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ORIGINAL ARTICLE

Fitness and stability of obligate cross-feeding interactions that emerge upon gene loss in bacteria

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Cross-feeding interactions, in which bacterial cells exchange costly metabolites to the benefit of both interacting partners, are very common in the microbial world. However, it generally remains unclear what maintains this type of interaction in the presence of non-cooperating types. We investigate this problem using synthetic cross-feeding interactions: by simply deleting two metabolic genes from the genome of *Escherichia coli*, we generated genotypes that require amino acids to grow and release other amino acids into the environment. Surprisingly, in a vast majority of cases, cocultures of two cross-feeding strains showed an increased Darwinian fitness (that is, rate of growth) relative to prototrophic wild type cells—even in direct competition. This unexpected growth advantage was due to a division of metabolic labour: the fitness cost of overproducing amino acids was less than the benefit of not having to produce others when they were provided by their partner. Moreover, frequency-dependent selection maintained cross-feeding consortia and limited exploitation by non-cooperating competitors. Together, our synthetic study approach reveals ecological principles that can help explain the widespread occurrence of obligate metabolic cross-feeding interactions in nature.

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Introduction

Microbial communities are genetically and metabolically highly diverse (Maharjan and Ferenci, 2005; Ley *et al.*, 2006), and this complexity is mainly caused by ecological interactions among its constituents (Little *et al.*, 2008; Maharjan *et al.*, 2012). When living together, microorganisms specifically modify their chemical environment by virtue of their metabolic activities. In this way, they create opportunity for new ecological interactions to emerge (Phelan *et al.*, 2012). The spectrum of interactions that results from this process includes—besides the competition for limiting resources (Foster and Bell, 2012) or the release of toxic (waste) products (Wilkinson *et al.*, 1974)—also interactions, in which one organism benefits from the biochemical activities of another one. For example, a metabolic by-product that is released by one genotype can benefit a recipient strain that utilises this resource (Rosenzweig *et al.*, 1994; Doebeli, 2002). This simple type of facultative cross-feeding is easy to understand from an evolutionary point of view, because the released metabolite incurs no costs to its producer and the receiver benefits from opportunistically exploiting this resource (Sachs *et al.*, 2004).

The situation, however, is different for metabolic interactions in which two or more microorganisms associate and perform costly biochemical functions that neither of them can perform alone. This type of cooperative interaction is very widespread among both Bacteria and Archaea and is frequently based on the reciprocal exchange of certain metabolites (Schink, 2002; McInerney *et al.*, 2008; Sieuwerts

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et al., 2008; Morris *et al.*, 2013). For those cases, where the interaction partners have been studied in more detail, characteristic features such as the loss of essential biosynthetic functions (McInerney *et al.*, 2007) or the formation of physical attachment structures (Ishii *et al.*, 2005; Wanner *et al.*, 2008) have been reported that may have arisen as specific adaptations to the symbiotic lifestyle. Unfortunately, the obligate interdependence of both partners makes it often difficult to study this type of interactions under laboratory conditions (Nauhaus *et al.*, 2002; Pernthaler *et al.*, 2008; McCutcheon and Moran, 2012). As a consequence, many open questions remain regarding the ecological and evolutionary consequences of entering into such an obligate cross-feeding interaction.

The existence of cooperative cross-feeding interactions represents an evolutionary conundrum: why should one organism produce a costly metabolite to benefit another organism and not use it for itself? An answer to this question is challenging because of two reasons: first, it requires knowledge on the fitness consequences that result from the evolutionary transition from an autonomous lifestyle to a metabolic dependency on another organism. For any derived metabolic interaction, such a comparison would require the availability of closely related cells that still retained the ancestral, non-cooperative state. Unfortunately, such test cases are rarely available (Hillesland and Stahl, 2010). Second, evolutionary theory predicts for cooperative cross-feeding interactions the rapid evolution of non-producing types that reap cooperative benefits without reciprocating (Axelrod and Hamilton, 1981; Bull and Rice, 1991). The fitness advantage that non-cooperating types gain relative to cooperators should ultimately result in a collapse of the cooperative interaction (Ferriere *et al.*, 2002). Again, investigating this type of question under laboratory conditions is hampered by the scarcity of well-characterised genotypes as well as the difficulties to cultivate most naturally evolved microbial consortia under laboratory conditions (Orphan, 2009). Overcoming these difficulties, however, is pivotal for understanding the evolutionary ecology of cooperative cross-feeding.

