 Cell lysis of auxotroph results in vesicle formation

Autolysis is the process in which, digestion of the peptidoglycan layer is catalyzed by murein hydrolases produced by the cell itself (Höltje 1995). Various factors like antibiotics, sporulation, DNA transformation or fruiting body formation, are known to trigger autolysis in bacteria (Lewis 2000). Auxotrophic cells that do not acquire the focal amino acid from another source (environment or donor) may undergo autolysis if nutrient-limited conditions prevail (Fig. 8c). Following autolysis the scattered cell material can aggregate to form membrane vesicles. These vesicles could then assist in metabolite exchange. Recent studies show that stress (phototoxic, antibiotic and genotoxic) in *Pseudomonas aeruginosa* leads to autolysis. Interestingly, after disintegration of the cell during the "explosive cell lysis", membrane fragments can reassemble to form vesicles (Turnbull *et al.,* 2016).

Alternatively errors in DNA replication due to starvation can lead to the formation of mini-cells. They are essentially smaller, spherical versions of a bacterial cell but devoid of genomic DNA and hence incapable of replication (Adler *et al.,* 1967, Ward and Lutkenhaus 1985). Plasmids however can be maintained in mini-cells and are used for protein expression (Meagher *et al.,* 1977) and can be found in mini-cells due to irregular cell division. However

cross-feeding genotypes did not test positive for an exchange of the plasmid used for cytoplasmic labelling (chapter 7). A mini-cell devoid of genetic material would still contain cytoplasmic contents, however if the mini-cell originates from the auxotroph then the focal amino acid would still be missing. One can consider use of mini-cells by cross-feeding genotypes which do contain the amino acid for exchange in the cytoplasm. The formation of mini-cells by cross-feeding genotypes points to altruistic behavior since the mini-cell is a dead end in terms of reproduction. However transport of amino acid via a mini-cell ensures growth of the partner. The possibility of this phenomenon needs to be further tested.

10.2.4 Disentangling the three models

Each of the above hypotheses requires the transcription of a group of genes to be initiated at different timepoints. Hence by RNA sequencing of the cross-feeding coculture at early and late timepoints of nanotube formation, we can identify the patterns in formation of different structures (vesicles, membrane lipids). Considering that the onset of cytoplasmic exchange is around 9 hours (chapter 7), the period before 9 hours would display a response to amino acid starvation wherein the expression of curli and PGA increases. Moreover chemotaxis, if involved, probably plays a significant role during this phase of finding a donor cell. The period, during and after cytoplasmic exchange, should have decreased levels of expression for cell adhesion and membrane vesicle formation genes.

Gene expression data alone is not sufficient to decode this complex phenomenon of nanotube-mediated cross-feeding. Structural details of the nanotubes need to be inferred through advanced microscopy. Cryo-electron tomography (cryo-TEM) is an ideal method to understand structures at the required level of structural detail (Al-Amoudi *et al.,* 2004). Tomography allows for determining even membrane proteins at the point of contact between nanotube and the cell membrane (Hoffmann *et al.,* 2008, Matias *et al.,* 2003, Matias and Beveridge 2005, Remis *et al.,* 2014). Furthermore a tilting of the axis of imaging provides a 3D view of the connection itself. On the other hand fluorescence microscopy paired with microfluidics will answer the question of how a nanotube grows out of a cell or are vesicles the precursors of nanotubes. Microfluidic chambers provide control over liquid flow as well as containing the interacting partners in a monitored space. It is possible to replicate the conditions of a constant flow of liquid (shaken liquid medium) to ensure nanotube formation. This can be controlled using plastic tubing connected to the flow chamber in the microfluidic device. A carefully designed chamber for growing the two partners will result in detecting the exchange process between cross-feeders in real-time.

10.3 Implications of contact-dependent cross-feeding

Metabolic dependency, availability of free functions, intercellular connections and metabolic coupling, can collectively result in, an inter-connected, biochemical network within a microbial community.

Consider an individual bacterial cell that is exposed to an external supply of a metabolite. By losing the gene required to synthesize this metabolite, the cell saves production cost which in turn allows it to grow fast. As this event of gene loss and metabolic dependency occurs in multiple cells in a given community, there is diversity in terms of nutritional requirements (Johnson *et al.,* 2012). Multiple auxotrophies in the same cell might also arise in such a community. When these auxotrophs acquire nutrients from neighboring cells, a transient network is built within the community (Zelezniak *et al.,* 2015).

Owing to the characteristic construction of metabolic pathways in bacterial cells, decrease in carbon flux through a given pathway, redirects intermediates and cofactors involved in this pathway to another pathway (Chubukov *et al.,* 2014, Waschina *et al.,* 2016). This property of biosynthetic pathways in bacteria is of significance when using bacteria for large scale production of amino acids or vitamins. By providing an excess amount of substrate or by deleting genes in biosynthetic pathways, one can obtain increased flux through the desired pathway (Bohl *et al.,* 2010). These metabolites produced in excess as compared to a wildtype cell, called by-products, can be secreted out of the cell for extraction purposes in industrial applications (Bylund *et al.,* 1998, Stelling *et al.,* 2002, Xu *et al.,* 1999). A similar redirection of flux through a pathway can also take place in auxotrophs and has been shown to support auxotrophic communities through experimental evolution studies (Wintermute and Silver 2010). Different auxotrophs can divide the cost of producing costly metabolites among cells in a community. When considered together, individual auxotrophic cells form a bigger biochemical network, with different pathways distributed in different cells. By dividing the cost of producing costly metabolites, cross-feeding cells have a fitness advantage over autonomous wildtype cells (Pande *et al.,* 2014).

10.4 Conclusion

Through this study I show how a single loss-of-function mutation can alter the metabolic state of the cell such that a novel mechanism of exchange emerges. It highlights the robustness and innovation of a bacterial cell to overcome perturbations in metabolism. In their natural habitats, bacteria face a greater number of perturbations such as other organisms, temperature, pH and pressure, which also positively or negatively affect metabolism. The fact that these bacteria survived all perturbations hints to the possibility of many, yet unknown, mechanisms of exchange employed by bacteria in nature. Contact-dependence is used to enhance interactions everywhere, (i) by eukaryotic cells to transmit signals, (ii) by pathogenic bacteria to improve colonization of host cell, (iii) by syntrophic aggregates to improve product exchange, (iv) by bacteria to exchange genetic material, (v) by bacteriophages to infect a host. The presence of contact-dependence as well as similarities in the structures used for maintaining contact implies that it is an evolutionarily conserved trait making it all the more important to study.

Chapter 11: Future directions

This thesis highlighted many key aspects of the process by which auxotrophic *E. coli* obtains nutrients from neighboring cells. The main questions of the thesis were answered using a tractable model system, microscopy, real-time sensing techniques and transcriptomics. However the analysis of observed results invoked additional questions about nutrient cross-feeding.

11.1 How carbon fluxes influence metabolic coupling

Bacteria take up and break down available carbon sources through catabolic pathways to produce building blocks (amino acids, nucleotides, and lipids), cofactors (vitamins) and energy (ATP). Catabolic pathways consist of multiple reactions to carry out a step-wise biochemical transformation of a substrate to the end product. The step at which a carbon source enters the pathway influences the flux through subsequent reactions (Stelling *et al.,* 2002). For example glycolytic substrates (maltose, fructose and xylose) increase the flux through initial steps of the tricarboxylic acid cycle (Krebs and Lowenstein 1960, Waschina *et al.,* 2016). An increased flux through these first steps results in higher production of specific amino acids connected to these reactions like histidine, tryptophan and glycine. The production profile of amino acids is dependent on the step at which a carbon source enters as well as the architecture of the biosynthetic pathway. In our experiments the carbon source used was fructose, which is a glycolytic substrate and results in increased flux to produce histidine and tryptophan.

It would be interesting to see how the carbon source would influence an interaction between cross-feeding genotypes. Gluconeogenic substrates like lactate, pyruvate and malate enter at later steps of the tricarboxylic acid cycle. An increased flux is seen through the biosynthesis of amino acids like lysine, threonine, glutamate and proline (Waschina *et al.,* 2016). Internal amino acid sensors demonstrated the changes in internal levels of lysine when a recipient cell interacts with a donor cell (chapter 8). In the presence of fructose, significant changes were observed in the donor internal lysine levels upon interaction with the recipient. If a gluconeogenic carbon source was used instead, for example malate, then lysine would be produced at increased quantities owing to the architecture of the pathway. Furthermore the entry point of malate being close to the pathway branching out for lysine production indicates a quicker turnover for lysine with malate as the carbon source. Would we see the same changes in the internal levels of lysine when the donor and recipient are grown in malate? Do the "stolen" amino acids from the donor cell get resupplied faster depending on the carbon source? What happens when multiple carbons sources are available to the donor cell?

11.2 Recognition of donor cell

Formation and stability of chemical gradients is challenging in a vigorously shaken environment. This raises questions about how the auxotrophic genotype is able to detect a donor genotype. There are two possibilities, (i) the auxotrophic genotype extends the outer membrane to form nanotubes which attach to any neighboring cell or (ii) the auxotroph detects donor cells via chemotaxis. A non-specific attachment of nanotubes would be plausible at the early stage of coculture when the donor cells number is high. However, in terms of multispecies communities a random attachment strategy might result in negative consequences (Jones *et al.,* 2017). Since attachment in case of cross-feeding is a cytoplasmic connection, antagonistic effectors could be exchanged leading to death. Chemotaxis may also be playing a role since the possibility of micro-gradients of amino acids around the cells might be possible (Azam and Malfatti 2007, Stocker 2012). Additionally the liquid media in which co-cultures were grown were shaken however at constant speed and the same direction (clockwise). A constant speed and direction can lead to the relative distance between particles to remain unchanged over time. Would micro-gradients be strong enough for chemotaxis to play a role in donor recognition? Where would these receptors for amino acid be present, at the tip of the nanotube or at intermediate points? By modifying the permeability of the cell membrane in the donor cell or by silencing the chemotaxis pathway in the recipient, this can easily be answered. Alternatively by introducing a recipient in a multi-membered community consisting of donors with varying amino acid production capacities, one could identify the preference for specific recipient-donor pairs.

