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ORIGINAL ARTICLE

# Privatization of cooperative benefits stabilizes mutualistic cross-feeding interactions in spatially structured environments

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**Metabolic cross-feeding interactions are ubiquitous in natural microbial communities. However, it remains generally unclear whether the production and exchange of metabolites incurs fitness costs to the producing cells and if so, which ecological mechanisms can facilitate a cooperative exchange of metabolites among unrelated individuals. We hypothesized that positive assortment within structured environments can maintain mutualistic cross-feeding. To test this, we engineered *Acinetobacter baylyi* and *Escherichia coli* to reciprocally exchange essential amino acids. Interspecific coculture experiments confirmed that non-cooperating types were selectively favoured in spatially unstructured (liquid culture), yet disfavoured in spatially structured environments (agar plates). Both an individual-based model and experiments with engineered genotypes indicated that a segregation of cross-feeders and non-cooperating auxotrophs stabilized cooperative cross-feeding in spatially structured environments. Chemical imaging confirmed that auxotrophs were spatially excluded from cooperative benefits. Together, these results demonstrate that cooperative cross-feeding between different bacterial species is favoured in structured environments such as bacterial biofilms, suggesting this type of interactions might be common in natural bacterial communities.**
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## Introduction

Bacteria are ubiquitous and exist within surface-bound biofilm communities rather than as isolated, planktonic cells (Tolker-Nielsen and Molin, 2000). This strong propensity to attach to surfaces suggests a strong adaptive value of an aggregated lifestyle relative to a planktonic, free-living state. Reasons that may account for the inclination of bacteria to form biofilms include protection from predation, immune response, desiccation, or toxic chemicals (Costerton *et al.*, 1999; Jefferson, 2004; Matz *et al.*, 2005).

Surface-attached bacterial communities are commonly densely packed, thereby drastically affecting ecological interactions among co-residents (Hall-Stoodley *et al.*, 2004). For example, a strong nutrient limitation can result in exploitative competition (Hansen *et al.*, 2007) or benefit genotypes, which secrete compounds that are toxic to neighbouring cells (Lenski and Riley, 2002; Rendueles and Ghigo, 2012). On the other hand, an increasing number of cooperative behaviours are being discovered among microorganisms (Velicer, 2003) that can significantly affect the fitness of interacting genotypes (Kreft, 2004).

One important type of microbial cooperation involves the production of so-called 'public goods'. These diffusible substances are released into the cell-external environment, thus benefiting not only the producer or its relatives, but potentially also other organisms in the vicinity. Examples involve digestive enzymes (Greig and Travisano, 2004), polymeric substances produced to form biofilms (van Gestel *et al.*, 2014), or chelating compounds that aid nutrient acquisition (Luján *et al.*, 2015).

Another type of interaction is metabolic cross-feeding or syntrophy (Schink, 2002; Sieuwerts *et al.*, 2008; Morris *et al.*, 2013), in which microorganisms release metabolites into the cell-external environment that are used by others as a nutrient or energy source (Honegger, 1998; Reeburgh, 2007; Morris *et al.*, 2013; Pande *et al.*, 2013; Udvardi and Poole, 2013).

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Although the production and utilization of by-products is likely incidental and not selected for, an active investment of one bacterial cell to benefit others is difficult to reconcile with natural selection. In particular, it is not clear how such interactions can resist the invasion of non-cooperating types, which reap the benefits without reciprocating (Herre *et al*., 1999; Strassmann *et al*., 2000; Sachs *et al*., 2004; Travisano and Velicer, 2004). Moreover, metabolic interactions are often obligatory (Morris *et al*., 2013). Thus, a cell that has lost its metabolic autonomy might face difficulties to encounter complementary genotypes to supply it with the metabolites that it requires to grow, thereby limiting the potential of cooperative cross-feeding to evolve in natural microbial communities (Oliveira *et al*., 2014).

Despite these predictions, an increasing number of cross-feeding interactions are being reported to have evolved in laboratory-based settings (Sieuwerts *et al*., 2008; Harcombe, 2010; Poltak and Cooper, 2011) or natural microbial communities (Schink, 2002; McCutcheon and Moran, 2007; Morris *et al*., 2013), in which the interacting partners seem to actively invest resources into their respective counterpart. One explanation to account for these observations could be a causal connection to the above-mentioned preference of bacteria to occur in surface-attached communities. Growth in spatially structured environments could enhance local feedbacks among cooperators and thus increase reciprocity (Sachs *et al*., 2004). As a consequence, the likelihood that cooperators interact with other cooperators might be increased over cooperator–non-cooperator interactions, and this so-called positive assortment (Fletcher and Doebeli, 2009) could help to maintain cooperative interactions (Nowak and May, 1992; Sachs *et al*., 2004; Kim *et al*., 2008; Nadell *et al*., 2010; Nahum *et al*., 2011; Estrela and Brown, 2013; Nadell *et al*., 2013; van Dyken *et al*., 2013).

Indeed, a recent study explored the dynamics of two engineered yeast populations (*Saccharomyces cerevisiae*) that reciprocally exchanged essential amino acids upon cell lysis (Shou *et al*., 2007). The authors provided compelling evidence that in their model system spatial assortment could indeed prevent non-cooperating types from overexploiting cooperative benefits (Momeni *et al*., 2013a). However, it remained unclear whether the observed positive assortment was a property that generally emerges when obligate cross-feeders interact in spatially structured environments, or if the finding was in any way specific to the focal model system.