Here we engineered a range of different metabolic cross-feeding interactions by simply deleting genes from the genome of *Escherichia coli*. Specifically, we aimed at implementing key features that characterise naturally evolved interactions such as (i) an obligate dependency of both partners and (ii) a cost of metabolite overproduction. The resulting cooperative interactions were based on the reciprocal exchange of essential amino acids between two strains that are both auxotrophic for one amino acid yet release other amino acids into the environment (Figure 1). We use these precisely defined cross-feeding interactions to address the following questions: (1) What fitness consequences result from the splitting of metabolic functions among two bacterial genotypes? (2) Are cooperative cross-feeding interactions vulnerable to the exploitation of non-cooperating genotypes? Our results provide evidence for a significant fitness advantage of obligate cross-feeding relative to metabolic autonomy and suggest that the metabolic cross-feeding interactions can stably coexist with other, non-cooperating genotypes—even in the absence of spatial structure.

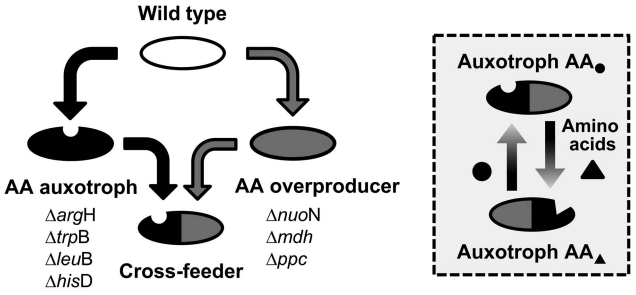

Figure 1 Design strategy of synthetic cross-feeding interactions. Overview over the genes deleted in *E. coli* wild type (WT) to yield one of four single-gene deletion mutants that are either auxotrophic for one amino acid (AA, that is, arginine, tryptophan, leucine and histidine) or overproduce AAs, as well as double deletion mutants (that is, 'cross-feeders'), in which the two mutations causing AA auxotrophy and overproduction were combined in all possible combinations. Coculturing two of those cross-feeders should result in reciprocal exchange of essential amino acids (inset).

Materials and methods

Strain construction

Genetic targets to generate amino-acid auxotrophs upon deletion of a single gene were identified as described (Bertels *et al.*, 2012). Amino-acid-overproducing mutants were identified using CASOP-GS (Supplementary Methods). *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 utilisation locus (Ara^+) from *E. coli* strain REL 607 (Lenski *et al.*, 1991) were introduced by P1 transduction (Thomason *et al.*, 2007). The ability to utilise arabinose was used as a phenotypic marker to identify strains in multi-strain competition experiments. 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 (Datsenko and Wanner, 2000).

Culture conditions and media

All cultures were incubated at 30 °C under shaking conditions and experiments were performed in MMAB medium (Vanstockem *et al.*, 1987) without biotin and using fructose (5 g l^{-1}) instead of malate as carbon source. Genotypes were precultured

overnight in the same medium that strains would experience in the main experiment or in MMAB medium to which 100 μM of the required amino acid was added. Overnight cultures were washed 3 × with MMAB, and cultures were diluted to an optical density at 600 nm ($OD_{600\,nm}$) of 0.1. Subsequent experiments were inoculated using 5 μl of these dilutions. For some experiments, a mixture of amino acids was added to the MMAB medium, whose relative composition mimicked the production levels of the overproducer *Δmdh*. The corresponding concentrations were determined by LC/MS/MS (Supplementary Methods). Final concentrations in the medium were: Arg: 7.5 μM, Trp: 7.7 μM, Leu: 7.3 μM, His: 18.5 μM, Ala: 7.5 μM, Asn: 7.5 μM, Gln: 7.5 μM, Glu: 10.7 μM, Gly: 11.0 μM, Lys: 18.5 μM, Met: 7.5 μM, Phe: 7.5 μM, Pro: 8.0 μM, Ser: 7.5 μM, Thr: 7.5 μM, Tyr: 3 μM, Val: 8.0 μM.