11.3 Altering the cell membrane

Experiments in this thesis have been performed within species (*E. coli*) and between species (*E. coli* and *A. baylyi*). These are both gram negative bacteria and hence have similar cell membrane structure and composition. By pairing auxotrophic *E. coli* with donors of different species like *Pseudomonas aeruginosa*, *Shewanella oniedensis*, *Bacillus subtilis*, in depth information regarding donor recognition can be obtained. *B. subtilis* is a gram positive organism and is devoid of the outer membrane but contains a thicker murein layer compared to *E.c oli.* Moreover, *B. subtilis* has shown to produce nanotubes on solid media for the exchange of plasmid DNA between and within species (Dubey and Ben-Yehuda). Will the two species cross-feed? What cell material will be employed to form connections between cells? Will an auxotroph detect differences in amino acid profiles of donors from different species in a tripartite community? Or will the auxotroph detect the differences in cell membranes?

11.4 Auxotroph-donor coculture to study parasitism

Auxotrophs develop a unidirectional exchange of required metabolite when cocultured with a donor genotype. This set-up is a type of commensal wherein the donor genotype does not have reduced growth. By controlling the number of auxotrophy mutations as well as the level

of amino acid production by the donor, the switch from commensalism to parasitism can be tested. The introduction of multiple auxotrophy mutations in the recipient resembles the endosymbiotic bacteria with reduced genomes also resulting in multiple auxotrophies (McCutcheon and Moran 2012). Moreover, genomic sequencing also reveals that the average number of auxotrophies in free-living eubacteria is >1% (D'Souza *et al.,* 2014). A coevolution of auxotrophic recipient and donor may reveal changes in donor cell membrane to prevent contact-dependent growth of auxotrophs.

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Chapter 13: Supplementary information

Manuscript I

Metabolic cross-feeding via intercellular nanotubes among bacteria

Shraddha Shitut, Samay Pande, Lisa Freund, Martin Westermann, Felix Bertels, Claudia Colesie, Ilka B. Bischofs and Christian Kost

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Supplementary Information

Metabolic cross-feeding via intercellular nanotubes among bacteria

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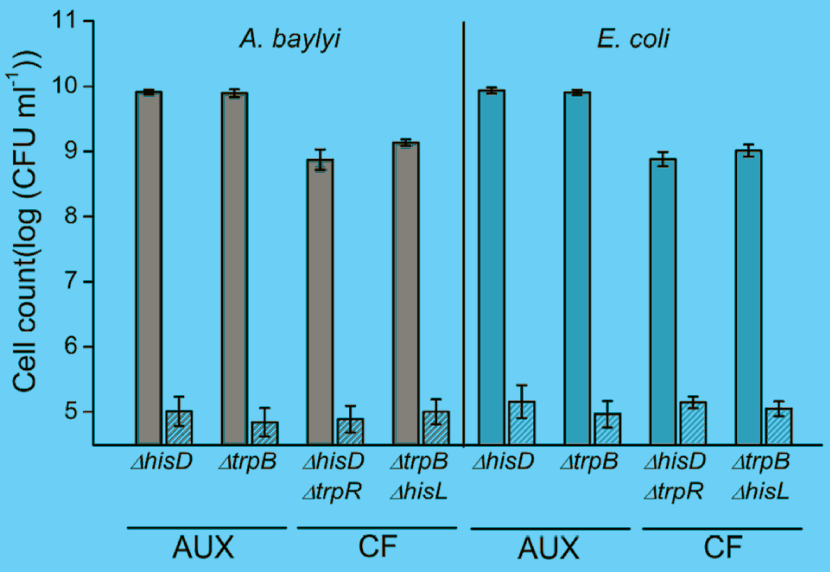
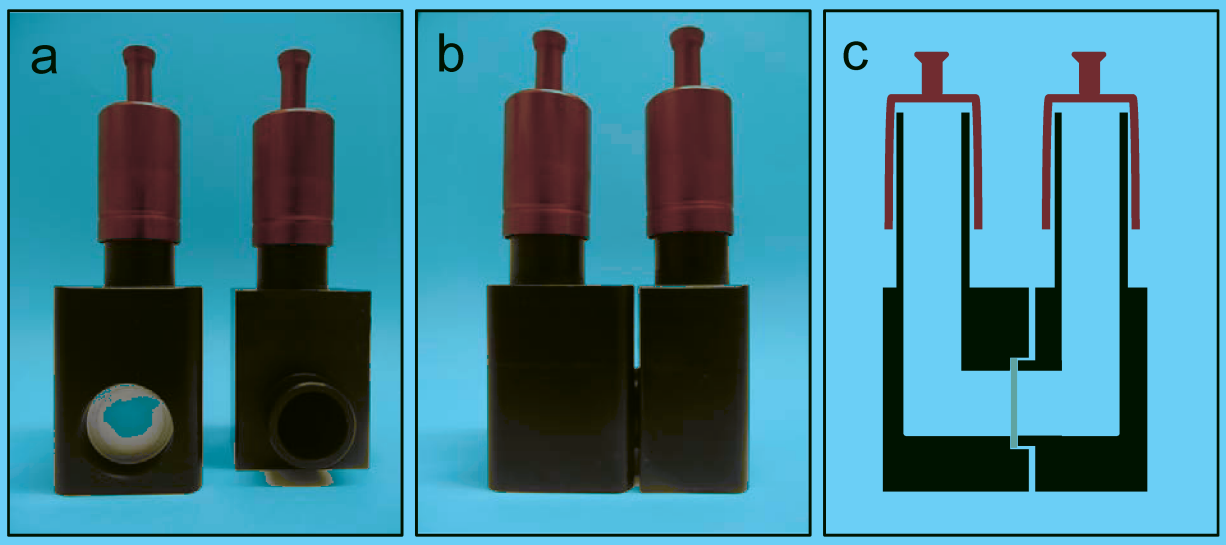
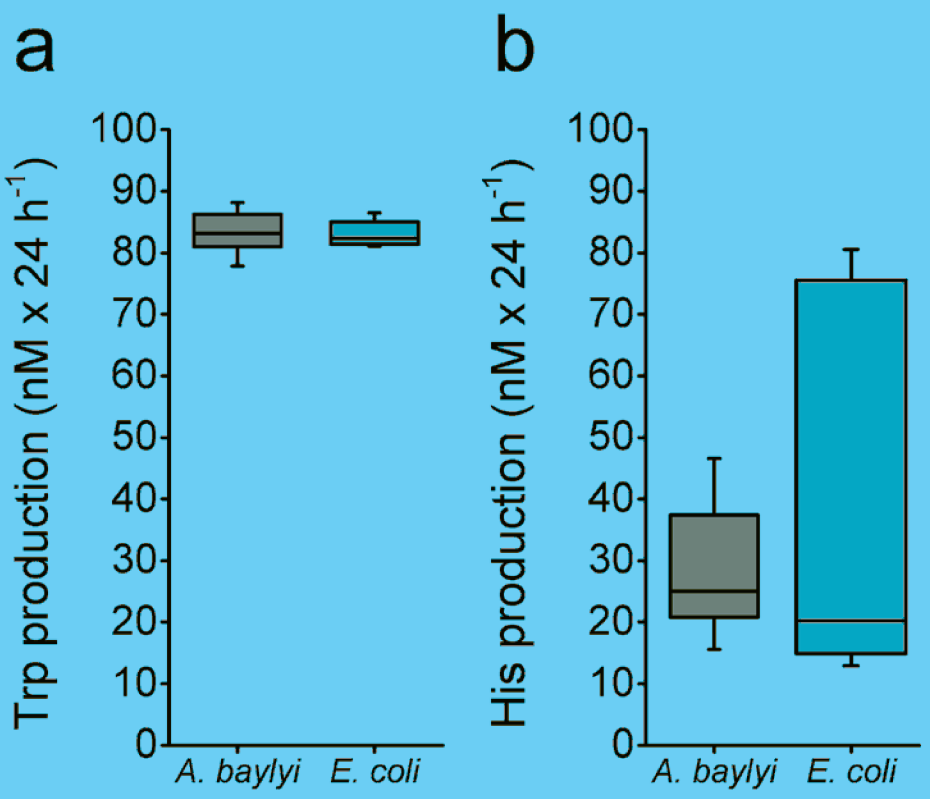


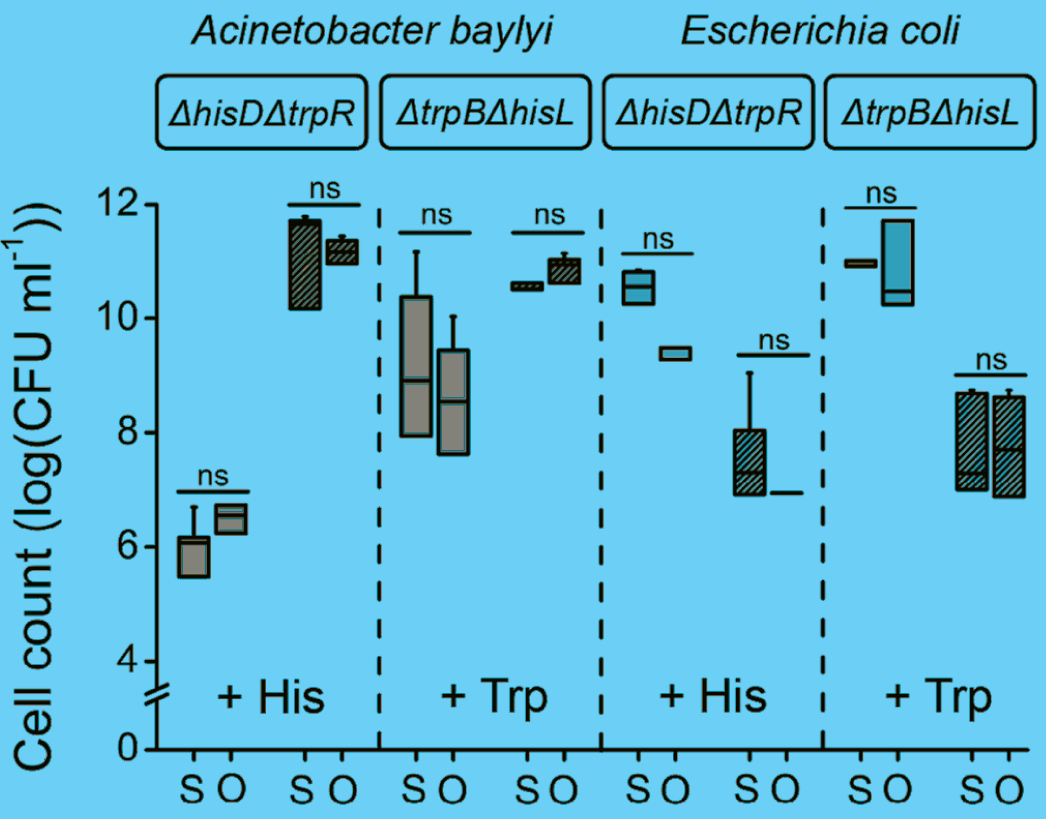
Supplementary Figure 1 | Auxotrophic genotypes of both *Acinetobacter baylyi* and *Escherichia coli* require amino acids to grow. Auxotrophic (Aux) and cross-feeding (CF) genotypes were grown in the presence of histidine (*ΔhisD*, *ΔhisDΔtrpR*, 100 µM) or tryptophan (*ΔtrpB*, *ΔtrpBΔhisL*, 100 µM) (grey bars) or in unsupplemented minimal medium (striped bars). The two species are colour-coded as *A. baylyi* (dark grey) and *E. coli* (light grey). `Cell count' refers to the number of colony forming units (CFU) after 24 h minus the initial number of CFU. Mean (± 95% confidence interval) values are shown. All comparisons (i.e. with and without amino acid) were significantly different (Paired t-test: $P<0.001$, $n=8$).