So far, our understanding of the importance of spatial structure for promoting cooperation among microorganisms is almost exclusively based on intraspecific interactions (Rainey and Rainey, 2003; Shou *et al*., 2007; Nahum *et al*., 2011; van Dyken *et al*., 2013; Drescher *et al*., 2014; Müller *et al*., 2014; van Gestel *et al*., 2014). Moreover, spatial structure has also been shown to reduce levels of cooperation (Hauert and Doebeli, 2004), for example, by intensifying competition among cooperators (Taylor, 1992; Wilson *et al*., 1992) or by limiting the exchange of cooperative benefits (Verbruggen *et al*., 2012). Given that bacteria frequently live in species-rich, surface-attached communities, clarifying how growth in spatially structured environments affects the exchange of metabolites among obligately dependent genotypes and, thus, the potential of cooperative cross-feeding to evolve and be maintained within these communities, is a major outstanding problem.

Here we studied the effect of spatial structure on maintaining cooperative amino acid cross-feeding using synthetically engineered interactions between the two bacterial species *Acinetobacter baylyi* and *Escherichia coli*. We demonstrate that non-cooperative genotypes are selectively favoured in unstructured environments, whereas they are selected against in structured environments. By combining individual-based modelling with experimental work, we show that segregation of cooperators and non-cooperators within a single bacterial colony can result in a spatial isolation of non-cooperating types, thus limiting their impact on the cross-feeding consortium. Moreover, spatially resolved laser-assisted desorption/ionization time of flight mass spectrometry imaging (MALDI-TOF MSI) of bacterial colonies revealed increased amino acid concentrations in cooperator-rich regions, whereas amino acid concentrations were generally low in areas populated with non-cooperators. Together, our results illustrate that spatial structure can stabilize cooperative cross-feeding interactions between two bacterial species by spatial segregation of cooperating and non-cooperating genotypes, which limits the access of non-cooperators to cooperative benefits.

## Materials and methods

*Strain construction and plasmids used*

Genetic targets, which would lead to auxotrophies for the two amino acids histidine (His) and tryptophane (Trp) or increased production rates of His and Trp (that is, 'overproducers'; Figure 1a) upon deletion from the genomes of *Acinetobacter baylyi ADP1* and *Escherichia coli* BW25113 were identified using the KEGG pathway database (Ogata *et al*., 1999). *E. coli* BW25113 (Baba *et al*., 2006) was used as wild type (WT), into which deletion alleles from existing strains (Baba *et al*., 2006) or the arabinose utilization locus ($Ara^+$) from *E. coli* strain REL 607 (Lenski *et al*., 1991) were introduced by P1 phage transduction (Thomason *et al*., 2007). To construct double-deletion mutants, previously constructed auxotrophic genotypes were used as receiver and amino acid overproducers as donor genotype. For this, the kanamycin-resistance cassette was removed from the receiver's genome (Datsenko and Wanner, 2000). *A. baylyi* ADP1 deletion mutants

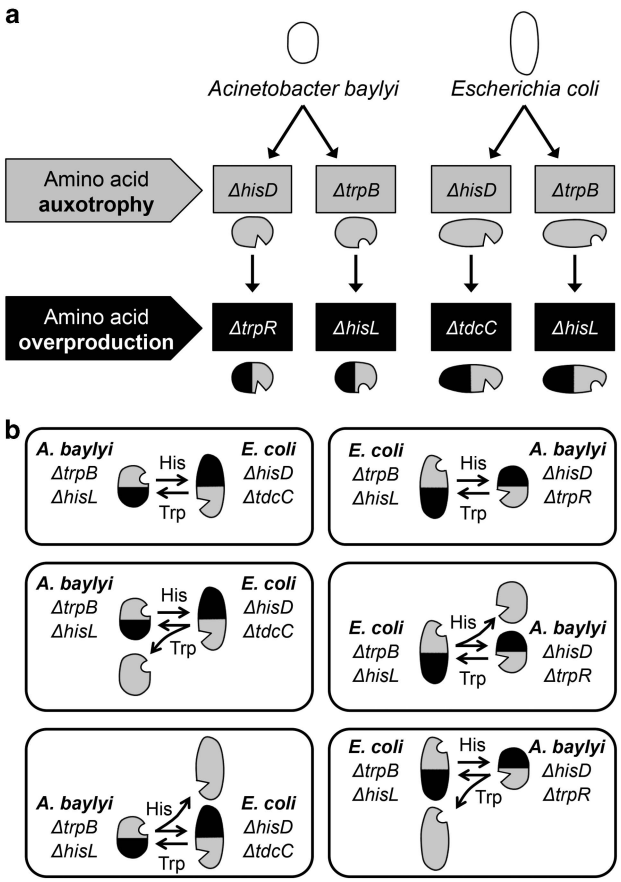


**Figure 1** Design of synthetic cross-feeding interactions and consortia of cross-feeding and auxotrophic genotypes used. (**a**) Overview over the genes deleted in *Acinetobacter baylyi* and *Escherichia coli* wild type to yield four double-deletion mutants (that is, 'cross-feeders'), in which a mutation causing overproduction of either histidine (His) or tryptophan (Trp) was combined with a mutation causing auxotrophy for the respective other amino acid. (**b**) Combinations of cross-feeders (cooperators) and auxotrophs (non-cooperators) used.

were constructed as described (de Berardinis *et al.*, 2008; Supplementary Methods).

All double-deletion strains were transformed with the plasmid pJBA24-*mCherry* that expresses the red fluorescent protein mCherry, while auxotrophic strains were transformed with the plasmid pJBA24-*EGFP* that expresses the green fluorescent protein EGFP (Pande *et al.*, 2015).