Coculture experiment

For coculture experiments, two strains were co-inoculated into 1 ml of MMAB medium. The total population size of the two-membered consortia was estimated by spreading cocultures on LB agar plates at 0 and 24 h. Fitness of WT and the cocultured consortia was expressed as the Malthusian parameter (Lenski *et al*., 1991). Realized growth rate over 24 h of the coculture (that is, Malthusian parameter *M*) was calculated as $M = (\ln(N_f/N_i)/24)$, where N_i is initial number of colony-forming units at 0 h and N_f is the final colony-forming unit count after 24 h. Net productivity (*P*) was calculated as: $P = N_f - N_i$. All coculture experiments were performed in eight replicates per genotype combination.

Determination of amino-acid production levels using auxotrophs as biosensors

To estimate the amount of free amino acids produced by a growing culture of either WT, auxotrophic, overproducing or cross-feeding genotypes, the corresponding donor strains were coinoculated (1:1) into 1 ml MMAB medium together with one of the four auxotrophs (that is, *ΔargH, ΔtrpB, ΔleuB, ΔhisD*). After 24 h, cocultures were plated on pure and kanamycin-containing LB plates to estimate population sizes of the auxotrophic strain.

Competitive fitness assays

Competition experiments were performed by inoculating two competing strains in equal density ($\sim 10^5$ cells each ml^{-1}) into the corresponding test medium and determining their frequency at 0 and 24 h by plating on LB agar with and without kanamycin. To verify the fitness consequences of both individual mutations of the cross-feeding strains, all single-gene deletion mutants (that is, auxotrophs and overproducers) were competed against WT in MMAB medium, which had been supplemented with culture supernatant of the amino-acid overproducing strain Δ*mdh*. For this, a single colony of the Δ*mdh* strain was inoculated into MMAB medium and cultivated for 20 h. After that, cells were spun down and the supernatant filter-sterilized (0.2 μm). For the competition experiments, this supernatant was replenished with fresh MMAB medium (1.25 ×) in an 8:2 ratio. To rule out that other factors in the Δ*mdh* supernatant caused the observed fitness effects, a second set of fitness experiments was performed. All single-gene deletion mutants were competed against WT in MMAB medium, to which an amino-acid mix (150 μM) has been supplemented that resembled the relative composition of the amino acid mixture produced by Δ*mdh*. These two experiments were replicated 8 and 10 times, respectively.

The fitness cost of amino-acid overproduction was quantified by competing the three overproducing mutants against WT for 24 h in MMAB medium. Competition experiments of the four auxotrophs against WT were performed in MMAB medium to which the one required amino acid (100 μM) has been added. Both experiments were replicated 10 times.

Fitness of two-membered consortia relative to WT was assessed in three-way competition experiments. For this, the three competing strains (that is, two overproducers versus WT; two cross-feeders versus WT) were inoculated in a 0.5:0.5:1 ratio into MMAB medium (initial density: $\sim 10^5$ cells ml^{-1}) and their frequency at 0 and 24 h determined by plating on LB agar with and without kanamycin. This experiment was replicated 10 times.

Competitors were differentiated using an antibiotic marker (kanamycin) that did not incur a fitness cost (paired *t*-test, $P > 0.05$, $n = 10$) and all competition experiments were performed with strains in which the antibiotic marker was swapped between competitors. Relative fitness was expressed as the ratio of Malthusian parameters of the strains involved (Lenski *et al*., 1991).

Invasion-from-rare experiment

The evolutionary stability of the cross-feeding interactions (that is, their coexistence as a mixed equilibrium) was investigated by performing reciprocal invasion-from-rare experiments. For this, each strain of the four representative cross-feeding consortia (Supplementary Figure S2) was inoculated into MMAB minimal medium in a frequency of 1:100 with respect to its corresponding partner. In addition, both strains were inoculated at a 1:1 initial ratio as a control. About 10^5 cells of each strain were inoculated per ml and cell numbers were determined by plating at 0, 24, 48, and 72 h on LB plates with and without kanamycin. This experiment was replicated eight times.