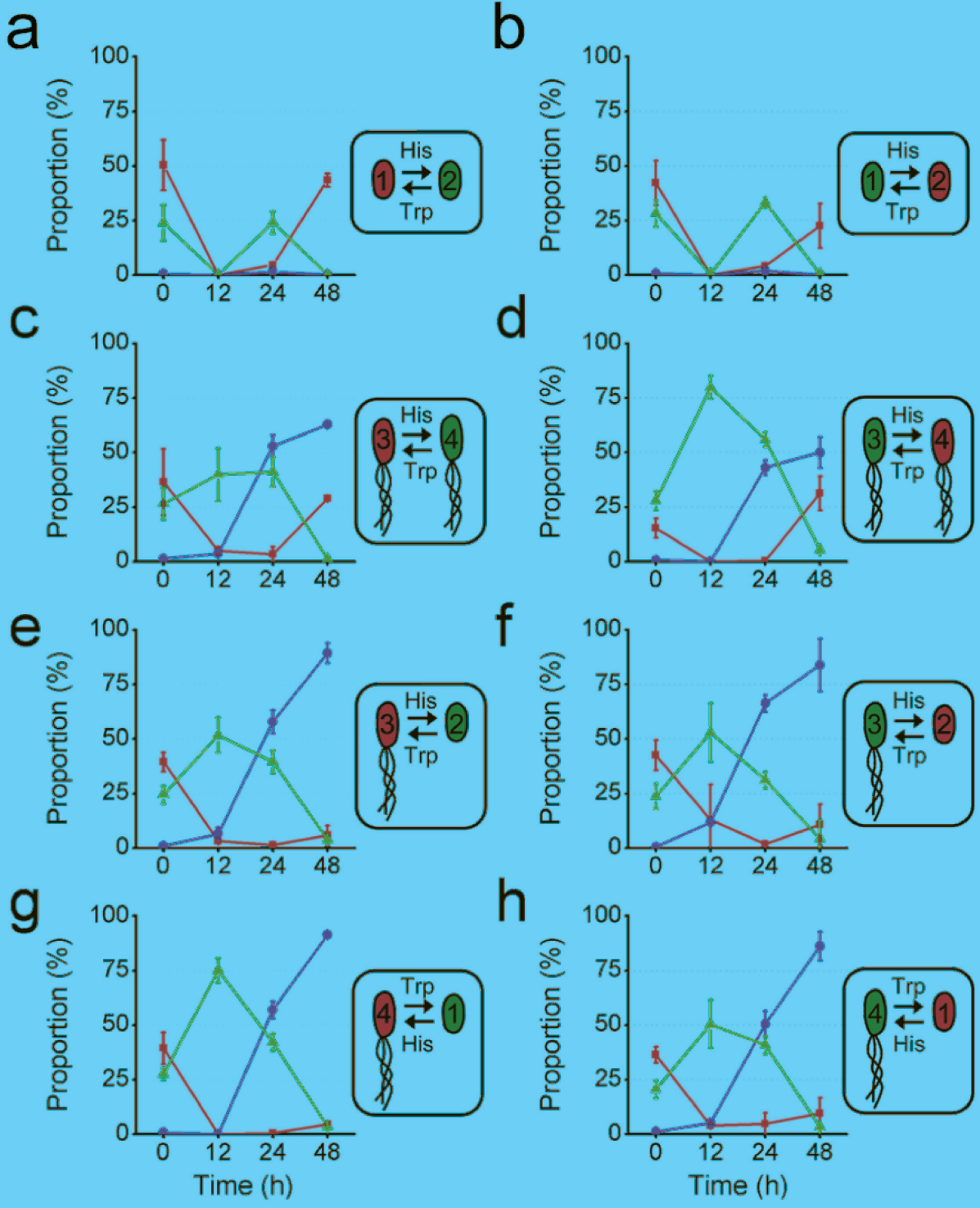
Supplementary Figure 2 | Nurmikko cell - a device that allows cultivating two bacterial populations separated by a membrane filter. The device is named after Veikko Nurmikko, a Finnish biochemist who developed a similar apparatus[1]. Cells were milled out of polyoxymethylene. (**a**) Front view of an opened Nurmikko cell. The white disk within the left part represents the filter membrane (0.2 µm). (**b**) Side view of Nurmikko cell with one growth chamber plunged into the other one (i.e. operating mode). (**c**) Sectional schematic of a Nurmikko cell in operating mode. The grey line represents the filter membrane that is pressed against the left growth chamber by the tubular protrusion of the right chamber. Pressing both chambers together seals the cell water-tightly.



Supplementary Figure 3 | Amino acid production levels of cross-feeding genotypes. Shown is the production level of (**a**) tryptophan by the *ΔtrpBΔhisL* cross-feeders or (**b**) histidine by the *ΔhisDΔtrpR* cross-feeding genotypes of both species. Amino acid production levels were determined by analysing the cell-free culture supernatants after 24 h of growth by liquid chromatography. Monocultures were grown in minimal medium supplemented with the amino acid the focal genotype required to grow (100 µM each). The two species are colour-coded as *A. baylyi* (dark grey) and *E. coli* (light grey). This experiment was replicated eight times.



Supplementary Figure 4 | Amino acids can freely pass through the filter membranes of the Nurmikko cells. Different cross-feeding mutants of both species were individually inoculated into one growth chamber of a Nurmikko cell (Supplementary Figure 2) and the required amino acid was either added on the same side of the filter membrane (S) or into the respective other growth chamber (O) to yield a final concentration of 100 µM. `*Cell count*' refers to the number of colony forming units (CFU) after 24 h minus the initial CFU number. The two species are colour-coded as *A. baylyi* (dark grey) and *E. coli* (light grey). Non-striped boxes are genotypes labelled with the *egfp*-containing plasmid and striped boxes with the plasmid containing *mCherry*. ns = not significant by Wilcoxon signed rank test: P>0.05, n=4.