*Culture conditions*

Cultures were generally incubated at 30 °C and all the experiments were performed in minimal medium for *Azospirillum brasilense* (MMAB; Vanstockem *et al.*, 1987) without biotin and using fructose (5 g $l^{-1}$) instead of malate as carbon source. Pre-cultures were grown in MMAB medium supplemented with the focal amino acid (100 μM). Replicate pre-cultures were started from individual colonies growing on freshly streaked LB agar plates. Overnight pre-cultures were diluted to an optical density of 0.1 at 600 nm and 10 μl (~$10^5$ colony-forming units (CFUs)) of this dilution were used to inoculate subsequent experiments. If necessary, kanamycin (50 μg $ml^{-1}$) or amino acids (100 μM His and/ or Trp) were supplemented to the growth medium.

*Amino acid quantification using biosensors*

Amino acid production of overproducing and WT strains of both species were estimated by coculturing the corresponding genotypes together with *E. coli* mutants auxotrophic for the corresponding amino acid (1:1 ratio) in MMAB medium (~$10^5$ CFU of each population). Growth of biosensors was estimated at 0 h and 24 h by plating on LB agar plates that did or did not contain kanamycin. Finally, the net cell count (CFU $ml^{-1}$) of the biosensor was determined by subtracting the CFU count at 0 h from the value determined after 24 h. This experiment was replicated eight times.

*Competitive fitness assays*

The fitness of two genotypes was compared by co-inoculating both strains (1:1 ratio, ~$10^5$ CFU each) and determining their frequencies at 0 h and 24 h by dilution-plating the culture on LB agar plates with or without kanamycin. Relative fitness was calculated as the ratio of Malthusian parameters (Lenski *et al.*, 1991). The cost of amino acid overproduction was determined by competing amino acid overproducers against the corresponding WT. Auxotrophs were competed against conspecific cross-feeders in the presence of the required amino acid (that is, His or Trp). These experiments were replicated eight times.

*Effect of spatial structure*

Two cross-feeders were co-inoculated (1:1 ratio, ~$10^5$ CFU each) in 1 ml MMAB medium or as a drop of 10 μl culture on a membrane filter (pore size 0.22 μm, Millipore GPWP0 1300, Merk KGaA, Darmstadt, Germany) and placed on MMAB agar in a 24-well flat-bottom microtiter plate (Nunc, Thermo Fisher Scientific, Schwerte, Germany). To ensure a random spatial distribution, all the genotypes were mixed in liquid medium before inoculation. The frequency of both cross-feeding strains was determined after 24 h by plating on tetrazolium arabinose indicator agar (Levin *et al.*, 1977) with or without kanamycin. The effect of non-cooperators on a cross-feeding community was analysed by coculturing a proportion of 0%, 20%, and 60% of one of the four possible auxotrophic strains together with the cross-feeding community. After 0 h and 24 h, the population-level frequencies of the auxotroph and both cross-feeding mutants were determined using the arabinose utilization marker ($Ara^+$/$Ara^-$) by plating on tetrazolium arabinose indicator agar with and without kanamycin. This experiment was performed with all possible three-partite

combinations of one auxotroph and two interspecific cross-feeders (Figure 1b).

To rule out a fitness disadvantage of auxotrophs growing on agar plates, interspecific cultures of two cross-feeding genotypes that contained initial population-level proportions of 0%, 20%, and 60% of one auxotroph were inoculated together on a membrane filter. This time, however, the filter was placed on MMAB agar, which was supplemented with both His and Trp. Finally, the population-level frequencies of auxotrophic and cross-feeding genotypes were determined as described above. All the experiments were replicated eight times.

*Fluorescence microscopy*

The spatial distribution of cross-feeders and auxotrophs was visualized by analysing colonies of EGFP-labelled auxotrophs and mCherry-labelled cross-feeders after 24 h of growth using fluorescence microscopy. For this, both cross-feeders (~$10^5$ CFUs of each strain) and auxotrophs (60% of the total population) were cocultured on a membrane filter as described above. After 24 h, the filter disc with the bacterial colony was transferred on a microscope slide. Samples were examined using an Axio Imager Z1 Zeiss microscope (Carl Zeiss, Jena, Germany). Images were analysed using the software AxioVision LE Rel. 4.4 (Carl Zeiss). Immediately after microscopic analysis, the colonies were subjected to MALDI MSI (Supplementary Methods).

The three-dimensional distribution of auxotrophs and cross-feeders in a colony were analysed using laser scanning confocal microscopy. For this, coculture colonies were grown for 24 h on filter membranes, which were subsequently transferred to a glass slide and scanned with a Zeiss LSM 710 NOL confocal microscope (Carl Zeiss) using a ×10 objective. Images were analysed using Zeiss LSM Image Browser (Carl Zeiss).

## Results

*Generation of synthetic cross-feeding interactions*

To construct amino acid cross-feeding interactions between *A. baylyi* and *E. coli,* mutants of both species were created that were auxotrophic for either histidine (His) or tryptophan (Trp). For this, the terminal genes of the His (*hisD*) and Trp (*trpB*) biosynthetic pathway were deleted from the WT background of both species (Figure 1a). Subsequently, genotypes were generated that produce increased amounts of His and Trp (hereafter: *overproducers*, Figure 1a). In both species, histidine overproduction was achieved by deleting *hisL* (that is, the operon leader peptide) to eliminate negative transcriptional regulation of the His pathway. Tryptophan overproduction was realized by deleting *trpR* (that is, the tryptophan repressor protein) from the genome of *A. baylyi* WT and *tdcC* (that is, an amino acid transporter) from the *E. coli* WT. Combining an amino acid auxotrophy-causing mutation together with a mutation resulting in overproduction of the respective other amino acid in a single genetic background yielded two interspecific pairs of double-deletion mutants with complementary metabolic requirements (hereafter: cross-feeders, Figure 1a).

*Characterization of synthetic cross-feeding interactions*

The engineered cross-feeders and auxotrophs needed to fulfil two key requirements to serve as a model system for studying the maintenance of obligate interspecific metabolic cooperation. First, all cross-feeding mutants needed to produce increased amounts of the amino acids relative to non-producing auxotrophs. Second, amino acid overproduction should incur a fitness cost.