A conceptually similar experiment was performed to verify the ability of cross-feeding consortia and auxotrophic mutants to invade the respective

other population. To this end, each of the four representative cross-feeding consortia (Supplementary Figure S2) and one of the two corresponding auxotrophs were inoculated into MMAB medium in a frequency of 1:100, 100:1 and 1:1. Both auxotrophs were tested for each cross-feeding consortium. After 0 and 24 h, cell numbers were determined by plating on LB plates with and without kanamycin.

Statistical analysis

Statistical differences between paired samples were assessed by paired *t*-tests and between multiple groups using univariate ANOVAs. Coculture data were analysed by a mixed-effects model using the 'interaction type' (that is, WT, auxotrophs, overproducers, and cross-feeders) as a fixed factor and the individually compared 'cocultured genotypes' as a random factor. Moreover, for the coculture data, the factor 'cocultured genotypes' was nested within factor 'interaction type'. LSD *post hoc* tests were used to determine between-group differences. One-sample *t*-tests were performed to test whether a genotype's relative fitness was significantly different from 1 (for example, WT fitness). Changes in the cell viability over time were confirmed by a paired Wilcoxon test. *P*-values of multiple comparisons were corrected by applying the false discovery rate procedure (Benjamini and Hochberg, 2000). The relationship between the initial frequency of a genotype or a consortium and its fitness after 24 h was investigated by fitting a quadratic regression model. All statistical analyses were done with SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Construction of cross-feeding interactions

In order to construct a synthetic cooperative interaction, we first generated strains that released increased amounts of amino acids into the environment. For this, we used a computational approach (Supplementary Methods) to predict the genetic targets that, upon deletion, would lead to the overproduction of amino acids (Bohl *et al*., 2010). Three genes, *nuoN*, *mdh*, and *ppc*, were identified in this way and deleted from the *E. coli* BW25113 WT genome (Supplementary Note 1). Next, each of the four genes involved in the biosynthesis of arginine (*argH*), tryptophan (*trpB*), leucine (*leuB*), and histidine (*hisD*) were independently deleted from the *E. coli* WT genome to yield four amino-acid auxotrophic mutants (Bertels *et al*., 2012) (Figure 1). Subsequently, every auxotrophy-causing mutation was combined with each one of the three mutations causing amino-acid overproduction, resulting in 12 double deletion mutants (Figure 1, hereafter: 'cross-feeders').

Characterisation of mutants

To investigate the amount of amino acids released by *E. coli* WT and all newly constructed genotypes, all genotypes were cocultured with each of the four auxotrophs (1:1) and the auxotrophs' productivity within 24 h was determined. This experiment revealed that in contrast to WT and the four auxotrophs, the three overproducers and all but one cross-feeding mutant released amino-acid levels sufficient to support growth of the four focal auxotrophs (Figure 2a; Supplementary Figure S1a). Notably, the amino-acid production levels of cross-feeders were on average four-times larger than what would have been expected from analysing the corresponding single-gene deletion mutants (Supplementary Figure S1b), thus indicating epistatic interactions among mutations.

To determine whether increased amino acid production also incurred fitness costs, the three single-gene deletion mutants *ΔnuoN*, *Δmdh*, and *Δppc* mutants were competed against WT. The results of this experiment indicated significant fitness costs of amino-acid overproduction of around 5–7% (Figure 2b).