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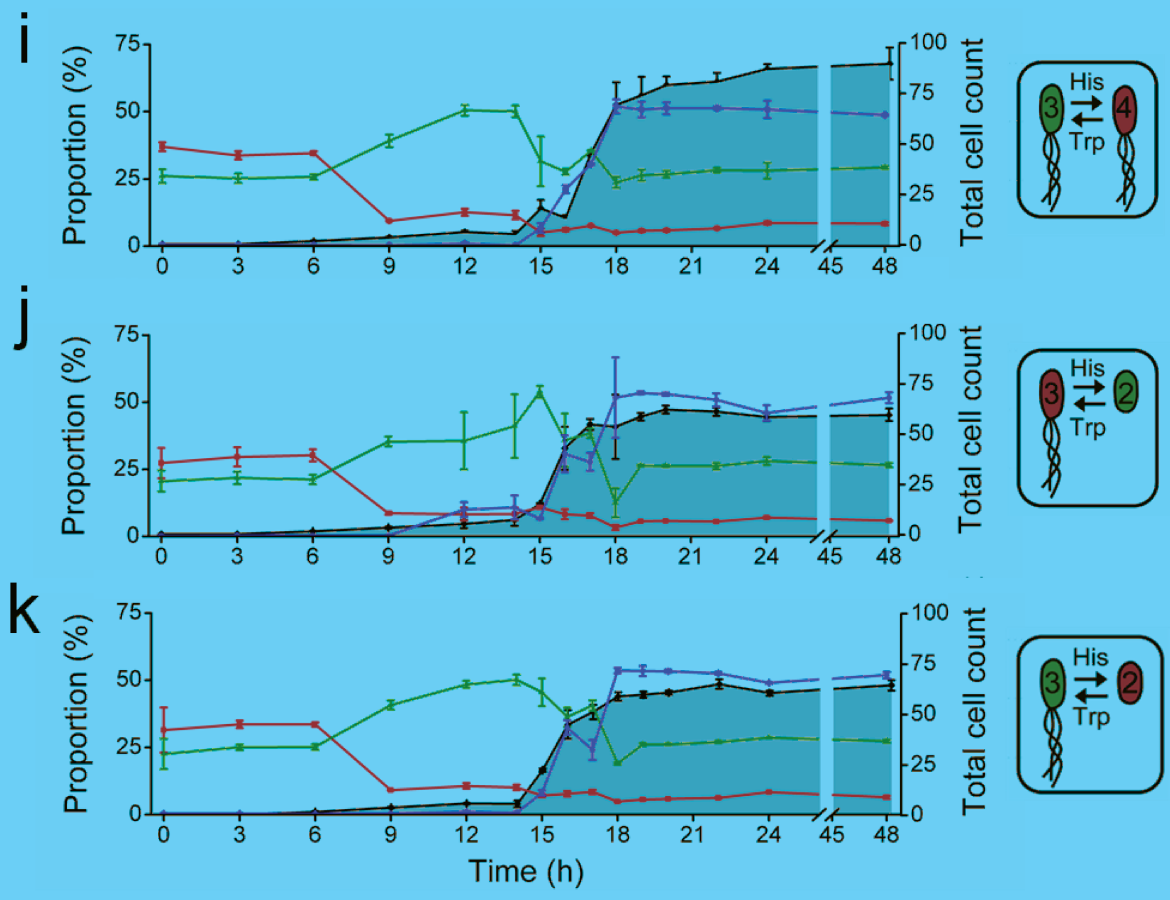
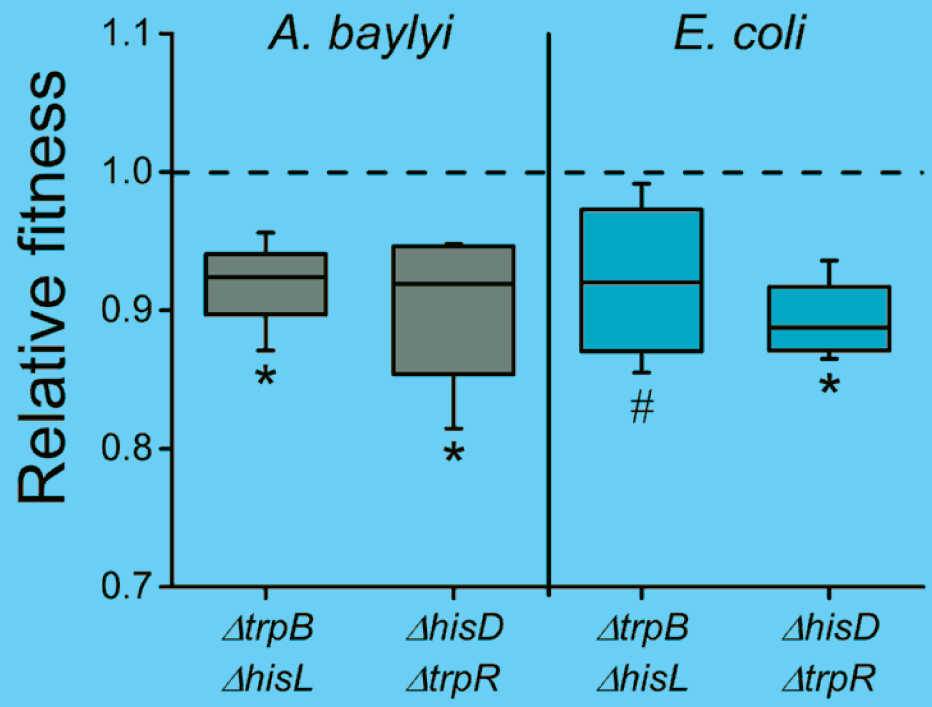
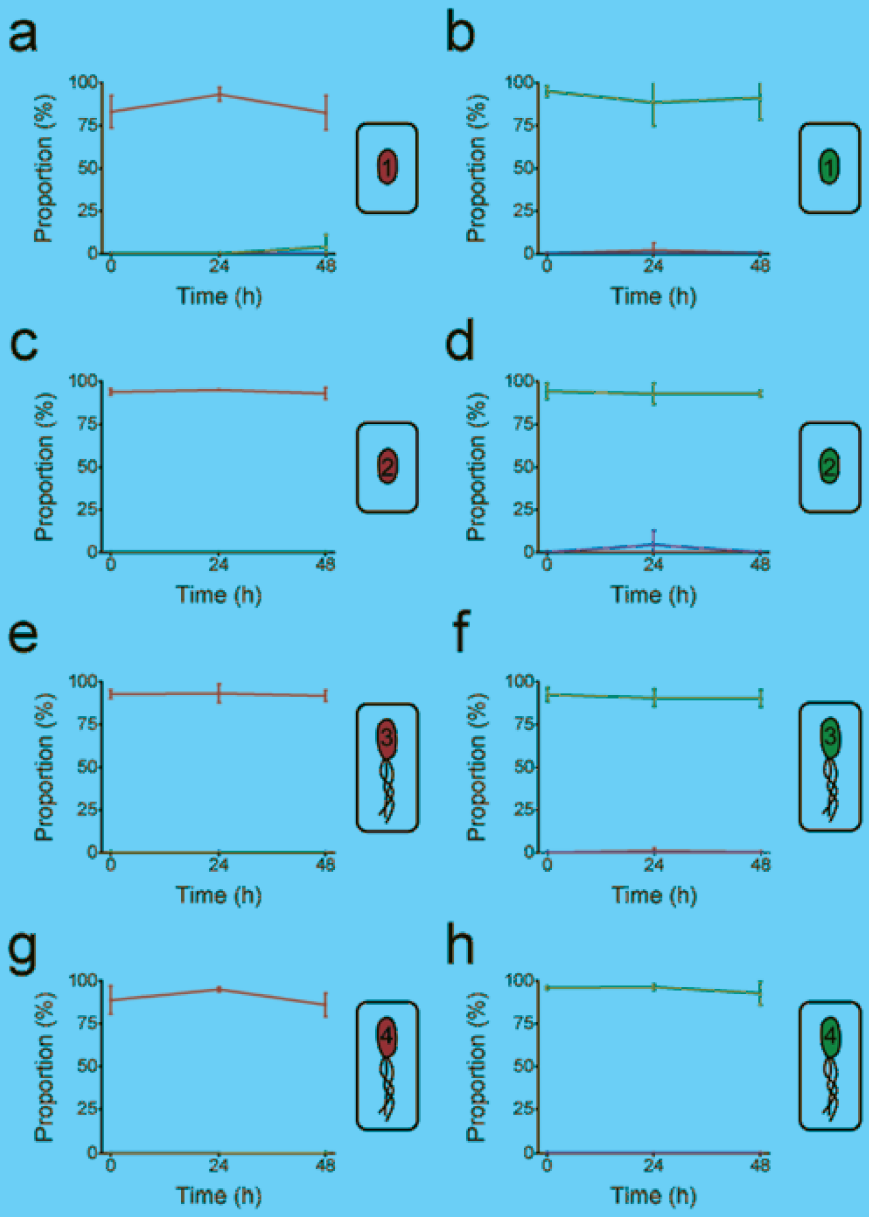


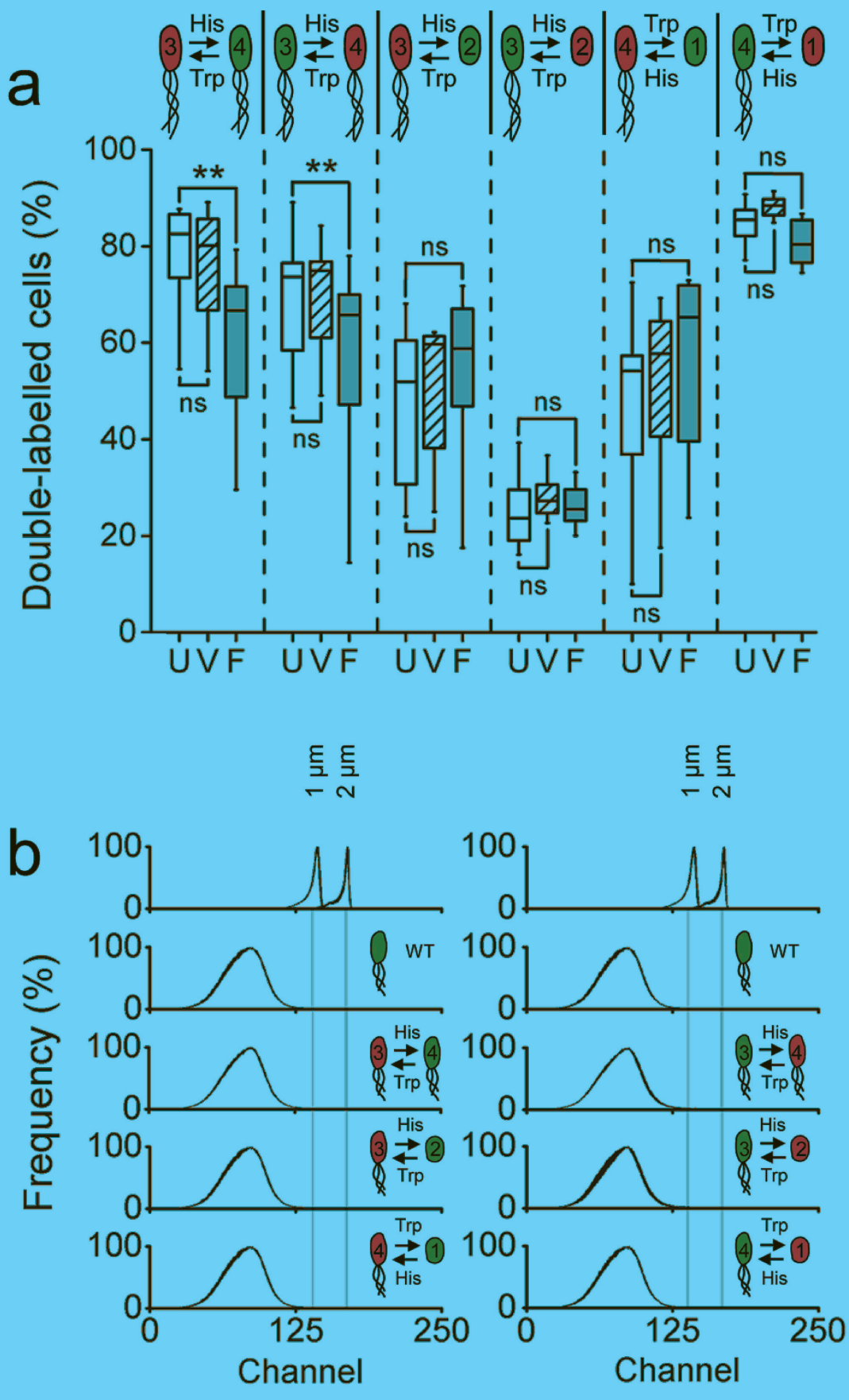
Supplementary Figure 5 | Dynamics of cytoplasmic exchange between two cross-feeding genotypes. (a-k) Proportion of cells labelled with EGFP (green line), mCherry (red line), or both markers simultaneously (blue line) and (**i-k**) total cell count (x 10^5) of the population as determined by flow cytometry (black line, grey area, secondary y-axis). The emergence of double-labelled cells (blue line) points to an exchange of cytoplasmic protein between cells. Mean values (± 95% confidence interval) of 8 replicates are shown. Genotype pictograms like in Figure 1a with green cells being labelled with EGFP and red cells with mCherry.