To verify the first condition, the amino acid overproducers of both species were individually cocultured with *E. coli* mutants auxotrophic for either His or Trp (initial ratio: 1:1). Growth of these auxotrophs during 24 h is indicative of the amino acids levels produced by the focal overproducers (Bertels *et al.*, 2012). This experiment confirmed that overproducers of both species produced amino acid levels that were significantly increased over wild-type levels (least significant difference (LSD) *post hoc* test: $P<0.05$, $n=8$, Figure 2a) and which were sufficient to support the growth of cocultured auxotrophs.

Next, the prediction that amino acid overproduction incurs a fitness cost was tested in two ways. First, overproducers of both species were competed against the respective wild-type strains (initial ratio: 1:1) in minimal medium supplemented with the respective other amino acid. This experiment revealed that even in the presence of the cooperative benefit, overproducers were significantly less fit (10–20%) than the corresponding wild type (one-sample *t*-test: $P<0.05$, $n=8$, Figure 2b). Second, directly competing cross-feeding genotypes against the cognate auxotrophs (for example, *ΔhisDΔtrpR* versus *ΔhisD*) in minimal medium supplemented with the focal amino acid revealed a significantly reduced fitness (4–8%) of cross-feeders relative to auxotrophs (one-sample *t*-test: $P<0.05$, $n=8$, Figure 2c). Thus, both experiments indicated a substantial cost of amino acid overproduction. Together, these results confirm that the introduced overproduction alleles resulted in increased amino acid production levels that were costly to the producing cells. This library of mutants allowed assembling two interspecific consortia of cells that reciprocally exchanged His and Trp. By introducing one of the two possible auxotrophic genotypes to these consortia (Figure 1b), it was possible to determine how the presence of non-producing genotypes affects the focal cooperative interaction.

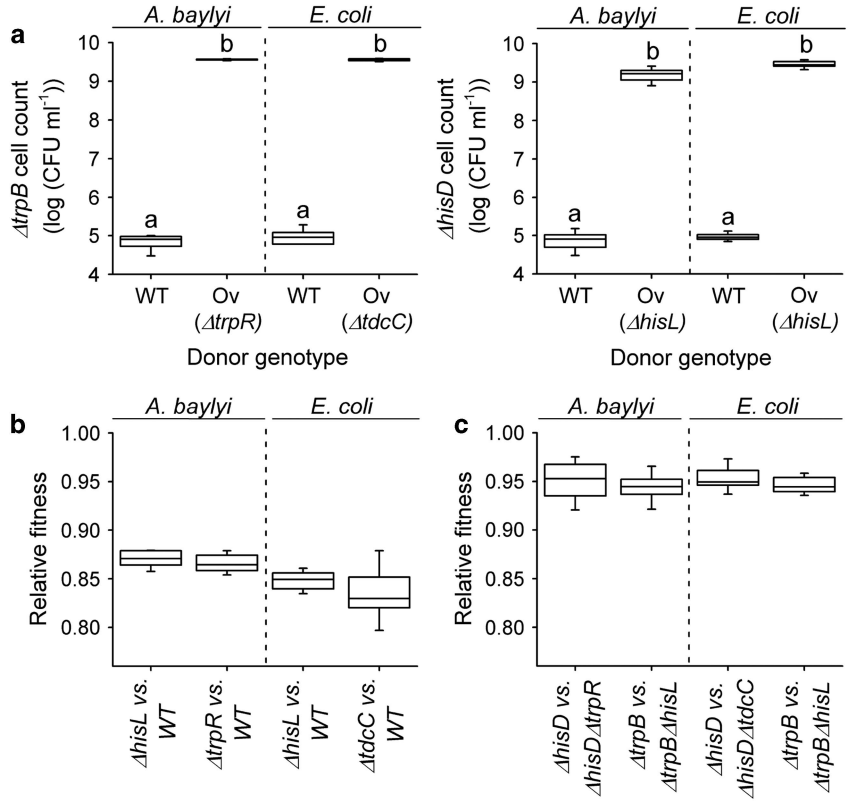


**Figure 2** Amino acid production levels of overproducing genotypes and cost of amino acid production. (**a**) Amino acid production of wild-type (WT) and overproducing mutants (Ov) of *A. baylyi* (left) and *E. coli* (right) as determined by coculturing each overproduction mutant together with an *E. coli* biosensor auxotrophic for either histidine (Δ*hisD*) or tryptophan (Δ*trpR*) (initial ratio: 1:1). Different letters indicate significant differences (LSD *post hoc* test: $P<0.05$, $n=8$). Shown is the log productivity of amino acid biosensors. (**b**) Competitive fitness of amino acid overproduction mutants relative to the respective WT. All strains tested were less fit than the corresponding WT (that is, below 1; one-sample *t*-test: $P<0.01$, $n=8$). (**c**) Competitive fitness of double-deletion mutants relative to the respective auxotrophic strain in minimal medium, which has been supplemented with the focal amino acid. The *A. baylyi* (left) and *E. coli* (right) double-deletion mutants were significantly less fit than the corresponding auxotrophs (that is, below 1; one-sample *t*-test: $P<0.05$, $n=8$).

*Spatial structure favours cooperative cross-feeding*

Significant fitness costs of amino acid overproduction suggested a susceptibility of the generated cross-feeding interactions to non-cooperating auxotrophs. In spatially unstructured environments, the released amino acids should be equally available to both cross-feeders and auxotrophs. Under these conditions, the resulting social conflict should lead to a collapse of the cooperative cross-feeding interaction. In contrast, spatially structured environments are expected to favour cooperative cross-feeding (Nowak and May, 1992; Nowak *et al.*, 1994; Sachs *et al.*, 2004; Nadell *et al.*, 2010; Momeni *et al.*, 2013a).