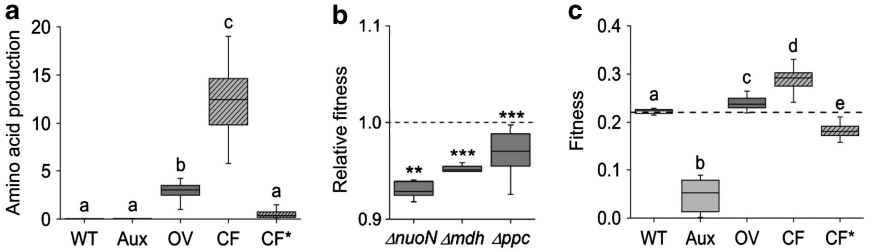

Figure 2 Characterisation of mutants and coculture experiment. (**a**) Amino acid (AA) production levels (that is, arginine, tryptophan, leucine, and histidine) of WT, four auxotrophs (Aux), three overproducers (OV), eleven cross-feeders (CF) and the cross-feeding genotype Δ*leuB*Δ*nuoN* (CF*) within 24 h determined as productivity of cocultured AA auxotrophs ($n=8$ for every auxotroph-genotype combination). Combinations with matching amino acid auxotrophies were excluded. (**b**) Competitive fitness of the three AA-overproducing mutants relative to WT within 24 h in minimal medium. Relative fitness is the ratio of Malthusian parameters and the dashed line indicates equality in fitness between WT and competitor. Asterisks indicate fitness values that were significantly different from 1 (that is, WT fitness, one-sample *t*-test, $^{**}P<0.01$, $^{***}P<0.001$, $n=10$). (**c**) Fitness given as the Malthusian parameter of WT, pairs of cocultured auxotrophs (Aux, 6 combinations), overproducers (OV, 3), cross-feeders (CF, 45), and cross-feeding consortia involving strain Δ*leuB*Δ*nuoN* (CF*, 9) within 24 h ($n=8$ for WT and every pair of genotypes). Different letters indicate significant differences (LSD *post hoc* test, $P<0.001$). Boxplots: median (horizontal lines in boxes), interquartile range (boxes), 1.5 × -interquartile range (whiskers).

Growth advantage of cross-feeding relative to prototrophic WT

To verify whether the newly constructed strains could cross-feed each other, all possible combinations between two amino-acid auxotrophs, overproducers, and cross-feeders were mixed (1:1) in minimal medium and their realised growth rate within 24 h (that is, the Malthusian parameter) determined as a measure of fitness. In this experiment, genotypes carrying the same amino-acid auxotrophy-causing mutation were not paired, and WT cells inoculated at a similar initial density served as control. The results indicated that consortia composed of two auxotrophic strains were significantly less fit than WT. Surprisingly, the fitness of two out of three pairs of overproducing strains as well as 43 of the 54 possible combinations of cross-feeding mutants significantly exceeded WT levels (Figure 2c; Supplementary Figure S2). Of the 11 cocultures of cross-feeding mutants that did not show this pattern, 9 involved the double deletion mutant *ΔleuBΔnuoN* (Figure 2c; Supplementary Figure S2), whose amino-acid production levels were also not sufficient to support growth of the four focal auxotrophs (Figure 2a; Supplementary Figure S1a).

Benefit of cross-feeding is larger than the costs

One explanation for the observed synergistic growth could be a division of metabolic labour among pairs of cross-feeding mutants. For this mechanism to operate, two conditions should be met: first, mutations causing amino-acid auxotrophies should—in the presence of the essential amino acid—confer a selective advantage over prototrophic WT cells. Second, the fitness benefit gained by the auxotrophy should exceed the costs for overproducing the other amino acids. We tested this hypothesis by determining the competitive fitness of all single-gene deletion mutants (that is, auxotrophs and overproducers) relative to WT in minimal medium supplemented with the culture supernatant of the overproducing strain *Δmdh*. Indeed, fitness of the auxotrophic mutants increased significantly by ~20% over WT levels, whereas overproducing amino acids resulted in a significant decrease of fitness by 5–8% in the corresponding mutants relative to WT (Figure 3a).

To rule out that other factors in the overproducer's culture supernatant caused the observed pattern, we repeated the same competition experiment, but this time supplemented the medium with an amino-acid mixture whose relative composition resembled the mixture produced by Δ*mdh*. In line with the previous experiment, fitness of the auxotrophic strains significantly increased by ~20% in the presence of amino acids relative to WT (Figure 3b), whereas amino-acid overproduction again reduced the fitness of the corresponding mutants relative to WT cells (Figure 3b). Moreover, competing each of the four auxotrophs against WT in the presence of the focal amino acid also revealed a significant growth advantage of ~20% of the single-gene deletion mutants relative to WT (Supplementary Figure S3), thereby corroborating that a single amino acid was sufficient to cause the observed gain in fitness.