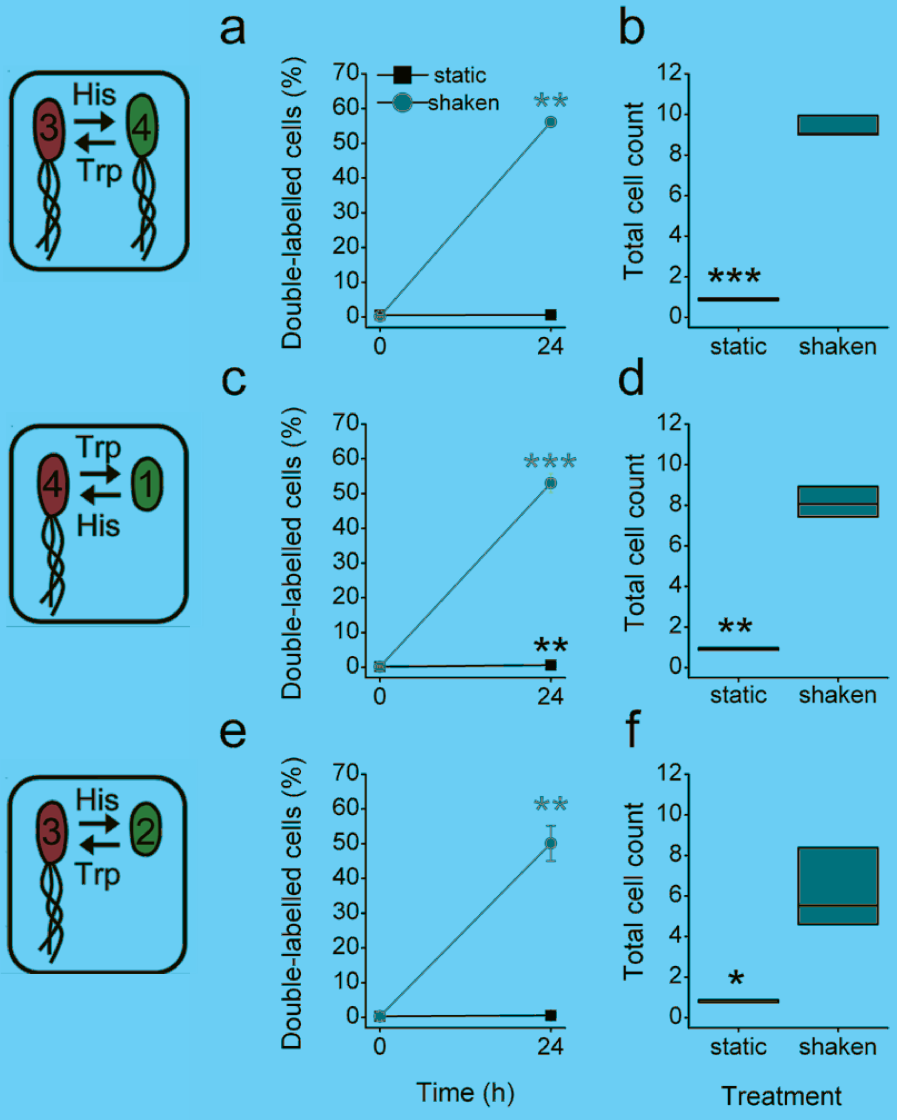
Supplementary Figure 6 | mCherry imposes a larger fitness cost than EGFP. Competitive fitness comparison of isogenic cross-feeding genotypes of both species that are labelled with the *egfp*- and *mCherry*-containing plasmid pJBS24, respectively. Relative fitness is expressed as the ratio of Malthusian parameters between both types and was determined by plating. Values below 1 indicate a fitness disadvantage of mCherry-labelled cells relative to EGFP-labelled cells. One-sample t-tests indicate significant differences from 1: * = $P<0.05$, # = $P = 0.087$). Boxplots show four replicates per comparison. The two species are colour-coded as *A. baylyi* (dark grey) and *E. coli* (light grey).



Supplementary Figure 7 | Autofluorescence cannot explain the emergence of double-labelled cells. Panels correspond to monocultures of cross-feeding genotypes of both species labelled with either an EGFP- (green cell) or mCherrry-expressing plasmid (red cell). The proportion of cells fluorescing at the wavelength expected for EGFP (green line), mCherry (red line), or both markers simultaneously (blue line) was determined by flow cytometry. Mean percentages (± 95% confidence interval) of 8 replicates are shown. Genotype pictograms like in Figure 1a.


a
Double-labelled cells (%)
100
80
60
40
20
0
**
**
ns
ns
ns
ns
ns
ns
ns
ns
ns
U V F
His
Trp
Trp
His
b
1 µm
2 µm
Frequency (%)
WT
0
125
250
Channel

Supplementary Figure 8 | Double-labelled cells are likely not due to the physical attachment of two differentially labelled cells. (**a**) Proportion of double-labelled cells after 24 h of growth as quantified by flow cytometry in untreated samples (U, white boxes), after vortexing for 1 min (V, striped boxes), or treatment with a commercially available cell dissociation solution (F, grey boxes). FDR-corrected paired t-test: ** = $P < 0.01$, ns = $P > 0.05$, n=8. (**b**) Size of wild type (WT) or double-labelled cells after 24 h of growth determined as forward scatter in the flow cytometer. The top row shows measurements of microspheres with a diameter of 0.1 µm and 0.2 µm. All other panels show the forward scatter of wild type (WT) or double-labelled cells that emerged in cocultures after 24 h of growth. Black lines of the curves represent the 95% confidence intervals (CI) of six replicate measurements. Grey lines mark the 95% CI of the microsphere measurements (n=6). Genotype pictograms like in Fig. 1a. Green cells represent those that contain the EGFP-expressing plasmid and red cells the mCherry-expressing plasmid.



Supplementary Figure 9 | Cytoplasmic exchange requires the mixing of cocultures. Cross-feeding consortia consisting of (**a,b**) two *E. coli* genotypes or (**c-f**) *A. baylyi* and *E. coli* were differentially labelled with plasmids expressing either EGFP (green cells) or mCherry (red cells) and cocultured for 24 h in liquid minimal medium under static (black lines/ boxes) or shaken conditions (grey lines/ boxes). Shown is (**a,c,e**) the proportion of double-labelled cells (i.e. cells containing both labels) at 0 h and 24 h as determined by flow cytometry as well as (**b,d,f**) the cell count (CFU x 10^6) of the corresponding populations achieved during this time minus the initial CFU count. Paired t-tests: *** = P <0.001, ** = P < 0.01, * = P > 0.05. Genotype pictograms like in Fig. 1a. This experiment was replicated three times.

Supplementary Tables

Supplementary Table 1 | Primers used for the construction of *Acinetobacter baylyi* auxotrophs.

Gene	Primer	Sequence (5'-3')
Kanamycin resistance cassette	UF	TGTAGGCTGGAGCTGCTTC
	UR	CATATGAATATCCTCCTTA
hisD	UF	TATGCAAGCCTTGGTGAGCA
	UR	GAAGCAGCTCCAGCCTACACAGCCTCTTCCACTTGA
trpB	UF	AACCACACACGCTTTTGCAG
	UR	GAAGCAGCTCCAGCCTACAGCTGATCCACATTGGACT
hisD	DF	TAAGGAGGATATTCATATGGTAACTGCTCTACGGGG
	DR	ATGCGTCTGCCTGATCTACC
trpB	DF	TAAGGAGGATATTCATATGACGTGATGTGGAAATGG
	DR	AGTTGGGGCTGGATGTCTTG
hisD	UF *kan*^{s}	TATGCAAGCCTTGGTGAGCA
	UR *kan*^{s}	CCCCGTAGAGCAGTTACCAGCCTCTTCCACTTGA
trpB	UF *kan*^{s}	AACCACACACGCTTTTGCAG
	UR *kan*^{s}	CCATTTCCACATCACGTGCTGATCCACATTGGACT
hisD	DF *kan*^{s}	GTAACTGCTCTACGGGG
	DR *kan*^{s}	ATGCGTCTGCCTGATCTACC
trpB	DF *kan*^{s}	ACGTGATGTGGAAATGG
	DR *kan*^{s}	AGTTGGGGCTGGATGTCTTG
trpR[1]	UF	GAGGTCTGGGTTGAGGTTGG
	UR	GAAGCAGCTCCAGCCTACATAACGCTGCATTTGCAC
hisL[2]	UF	TATGCAAGCCTTGGTGAGCA
	UR	GAAGCAGCTCCAGCCTACACAGCCTCTTCCACTTGA
trpR	DF	CCGTTTACAGGGCTCAGTGT
	DR	GAAGCAGCTCCAGCCTACATCACCCAATCCTGTCAC
hisL	DF	AACCACACACGCTTTTGCAG
	DR	GAAGCAGCTCCAGCCTACACAGCCTCTTCCACTTGA

UF = upstream forward, UR = Upstream reverse, DF = downstream forward, DR = downstream reverse, kan^s = primers to generate kanamycin-sensitive genotypes. [1] gene identity number: 49529273 (3095051-3095650); [2] gene identity number: 49529273 (56394-56552)

Supplementary Table 2 | Strains and plasmids used.