These predictions were verified by coculturing both cross-feeding consortia in the absence or presence of one of the two possible auxotrophs at different initial ratios (0%, 20%, or 60% of the total population) in either spatially unstructured (that is, shaken, liquid medium) or structured (that is, agar surface) environments. In line with expectations, these experiments confirmed that in unstructured environments, the presence of non-cooperating auxotrophs resulted in a significantly reduced productivity of the entire cross-feeding community (LSD *post hoc* test: $P<0.05$, $n=8$, Figure 3a), whereas the adverse effect of auxotrophs was limited in spatially structured environment (LSD *post hoc* test: $P<0.05$, $n=8$, Figure 3a). After 24 h, auxotrophs reached—independent of their initial proportion—a community-level frequency of ~60% in spatially unstructured environments, while their proportion in spatially structured environments did not exceed 10–25% (Figure 3b). This finding suggests that in contrast to expectations, the presence of non-cooperating auxotrophs did not result in a collapse of the cooperative system. Instead, the community-level frequency of auxotrophs was determined by negative frequency-dependent selection and stabilized at different proportions depending on the degree of spatial structuring in the environment.

An explanation for the limited effect of non-cooperating auxotrophs in spatially structured environments could simply be a reduced fitness of auxotrophic mutants when growing on solid media. To exclude this possibility, each cross-feeding consortium was cocultured with one of the two possible auxotrophs in a spatially structured environment that has been supplemented with both amino acids. By uncoupling the obligate interaction in this way, auxotrophs should increase in frequency, because

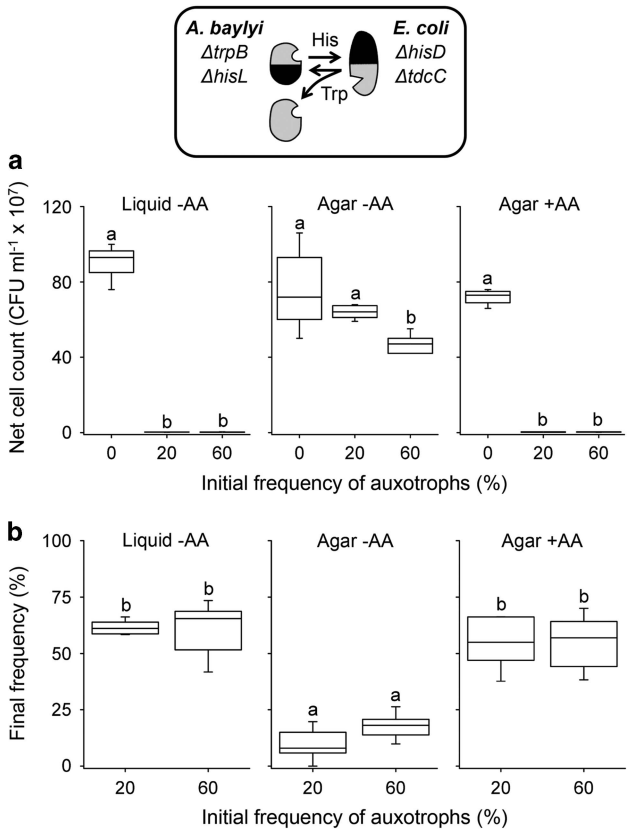


**Figure 3** Effect of non-cooperating auxotrophs on consortia of cross-feeding genotypes. (**a**) Productivity of pairs of cross-feeding genotypes that were co-inoculated with 0%, 20%, or 60% of an initial frequency of auxotrophs. Consortia were cultured in liquid culture or on agar plates containing either unsupplemented minimal medium (−AA) or minimal medium, to which both amino acids (100 μM of histidine and tryptophan) have been added (+AA). Net cell count is the number of colony-forming units (CFUs) determined after 24 h minus the count at the beginning of the experiment (0 h). Different letters indicate significant differences (LSD *post hoc* test: $P<0.05$, $n=8$). (**b**) Frequency of auxotrophic genotypes after 24 h of coculture when initially inoculated at a frequency of 20% or 60% together with pairs of cross-feeding genotypes. Different letter indicate significant differences (LSD *post hoc* test: $P<0.05$, $n=8$). Shown are representative results of a combination of cross-feeding and auxotrophic genotypes (top panel). Qualitatively similar results were obtained from analysing the other combinations as well (Supplementary Figures 1 and 2).

they benefit from the available amino acids yet do not invest resources into their production. Indeed, amino acid supplementation significantly decreased the productivity of the entire cross-feeding consortia within 24 h when non-cooperating auxotrophs were present (LSD *post hoc* test: $P<0.05$, $n=8$, Figure 3a). Moreover, independent of their initial inoculum size, auxotrophs increased to a final community-level frequency of ~60% after 24 h, which was statistically indistinguishable from the proportion auxotrophs had reached in a spatially unstructured, liquid environment (LSD *post hoc* test: $P<0.05$, $n=8$, Figure 3b). These findings ruled out that non-cooperating auxotrophs were generally less fit when growing on solid agar surfaces. Furthermore, this result implied that alleviating the requirement for external amino acids provided non-producing auxotrophs with a selective advantage over producing types.

Repeating the same series of experiments with all other possible three-partite combinations of two amino acid cross-feeders and one auxotroph yielded qualitatively similar results in all the cases (Supplementary Figures 1 and 2).

Together, these experiments demonstrated that non-cooperating auxotrophs were selectively favoured in spatially unstructured environments, but selected against in structured environments. Furthermore, these results identified the obligate nature of the cross-feeding interaction as a key component of the ecological mechanism that hampered the invasion of amino acids auxotrophs in spatially structured environments.