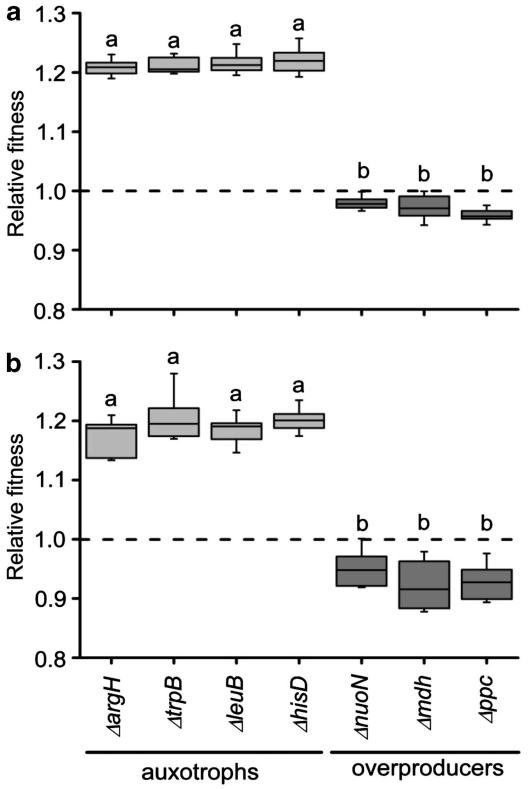

Figure 3 Competition of single-gene deletion mutants against WT. (**a**) Fitness of amino-acid auxotrophic and overproducing single-gene deletion mutants relative to WT within 24 h in minimal medium supplemented by either culture extract from the *Δmdh* mutant, or (**b**) a mixture of amino acids (150 μM) that resembled the relative composition of the amino-acid mixture produced by *Δmdh*. The dashed line indicates equality in fitness between WT and the corresponding competitor. Different letters denote significant differences (LSD *post hoc* test, $P<0.05$). All fitness values were significantly different from 1 (one-sample *t*-test, $P\leqslant 0.05$, $n\geqslant 8$).

Quantifying the number of dead cells (Supplementary Methods) in the populations consisting of either WT or one of the four representative pairs of cross-feeding mutants (Supplementary Figure S2) within 24 h demonstrated a significantly increased cell viability (paired Wilcoxon test, $P\leqslant 0.07$) and no significant differences in the cell viability among different consortia (LSD *post hoc* test, $P>0.05$). Hence, the growth advantage of cross-feeding mutants was due to a reciprocal exchange of released amino acids and not caused by increased rates of cell lysis. Altogether, these experiments support the above-mentioned division of labour hypothesis to explain the unexpectedly strong synergistic growth advantage observed among cross-feeding strains.

Negative frequency-dependent selection stabilises cross-feeding interactions

Next, we asked whether the relative fitness of a given cross-feeder depends not only on its own frequency, but also on the frequency of another, complementary cross-feeding genotype. This so-called 'frequency-dependent selection' (Vellend, 2010) could stabilise, and thus help to maintain, cross-feeding. If this was the case, each of the two cross-feeding genotypes should have a higher relative fitness when rare and increase in frequency until an equilibrium is reached (Damore and Gore, 2012). To test this, we performed reciprocal invasion-from-rare experiments to examine the ability of one cross-feeding genotype to invade a population of its respective partner when rare (1:100). Four representative pairs of cross-feeding mutants were used for this experiment (Supplementary Figure S2). All mutants tested had a significant fitness advantage when rare (relative fitness during the first 24 h $\geqslant 1.2$, one-sample *t*-test, $P<0.05$) and converged to a 1:1 ratio within 3 days (Figure 4), suggesting that these interactions are likely stabilised by negative frequency-dependent selection.