Strains and plasmids	ID	Genotype	Phenotype	Reference
Strains				
A. baylyi WT		ADP1	P	2
A. baylyi ΔhisD		*A. baylyi* ADP1 *ΔhisD*	A_{His}	This study
A. baylyi ΔtrpB		*A. baylyi* ADP1 *ΔtrpB*	A_{Trp}	This study
A. baylyi ΔhisL		*A. baylyi* ADP1 *ΔhisL*	O_{His}	This study
A. baylyi ΔtrpR		*A. baylyi* ADP1 *ΔtrpR*	O_{Trp}	This study
A. baylyi ΔhisD ΔtrpR	2	*A. baylyi* ADP1 *ΔhisD, ΔtrpR*	CF (A_{His}, O_{Trp})	This study
A. baylyi ΔtrpB ΔhisL	1	*A. baylyi* ADP1 *ΔtrpB, ΔhisL*	CF (A_{Trp}, O_{His})	This study
E. coli WT		BW25113 *Δ(araD-araB)56, ΔlacZ4787(::rrnB-3), λ-, rph-1, Δ(rhaD-rhaB)568, hsdR514*	P	3
E. coli ΔhisD		BW25113 *ΔhisD*	A_{His}	This study
E. coli ΔhisD (Biosensor)		BW25113 *ΔhisD::kan*^{r}	A_{His}	This study
E. coli ΔtrpB		BW25113 *ΔtrpB*	A_{Trp}	This study
E. coli ΔtrpB (Biosensor)		BW25113 *ΔtrpB::kan*^{r}		This study
E. coli ΔhisL		BW25113 *ΔhisL*	O_{His}	This study
E. coli ΔtrpR		BW25113 *ΔtrpR*	O_{Trp}	This study
E. coli ΔhisD ΔtrpR	4	BW25113 *ΔhisD, ΔtrpR*	CF (A_{His}, O_{Trp})	This study
E. coli ΔtrpB ΔhisL	3	BW25113 *ΔtrpB, ΔhisL*	CF (A_{Trp}, O_{His})	This study
Plasmids				
pJBA24		Amp^r; pUC18NotI-$P_{A1/04/03}$-RBSII-T0-T1		4
pJBA24-*egfp*		pJBA24; EGFP		5
pJBA24-*mCherry*		pJBA24; mCherry		This study

Supplementary Table 2 (continued)

Strain-plasmid combinations	*Strain*	*Plasmid*
	A. baylyi WT	pJBA24-*egfp*
	A. baylyi WT	pJBA24- *mCherry*
	A. baylyi ΔhisD	pJBA24-*egfp*
	A. baylyi ΔhisD	pJBA24- *mCherry*
	A. baylyi ΔtrpB	pJBA24-*egfp*
	A. baylyi ΔtrpB	pJBA24- *mCherry*
	A. baylyi ΔhisL	pJBA24-*egfp*
	A. baylyi ΔhisL	pJBA24- *mCherry*
	A. baylyi ΔtrpR	pJBA24-*egfp*
	A. baylyi ΔtrpR	pJBA24- *mCherry*
	A. baylyi ΔhisD ΔtrpR	pJBA24-*egfp*
	A. baylyi ΔhisD ΔtrpR	pJBA24- *mCherry*
	A. baylyi ΔtrpB ΔhisL	pJBA24-*egfp*
	A. baylyi ΔtrpB ΔhisL	pJBA24- *mCherry*
	E. coli WT	pJBA24-*egfp*
	E. coli WT	pJBA24- *mCherry*
	E. coli ΔhisD	pJBA24-*egfp*
	E. coli ΔhisD	pJBA24- *mCherry*
	E. coli ΔtrpB	pJBA24-*egfp*
	E. coli ΔtrpB	pJBA24- *mCherry*
	E. coli ΔhisL	pJBA24-*egfp*
	E. coli ΔhisL	pJBA24- *mCherry*
	E. coli ΔtrpR	pJBA24-*egfp*
	E. coli ΔtrpR	pJBA24- *mCherry*
	E. coli ΔhisD ΔtrpR	pJBA24-*egfp*
	E. coli ΔhisD ΔtrpR	pJBA24- *mCherry*
	E. coli ΔtrpB ΔhisL	pJBA24-*egfp*
	E. coli ΔtrpB ΔhisL	pJBA24- *mCherry*

ID refers to the numbering of genotypes as introduced in Fig. 1a. A_{His} = histidine auxotroph, A_{Trp} = tryptophan auxotroph, O_{His} = histidine overproducer, O_{Trp} = tryptophan overproducer, CF = cross-feeder, P = prototroph, WT = wild type.

Supplementary References

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Chapter 13: Supplementary information

Manuscript II

Metabolic coupling in bacteria

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Supplementary information for

Metabolic coupling in bacteria

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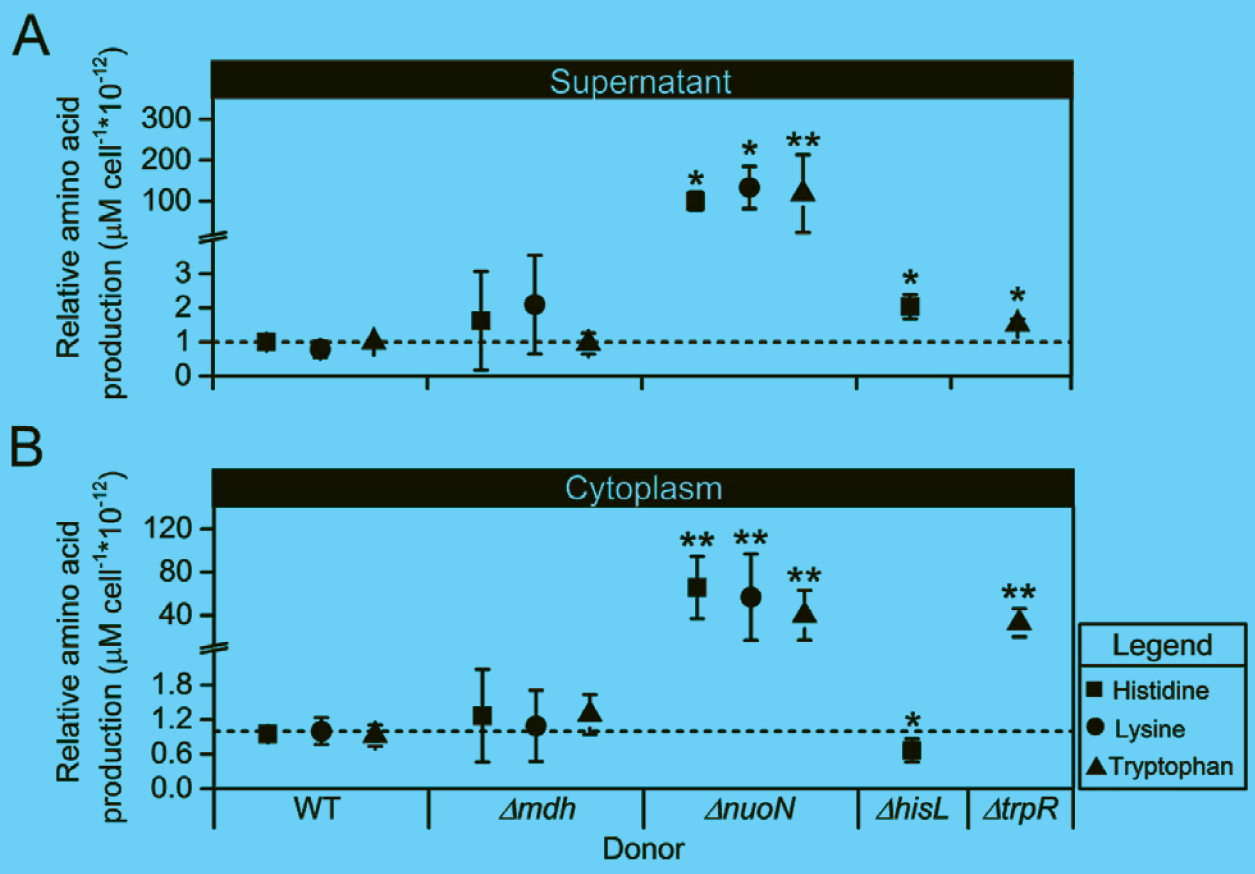
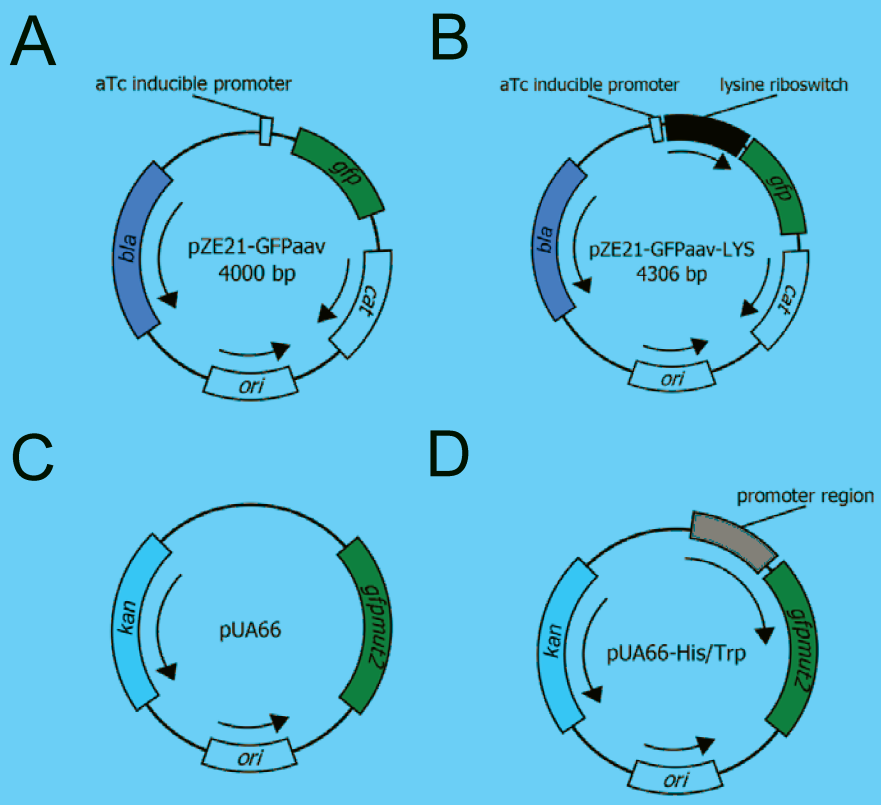
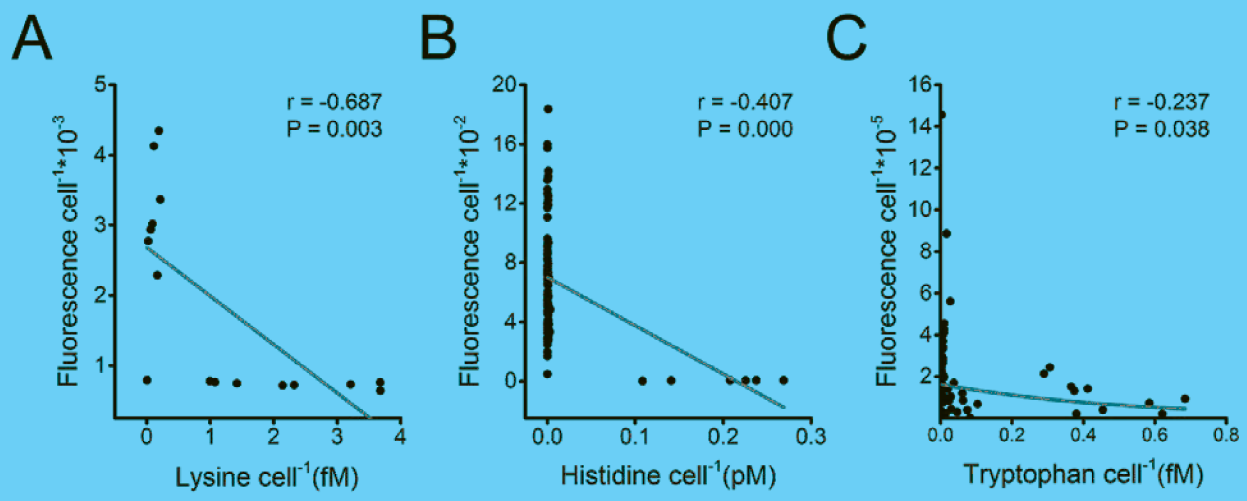
Supplementary figures