### *Cross-feeding genotypes show intense population intermixing*

An individual-based model was devised to identify the ecological mechanism that limited the exploitation of cooperative cross-feeding interactions by non-cooperating auxotrophs in spatially structured environments (Supplementary Methods). In parallel to the previous experiments, the model included three interacting types (two cross-feeders and one non-cooperating auxotroph), which were parameterized using experimentally determined parameter values. Simulations included a diffusion of released amino acids in a two-dimensional world and fitness of cell types depended on the local distribution of amino acids they required to grow.

At first, replicate simulations were initiated with the two cross-feeding genotypes only. The results of these simulations revealed a high degree of intermixing between both partners (Figure 4a). To empirically validate these simulation results, both cross-feeders were individually labelled with mCherry (*A. baylyi ΔhisDΔtrpR*) and EFGP (*E. coli ΔtrpBΔhisL*) and the resulting interspecific colony was analysed using fluorescence microscopy. After 24 h, both cross-feeding types showed such a high degree of population intermixing that the resulting colonies appeared yellow under the fluorescence microscope (Figure 4b).

### *Positive assortment of cross-feeders in spatially structured environments*

Which ecological mechanism disfavoured non-cooperating auxotrophs in spatially structured environments? To answer this question, simulations were initiated in a spatially structured environment that involved two cooperating cross-feeders and one non-cooperating auxotroph. Multiple independent simulation runs revealed that without amino acids supplementation, non-cooperating auxotrophs of both types persisted exclusively at the edge of the expanding colony

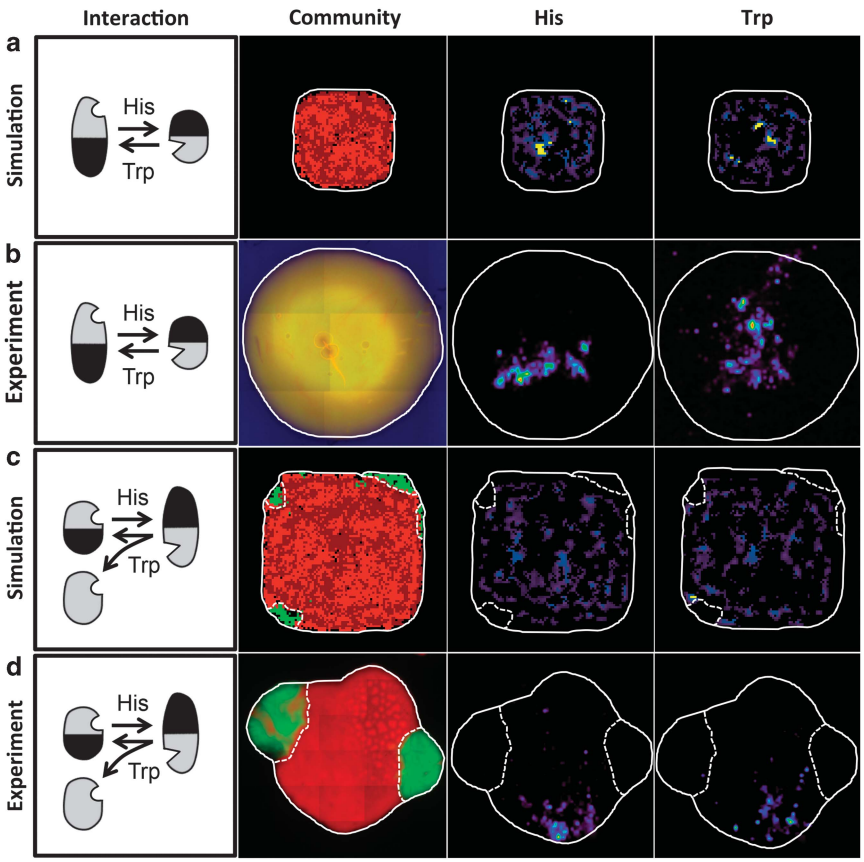


**Figure 4** Population intermixing and exclusion of non-cooperating genotypes is governed by the spatial distribution of available amino acids. Shown are sketches of the focal interactions (first column), the two- or three-partite bacterial communities consisting of cooperative cross-feeders (red colour) and non-cooperating auxotrophs (green colour) after 30 simulation steps or 24 h of growth in spatially structured environments (second column), as well as the spatial distribution of the two amino acids histidine (His) or tryptophan (Trp) within bacterial colonies at the end of the simulations or as experimentally determined by MALDI-TOF MS imaging (third and fourth column). Different intensities of blue (columns three and four) indicate different concentrations of both amino acids within a colony. White lines represent the boundary of the respective colony and the dashed lines delimit the spatial distribution of non-cooperating auxotrophs. (**a**, **b**) In the absence of non-cooperating auxotrophs, cross-feeding cells intermix to a high degree. (**a**) Simulation results after 30 time steps and (**b**) experimental result after 24 h of growth using the two cross-feeders *A. baylyi* (*ΔhisDΔtrpR*) and *E. coli* (*ΔtrpBΔhisL*). (**c**, **d**) Communities consisting of auxotrophic and cross-feeding genotypes show strong assortment during 24 h of growth in spatially structured environments. (**c**) Simulation results after 30 time steps and (**d**) experimental result after 24 h of growth using two cross-feeders of *A. baylyi* (*ΔtrpBΔhisL*) and E. coli (*ΔhisDΔtdcC*) as well as an auxotrophic *A. baylyi* strain (*ΔtrpB*). Qualitatively comparable results were obtained from other combinations of cross-feeding consortia and auxotrophs as well (Supplementary Figure 6).