Cross-feeding consortia can persist in the presence of non-cooperators

To assess whether cross-feeding consortia would be able to persist in the presence of non-cooperating WT cells, three-way competition experiments were performed in which four representative combinations of cross-feeders (Supplementary Figure S2) were competed against WT cells (initial ratio: 0.5:0.5:1). The results showed that three of four consortia tested showed enhanced fitness over WT cells (Figure 5), demonstrating that cross-feeding consortia was not destabilised by the presence of non-cooperating WT cells.

As the overproduction of amino acids was costly (Figure 2b), auxotrophs should have a selective advantage in direct competition with a cross-feeding consortium, because they reap the benefits of taking up free amino acids yet do not contribute to their production. According to evolutionary theory, the resulting conflict between 'cooperators' (that is, cross-feeders) and 'non-cooperators' (that is, auxotrophs) should collapse the cross-feeding interaction in a well-mixed (that is, spatially unstructured) environment, where the public good is equally accessible to both competitors (Amarasekare, 2003). To test this prediction, cocultures between the four representative cross-feeding consortia and one of the two corresponding auxotrophs were inoculated together at different initial ratios (that is, 1:100, 1:1, 100:1) and the fate of both populations followed during the first 24 h. This experiment revealed that fitness was negative frequency-dependent and that both auxotrophs and cross-feeders could invade a population of the respective other when rare (Figure 6), suggesting stable coexistence of both populations. Interestingly, cross-feeding consortia showed a much stronger ability to invade a population of auxotrophs than vice versa. This finding indicates that the stable equilibrium reached is likely numerically dominated by cross-feeding types. Together, these results imply that cooperative cross-feeding interactions can likely persist in the

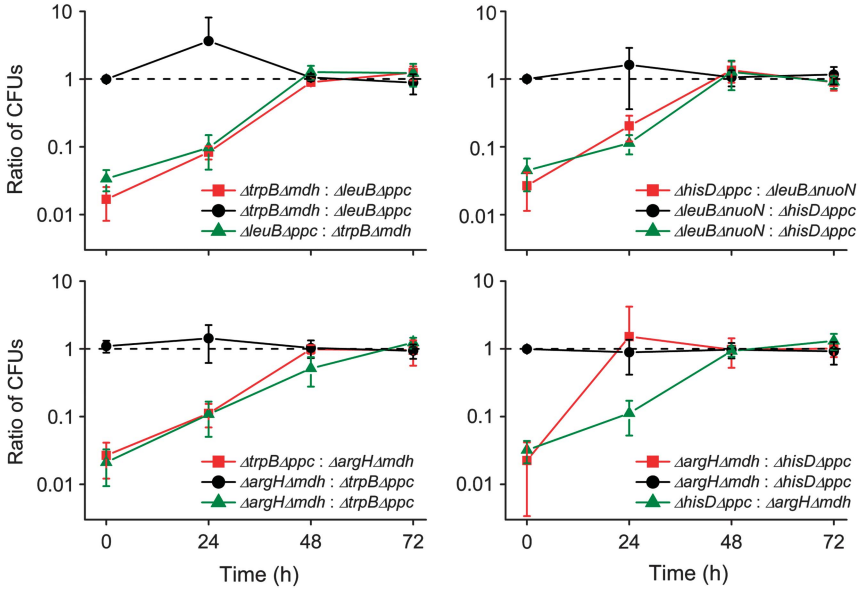

Figure 4 Reciprocal invasion-from-rare experiments with four cross-feeding consortia. In every case, each cross-feeding mutant (Supplementary Figure S2) was inoculated 1:100 to its respective partner and the number of colony-forming units (CFUs) of both competitors followed over time (red and green symbols). Consortia inoculated at 1:1 ratio (dashed line) served as controls (black symbols). Mean number of CFUs (±95% CI) are given ($n=8$ for each comparison).