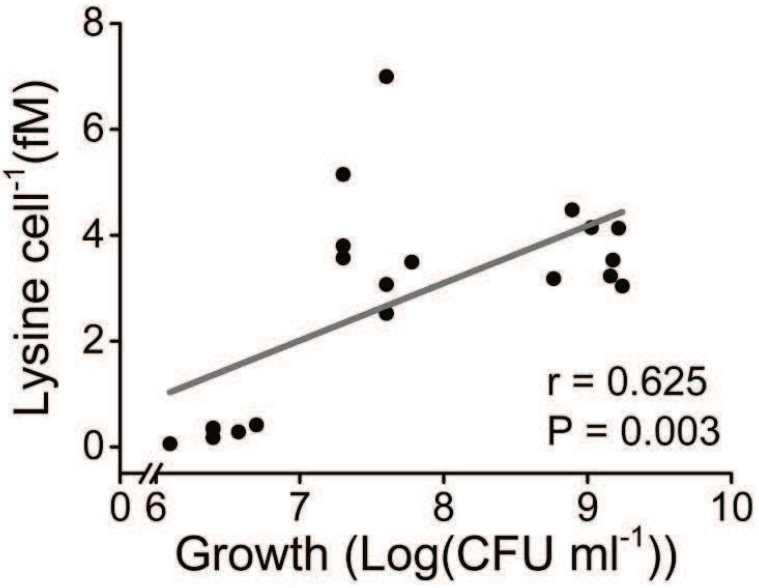
Supplementary figure 1: Amino acid production levels of different donor genotypes as determined by LC/MS/MS analysis. Values represent mean (± 95% CI) concentrations of histidine (boxes), lysine (circles), and tryptophan (triangles) per cell (i.e. number of CFUs) after 24 h of growth. Amino acid concentrations 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: ** $P < 0.01$, * $P < 0.05$, 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.



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



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).

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Chapter 13: Supplementary information

Manuscript III

Transcriptional insights from metabolite cross-feeding bacteria

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Under preparation

Supplementary information for

Transcriptional insights from metabolite cross-feeding bacteria

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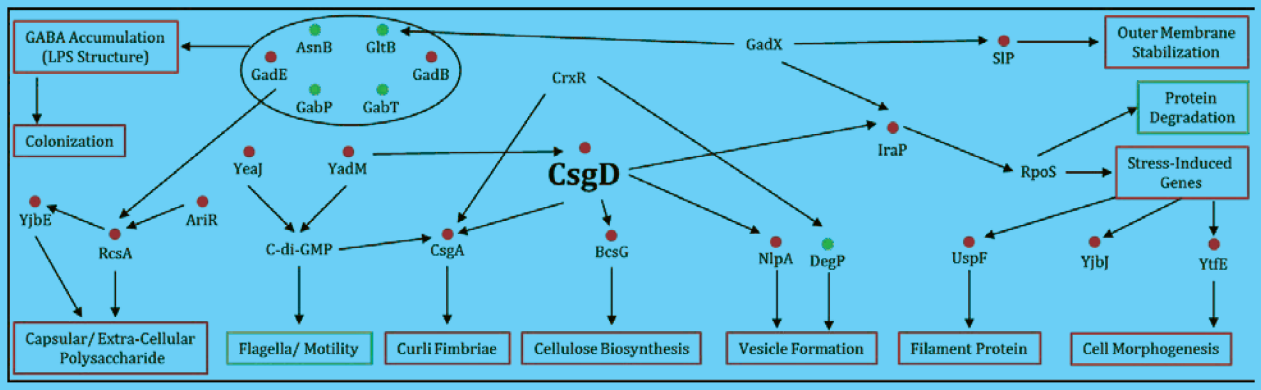
Supplemental figures and table



Supplementary figure 1: Over view of protein network obtained from transcriptional analysis of cross-feeding genotypes. Red indicates upregulated functions and green indicates downregulation. CsgD is the global transcriptional regulator that seems to connect many pathways and effect the formation of cell adhesion proteins (Curli fimbriae, cellulose biosynthesis). Other membrane structures are also upregulated and play a potential role in cell-cell attachment like GABA accumulation, extra-cellular polysaccharide. Flagella biosynthesis and motility is downregulated probably due to the fact that movement of cells during nutrient exchange is not favored.

Table 1: List of primers used for PCR based confirmation of double mutants

		PRIMERS	
Accession ID	**Gene name**	**fwd (start)**	**rev (stop)**
b3163	*nlpI*	TTTGCCCTCCGCTGCGGCGG	GTGTGAACCGGCTCAAAGTA
b3661	*nlpA*	CACTTTTATTCCTTTTTATT	GCTGGGAACGCTGCTGATTC
b0161	*degP*	TTTTACCTTTTGCAGAAACT	TGCAAATGCCTAAAGGATGA
b3870	*glnA*	TTGGCACAGATTTCGCTTTA	CCGGATAAGACGCATTTGCG
b2908	*pepP*	GCGCTGTTATGCCACGACAC	GTCGCTTCAATCAAATGTAC
b3839	*tatC*	AGAAGCCAGAAACCACGCCA	ACATCATCACGGTCTTTCGC
b1071	*flgM*	TGGGAATATTCTTATAAACC	CCCCATAGAGAGATGTTGCT
b1278	*pgpB*	ACGACGCGTCGATCGTTCAC	CGCAATAGCCCTTTAAAGTA
b3226	*nanR*	GGTAATGATGAGGCACAAAG	TGTCTGATAAACGATATACC
b0240	*crl*	CACCGACTGGATCGAAAAAC	GTCACGAAAATCATCCGCC
b1042	*csgA*	AAAACGGCAATTGTAGTGCA	TATCTGACTGGAAAGTGCCG
b1041	*csgB*	TTTCCATCGTAACGCAGCGT	TGCCAGAGCGCTACCGGAGA
b1039	*csgE*	ACGGTTAAAACGCATCTTTA	ACTGGAAAGTCATGGTTCCA
b4314	*fimA*	AAAACTGTGCAGTGTTGGCA	CAACGACGCTAATAGAAATA
b4313	*fimE*	CCGTGTGTGGTTATCTTTTT	TGCTTGAGAAAAAATACGTA
b4315	*fimI*	AATGCGGATGCGACCTTCAA	TACCTGCCAGCAAGCAGAAT
b2826	*ppdA*	ATTTTGCAACGTCCTGCAAC	TCACGCTGTAACGCAGGCAG
b1502	*ydeQ*	ACGATCGAGGCGCTAATCAA	TGCGCGGATTTTCTTACAGG
b1090	*plsX*	GCAAGGTCATCGCTAAGTAA	ATAGCTGCCAGTACCAATAA
b3525	*yhjH*	GAGGAAATTTACCCCGGCCA	TCGCAGCGGTTATTTTGCCT
b1194	*ycgR*	TTTAAGAACAACATGTAAAA	GATGCTGACGAGTTCCTCGA

Chapter 14: Acknowledgements

"Why are you doing a PhD?" is a question I have been asked many times by others, and in times of despair I too have wondered why myself. An honest answer from me would be: curiosity. It was a paper I read during my master's studies in Baroda which described cheater resistance strategies in Amoeba. This paper took me down a rabbit hole, at my own will, to discover that bacteria also show such complex interactions. The logic was simple, if I can willingly spend half a year researching a topic out of syllabus just out of curiosity, then another four years with the added bonus of doing experiments sounded perfect. Alas I realized that curiosity can only help you make that decision but it isn't enough to get you all the way through it. I have been lucky to have people in my life that helped me get through these 'PhD days' and I would like to give them a sincere thanks.

To **Christian**, for believing in me to see this project through. His supervision was a good balance between giving me independence to direct the project and, giving timely guidance when I got lost. Thanks to him I have learnt to look at the bigger picture and question why a particular approach is the right one before applying it. His suggestions, critique and advice to aim high, has helped me improve the way I present my research and also how I write about it. Lastly, thanks to his, at times annoying, perfectionism, I shall never have misalignment in my figures again.

To **Prof. Kothe**, for the discussions and helpful suggestions during committee meetings as well as providing a different perspective to the project.

To **Martin Kaltenpoth**, for his infinite enthusiasm about everything, especially science. Discussions with you have been a pleasure and always insightful.