(Figure 4c, Supplementary Movie 1) and reached significantly lower densities than each of the two cross-feeding genotypes (Mann–Whitney *U*-test after 30 simulation steps: $P<0.001$, $n=100$, Supplementary Figure 3, Supplementary Movie 2). In contrast, when growth was not limited by the two focal amino acids, auxotrophic types intermixed with both cross-feeders and reached population densities that significantly exceeded the cross-feeding type that competed for the same amino acid (Mann–Whitney *U*-test after 30 simulation steps: $P<0.001$, $n=100$, Supplementary Figure 3). Thus, these simulations suggested the obligate relationship caused a spatial segregation of cross-feeders and non-producing auxotrophs, thereby resulting in a positive assortment among cross-feeding types.

To investigate whether these theoretical predictions were indeed causing the selective disadvantage non-cooperators experienced in spatially structured environments, two mCherry-labelled cross-feeding genotypes were cocultured with one of the two possible EGFP-labelled auxotrophs in a spatially structured environment and the resulting colonies analysed using fluorescence microscopy. In line with the above predictions, a top view of these colonies after 24 h of growth revealed that despite an initially random distribution of all genotypes, the growth of non-cooperating auxotrophs was restricted to small zones at the edge of cross-feeder-rich regions (Figure 4d). In contrast, non-cooperators swept through the entire population when His and Trp were supplemented (Supplementary Figure 4b). In fact, the mCherry-labelled cross-feeders and EGFP-labelled auxotrophs again intermixed to such a high degree that the resulting colony appeared yellow under the fluorescence microscope (Supplementary Figure 4b). To rule out that

auxotrophs were located below cross-feeding genotypes, 24-h-old colonies of two cross-feeders and one auxotroph were analysed using laser scanning confocal microscopy. These experiments confirmed that cooperator-rich regions were indeed devoid of auxotrophic non-cooperators (Supplementary Figures 4a and c). Altogether, these results imply that in spatially structured environments, cross-feeding cells interacted preferentially with other cross-feeders, thereby spatially excluding non-cooperating auxotrophs.

*Segregation is caused by the spatial distribution of amino acids*

One explanation for the spatial exclusion of non-cooperating auxotrophs in spatially structured environments could be a limited access to free amino acids. If so, regions containing both cross-feeders should exhibit higher local amino acid concentrations relative to zones containing auxotrophs. To test this, the simulation runs in spatially structured environments were revisited, but this time, the spatial distributions of amino acids that caused the emergent community patterning were visualized. Interestingly, these results illustrated that regions occupied by cross-feeders were generally characterized by a patchy distribution of both amino acids (Figures 4a and c). This pattern materialized both in the absence (Figure 4a) and presence (Figure 4c) of non-cooperating auxotrophs. Comparing the concentrations of His and Trp in regions populated with cross-feeders to regions occupied by non-cooperating auxotrophs corroborated that amino acids were indeed less available to non-cooperating auxotrophs (Figure 4c). In addition, simulations revealed that when two cross-feeders interacted in spatially structured environments, the local concentrations of free amino acids available to cross-feeders increased over time (Supplementary Figure 5a). Interestingly, this pattern did not change when non-cooperating auxotrophs were present. Under these conditions, amino acids concentrations were again increased in regions populated by cross-feeders, yet reached only ~10% of these concentrations in areas occupied by auxotrophs (Mann–Whitney *U*-test after 30 simulation steps: $P<0.001$, $n=100$; Supplementary Figure 5b). Together, these simulation results revealed amino acids are patchily distributed in cross-feeder-rich regions, yet are significantly less available to non-cooperating auxotrophs.

To verify these predictions, cross-feeding consortia were cocultured in spatially structured environments in the absence or presence of one of the two possible auxotrophs and after 24 h of growth, the spatial distribution of His and Trp was visualized using MALDI MSI. In line with the simulation results, amino acids were heterogeneously distributed in areas where cross-feeders co-occurred (Figures 4b and d). In addition, regions occupied by non-cooperating auxotrophs were consistently devoid of detectable amino acids (Figure 4d, Supplementary Figure 6). To visualize the spatial distribution of His and Trp, colonies were dried using blotting paper and overnight desiccation. The colonies processed in this way showed in many instances the formation of crystalline deposits that occurred only in cross-feeder-rich regions. Analysing these surface crystals via FT-MS (Supplementary Methods) confirmed these crystals consisted indeed of His (*m/z* 154.0611 [$(M\text{-}H)^-$]) and Trp (*m/z* 203.0820 [$(M\text{-}H)^-$]). A perfect match of the high-resolution masses obtained from crystals and amino acid standards further corroborated this interpretation (Supplementary Figure 7). Altogether, these analyses established that a spatial exclusion of non-cooperating auxotrophs from cooperative benefits (here: amino acids) helped to maintain mutualistic cross-feeding interactions in spatially structured environments.

## Discussion

Metabolic cross-feeding interactions that involve multiple bacterial species are common in nature (Schink, 2002; Morris *et al.*, 2013). Evolutionary theory, however, predicts that interactions, in which the production and exchange of metabolites incurs fitness costs, should be prone to the invasion of non-cooperating individuals, which reap cooperative benefits without reciprocating (Herre *et al.*, 1999; Sachs *et al.*, 2004; Travisano and Velicer, 2004). The persistence of cooperative cross-feeding interactions hinges therefore on the existence of mechanisms that resolve these conflicts of interests. We hypothesized that spatially structured environments should enhance partner fidelity feedbacks within cooperator-rich patches (Trivers, 1971), possibly resulting in a positive assortment among cooperative individuals (Fletcher and Doebeli, 2009; Mitri *et al.*, 2011; Estrela and Brown, 2013), which could maintain these interactions in the long run.

To test this hypothesis, we engineered an obligate cooperative cross-feeding interaction between *A. baylyi and E. coli,* in which growth of both partners obligately depended on a reciprocal exchange of His and Trp. Analysing the stability of this interaction, we find that spatial structure favoured cooperative cross-feeding over unstructured environments. Despite an initially random distribution of auxotrophs and cross-feeders, both populations self-organized into a spatially inhomogeneous distribution after 24 h, in which auxotrophs occurred exclusively in smaller patches at the edges of colonies that otherwise consisted of cross-feeding types. A chemical imaging technique (that is, MALDI MSI) indicated that this pattern was caused by an inhomogeneous distribution of amino acids, which were only present in cross-feeder-rich regions and therefore unavailable to auxotrophs. The resulting segregation of auxotrophic and cross-feeding genotypes in spatially structured environments limited

the access of non-cooperators to cooperative benefits and thus maintained cooperative cross-feeding interactions in the focal bacterial communities.

Surface-attached biofilms are prime examples of spatially structured communities that can consist of multiple different bacterial species (Ley *et al*., 2006; Sudakaran *et al*., 2012). Strikingly, many biofilm communities are spatially stratified (Tolker-Nielsen and Molin, 2000; Stoodley *et al*., 2002) and several factors have been identified as possibly driving this patterning. First, physiological differentiation can result from environmental factors such as gradients of nutrients or oxygen (Serra and Hengge, 2014). For example, spatial stratification has been demonstrated in nitrifying biofilms (Okabe *et al*., 1999) or anaerobic granular sludge biofilms (Harmsen *et al*., 1996). Second, also ecological interactions among co-occurring bacteria can contribute to their spatial self-organization. Layered structures that physically separate cells of two species can emerge as a consequence of both synergistic and antagonistic interactions. Examples involve an exchange of metabolic by-products (Christensen *et al*., 2002) or competition for oxygen (Hansen *et al*., 2007), respectively.

Alternatively, strong metabolic interactions that involve an exchange of metabolites between different cells have been demonstrated to result in enhanced population intermixing (Thiele *et al*., 1988; Nielsen *et al*., 2000; Breugelmans *et al*., 2008; Estrela and Brown, 2013; Momeni *et al*., 2013b; Müller *et al*., 2014) —an observation that is consistent with the results of our study (Figures 4a and b). Notwithstanding the factors that caused the patterning in the first place, the results of our study suggest that when two organisms interact synergistically in a spatially structured environment, repeated interactions should reward mutants that increase their cooperative investment to benefit their respective partners. The automatic feedback that arises as a consequence of this process should intensify cooperative interactions in the long run. Whether and to which extent the synergistic metabolic interactions as well as the pronounced spatial heterogeneity in the concentrations of metabolites (Ramsing *et al*., 1993; Schramm *et al*., 1996) that are frequently observed within bacterial biofilms are a direct consequence of this process, however, is not known and should be subject to future studies.

Cooperation is expected to be evolutionary stable when cooperators are more likely to interact with other cooperators than with non-cooperating individuals (Fletcher and Doebeli, 2009). In the absence of derived mechanisms, with which cooperators can either recognize and thus preferentially interact with other cooperators (for example, 'partner choice'; Bull and Rice, 1991) or enforce cooperation by sanctioning non-cooperators (for example, 'policing'; Frank, 1995), alternative means are required to prevent non-cooperating individuals from overexploiting cooperative benefits (Travisano and Velicer, 2004). Evolutionary theory has suggested that spatial environments offer a possible solution to this dilemma (Mitri *et al*., 2011; Estrela and Brown, 2013): spatial self-organization can result in positive assortment of cooperators and thus help to maintain cooperative interactions.

Previous experimental work on this issue has largely focussed on intraspecific interactions. For example, *Bacilus subtilis* strains that produced extracellular polysaccharides (EPS, a substance that promotes biofilm expansion) and competed against EPS-deficient cells have been shown to segregate when growing on a two-dimensional surface—a mechanism that prevented EPS-non-producers from overexploiting the benefits of EPS production (van Gestel *et al*., 2014). Moreover, engineered yeast strains that reciprocally exchanged essential amino acids, showed positive assortment among cooperators when the interaction was staged in spatially structured environments (Momeni *et al*., 2013a). The authors of this study attributed the observed stabilizing effects to an asymmetric distribution of the released metabolites in the environment. A similar argument has been proposed by Drescher *et al*., 2014 to explain why thicker biofilms selectively benefited cooperative chitinase producers of *Vibrio cholerae* over conspecific non-producers. Most likely, a thicker biofilm matrix limited diffusion of the public good (that is, chitinase) and thus the availability of the chitin degradation products *Vibrio* needs to grow. These findings are in line with the main conclusion of our study, namely that limited diffusion of cooperative benefits in spatially structured environments confines their availability to non-producing individuals, which results in a positive assortment of cooperative producers. Building on these previous studies, our work is the first one to demonstrate experimentally that similar mechanisms also operate in reciprocal interspecific interactions and to causally link the exclusion of non-cooperators to the underlying distribution of the exchanged metabolites.

## Conclusion

Our work has identified a powerful mechanism to maintain cooperative cross-feeding interactions between two bacterial species: a limited diffusion of released metabolites in spatially structured environments results in a positive assortment of genotypes that invest into the costly production of exchanged metabolites. As a consequence, non-producing cells are spatially excluded from cooperative benefits, which limits their impact on the population of cooperators. Given that bacteria commonly live in surface-bound polymicrobial communities, our results suggest obligate cooperative cross-feeding interactions within these communities might be more widespread than previously thought.

## Conflict of Interest

The authors declare no conflict of interest.

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## Author contributions

SP and CK conceived and designed the study. SP performed all the experiments. SP and CK analysed and interpreted the results. SP, FK and AS performed the MALDI MSI, FT-MS experiments. SP and CK wrote the manuscript. All the co-authors amended the manuscript.

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