To all my coauthors and collaborators without whom this work would have taken much longer to reach completion. **Samay**, **Lisa**, **Ilka Bischofs**, **Martin Westermann**, **Tobias Ahsendorf**, **Ramya** and **Muhammad**, your contributions, efforts and time has helped in molding the projects into the masterpieces they are now.

To the **IMPRS** for giving me the opportunity to come interview for this position. To **Karin** and **Grit** for ensuring that my transition from India to Germany was smooth and a positive experience.

To the EEEs for making life in the institute fun. **Samay**, for the endless wisdom, patience, stories and pictures of "superb food". **Holger**, for the help with experiments and tough questions at lab meetings. **Glen**, for making my first few months in Jena a happy experience despite the big change from Pune. **Daniel**, for not telling me that you were interviewing for the same position as me, for the "eheehee" s and monster madness. **Silvio**, for good music in the lab and good beer outside the lab. **Anne**, for the timely "what's up" and "haaallo" to cheer me up. **Ghada**, for keeping company during long nights. **Samir**, for making sure I

don't starve by cooking food that's "so good". **Ramya**, for being the best house elf a muggle could wish for.

To **Michael** for help with all the amino acid measurements. **Jette**, for training me in RNA quantification and the microarray experiment. **Julio Ortiz**, for giving me a hands-on experience of the TITAN (cryo-TEM). **Daniel** (from the workshop), for building the Nurmikko cells in such a short time.

To everyone in the **Boland department**, the **insect-symbiosis group** and the institute for all kinds of emotions and memories made during these 4.5 years. The symposiums, the retreats, the parties and the hangovers have all been fun.

To some of my favorite Indians in Jena, **Abith**, **Tulsi**, **Sailen**, **Latha**, **Govind**, **Eisha**, **Tilo**. You guys have taken care of me (which Latha did at times by just asking how I am doing), in one case punched me (yes I'm talking about you Eisha), gave me the most memorable birthdays (no one can beat you at that Tulsi), pushed me to excel (I am lucky to have you Abith).

To **Karen**, for listening to all the bullshit about almost everything over the past four years and sharing the love for chocolate. To **Lisa**, for being there at some very difficult times and also very happy times, the Jena-part of my life is incomplete without you. To **Kishan**, in the form of the tiny hangout bubble, for being a source of nonsense as well as inspiration. To **Jan** and **Laura**, for all the sarcasm, for helping me vent out post-experiment frustrations and for letting me cook all that indian food for you.

To the zoukers, **Stefan**, **Hannes, Franzii**, **Anna**. Dancing zouk, going for workshops to dance more zouk and then teaching zouk, was all possible because of you guys. Zouk provided a portal for me to leave behind work issues and stress so much that you guys made me look forward to a Monday night.

The final thanks, to my family, for being awesome. **Baba**, for the honest (and sometimes harsh) criticism, for pushing me to do better, for the terrible jokes and the timely reminder "man chaha hua toh accha hua, mann chaha na hua toh aur bhi accha hua, kyun ki jo hota hai accha ke liye hota hai". **Aai**, for being my punching bag and also my pillow, for always matching my "vedepana" with yours. **Sid**, for being protective, for asking questions and for the new shoes you will buy for me when I come visit. **Jui**, for being supportive, fun and visiting me so many times.

To all those I have missed out as a consequence of the past few sleepless nights, I apologize. I am thankful for any little way in which you helped me get through and enjoy my time in Jena.

Chapter 15: Curriculum vitae

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EDUCATION:

2013- present **Doctorate** at Experimental Ecology and Evolution group, Max Planck Institute for Chemical Ecology (MPI-CE), Jena, Germany
Thesis: Mechanistic and metabolic basis of bacterial cross-feeding

2009 – 2011 **Master of Science** in Microbiology at the Maharaja Sayajirao University of Baroda, Vadodara, India

2006 – 2009 **Bachelor of Science** in Industrial Microbiology at Abasaheb Garware College, University of Pune, Pune, India

RESEARCH EXPERIENCE:

2013- present **Doctorate** at Experimental Ecology and Evolution group, Max Planck Institute for Chemical Ecology (MPI-CE), Jena, Germany
Thesis: Mechanistic and metabolic basis of bacterial cross-feeding

2011 - 2012 **Research trainee** at the Indian Institute of Science Education and Research, Pune, India
Topic: Variability in cell length in *E.coli* populations and the effect of replication fork stalling on the cell length.
*This work was acknowledged in the publication that ensued (Gangan, M. S. & Athale, C. A. Threshold effect of growth rate on population variability of *Escherichia coli* cell lengths. *R. Soc. Open Sci.* **4**, (2017)).

2009 - 2011 **Thesis project** during M.Sc. at the Center for Genome Research in Vadodara, India
Topic: Generating a GFP expressing strain of *Magnaporthe oryzae* and study of the homologue of Flavohemoglobin (flavoHbs) gene in *M.oryzae*.

2007 - 2007 **Research project** during B.Sc. at Abasaheb Garware College, Pune, India
Topic: Antibacterial activity of a soil isolate of Actinomycetes.

AWARDS:

- iNext grant for imaging nanotubes at the Netherlands Centre for Electron Nanoscopy (**NeCEN**)
- Travel award (1984 €) granted by the Deutscher Akademischer Austauschdienst (**DAAD**) for the ASM conference on Experimental microbial evolution.
- Travel award (800 €) granted by the GlaxoSmith Kline foundation (**GSK**) for the ASM conference on Experimental microbial evolution.
- 2nd position at the 6th Jena **Science slam**, Friedrich Schiller University, Germany.

ADVANCED TRAINING & WORKSHOPS:

2014 December	**Adobe illustrator** training, Max Planck Institute for Chemical Ecology, Germany.
2014 August	**Analysis of Transcriptome Data,** Max Planck Institute for Chemical Ecology, Germany.
2014 June	**Research Funding - Third Party Grant Proposals,** Max Planck Institute for Chemical Ecology, Germany.
2014 January	**Statistics with R** course, Max Planck Institute for Chemical Ecology, Germany.
2013 November	**Fluorescence-in-situ hybridization** course, Max Planck Institute for Chemical Ecology, Germany.
2013 June	**Evolutionary Biology Workshop** in the Alps, Riederalp, Switzerland.
2008	**Industrial training** at Elder Pharmaceuticals, Nerul, Navi Mumbai, India.

COLLABORATION VISITS:

2015 September	Max Planck Institute for Biochemistry, Molecular structural biology, **Munich**, Germany.
2014 March	Center for quantitative analysis of molecular and cellular bio-systems, University of **Heidelberg**, Germany.

ORGANIZATIONAL SKILLS:

- Member of the **organizing committee** of the 5th International Student conference on Microbial Communication (MiCom) in Jena, Germany.
  Responsibilities: Event branding and website design, organization of workshops.
- **Instructor** for two semester courses (October 2016- March 2017) at the Friedrich Schiller University for beginners in Brazillian zouk dance.

Chapter 16: List of publications

- Pande, S*., **S. Shitut***, L. Freund*, M. Westermann, F. Bertels, C. Colesie, I. B. Bischofs and C. Kost. Metabolic cross-feeding via intercellular nanotubes among bacteria. *Nat Commun* **6**, doi:10.1038/ncomms7238 (2015)
  *indicates shared authorship

- **Shitut, S**., Ahsendorf, T., Pande, S., Egbert, M. & Kost, C. Metabolic coupling in bacteria. Under revision at The ISME Journal, preprint on *bioRxiv*, doi:10.1101/114462 (2017)

- **Shitut, S.**, Ganesan, R., Atiqur, M., Westermann, M., Vogel, H., Kost, C. Transcriptional insights from metabolite cross-feeding bacteria. (Under preparation)

- D'Souza, G., **Shitut, S**., Preussger, D., Waschina, S., Abdelsalam, G., and Kost, C. Ecology and evolution of metabolic cross-feeding interactions in bacteria. (Invited review under preparation, Natural Product Reports)

Chapter 17: Conference presentations

Oral presentations

Shitut S., Kost C. The bacterial network. 14th Annual **IMPRS symposium**, Jena, Germany, February 2015

Shitut S., Kost C. Metabolite production in crossfeeding cells based on Le Chatelier's principle. **MPI-CE Institute symposium**, Jena, Germany, September 2014

Poster presentations

Shitut S., Ahsendorf, T., Pande, S., Egbert, M. & Kost, C. The bacterial network: Nutrient exchange via nanotubes. 2nd American Society for Microbiology (**ASM**) conference on Experimental Microbial Evolution , Washington DC, USA, August 2016

Shitut S, Pande, S., Westermann M., Bischofs I. and Kost C. The bacterial network: Nutrient exchange via nanotubes. **Annual Conference 2016 of the Association for General and Applied Microbiology (VAAM), Jena, Germany, March 2016**

Shitut S, Pande, S., Westermann M., Bischofs I. and Kost C. The bacterial network: Nutrient exchange via nanotubes. 15th Congress of the European Society for Evolutionary Biology **(ESEB)**, Lausanne, Switzerland, August 2015

Shitut S., Kost C. Intercellular cytoplasmic coupling facilitates metabolic crossfeeding. 5th International Student conference on Microbial Communication **(MiCom)**, Jena, Germany, April 2015

Shitut S., Kost C. Supply on demand: Intercellular control of amino acid production in *E.coli*. 13th Annual IMPRS symposium, Jena, Germany, February 2014

Shitut S., Parvate A., Rahane C., Panse M., Bhedasgaonkar V., Deshpande N. Antibacterial activity of soil isolate of Actinomycetes. 48th annual conference of Association of Microbiologists of India **(AMI)**, Chennai, India, December 2007

Chapter 18: Declaration/Eigenständigkeitserklärung

Declaration of authorship/Selbständigkeitserklärung

Ich erkläre, dass ich die vorliegende Arbeit selbständig und unter Verwendung der angegebenen Hilfsmittel, persönlichen Mitteilungen und Quellen angefertigt habe.

I certify that the work presented here is, to the best of my knowledge and belief, original and the result of my own investigations, except as acknowledged, and has not been submitted, either in part or whole, for a degree at this or any other university.

Shraddha Satish Shitut Jena, 29.06.2017