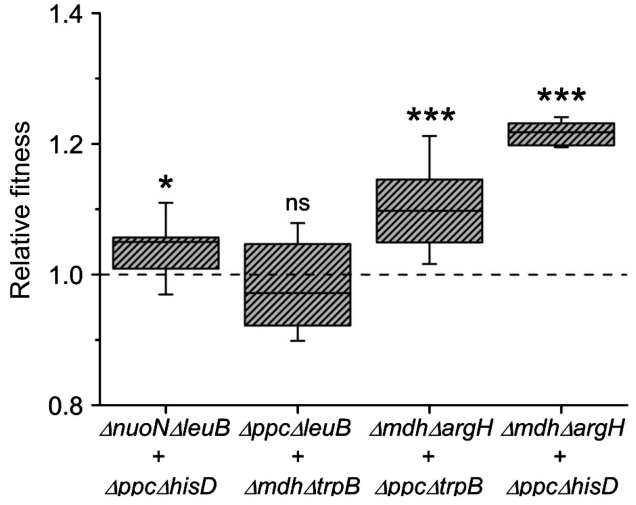

Figure 5 Competition of cross-feeding consortia against wild type. Representative pairs of cross-feeding mutants (Supplementary Figure S2) were competed against WT for 24 h in liquid minimal medium. Relative fitness is the ratio of Malthusian parameters and the dashed line indicates equality in fitness between WT and the cross-feeding consortia. All fitness values were significantly different from 1 ($P \leqslant 0.03$, $n = 10$), except the ones labelled with 'ns'.

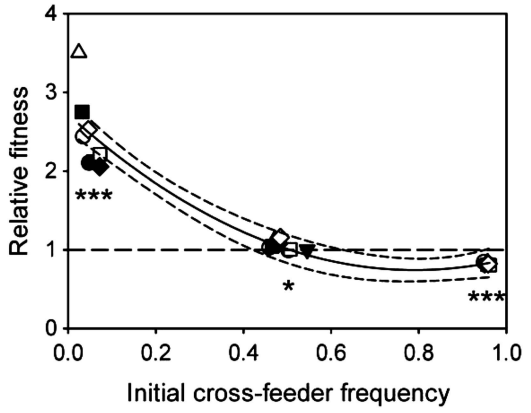

Figure 6 Reciprocal invasion-from-rare experiments with four cross-feeding consortia and the corresponding auxotrophs. Competition experiments with each of the four representative cross-feeding consortia (Supplementary Figure S2) and one of the two corresponding auxotrophs were initiated at a ratio of 1:100, 100:1 and 1:1 and the fitness of the cross-feeding populations relative to auxotrophs within 24 h determined. Shown is the percentage of the cross-feeding consortia at the onset of the experiment (*x*-axis) and their fitness relative to the focal auxotrophs after 24 h (*y*-axis). Different symbols correspond to the eight different combinations tested and every point is the mean of eight replicates. The solid line shows the quadratic regression of fitness on frequency ($R^2 = 0.91$, $P < 0.0001$) and the dotted line the 95% confidence interval of this regression. Asterisks indicate significant differences from 1 (that is, fitness of auxotrophs (dashed line): one-sample *t*-test: $***P < 0.001$, $*P < 0.05$, $n = 8$).

presence of non-cooperating individuals (that is, both prototrophic and auxotrophic cells) and negative frequency-dependent selection stabilises this type of ecological interactions within microbial communities—even in a well-mixed environment.

Discussion

Cooperative cross-feeding interactions are very common in bacterial populations and thus of critical importance to our understanding of microbial communities. Methodological difficulties to cultivate and study naturally evolved interactions, however, have so far impeded a mechanistic understanding of the costs and benefits associated with this type of symbiotic lifestyle as well as its robustness in the presence of non-cooperating genotypes. Here we used an approach of synthetic microbial ecology to experimentally determine the fitness consequences of an obligate and reciprocal exchange of metabolites between two partners as well as the effects of non-cooperating genotypes on the ecological stability of cooperative cross-feeding interactions. We have shown that the loss of two metabolic genes from a bacterial genome was sufficient to turn a prototrophic bacterial cell into a cooperating strain that was unable to produce a certain amino acid, yet released increased amounts of others into the environment. The ecological interactions resulting from the cross-feeding of essential amino acids among two deletion mutants increased their combined fitness relative to their prototrophic ancestor. This unexpected fitness advantage could be attributed to a division of metabolic labour: The fitness costs of amino-acid overproduction were more than compensated by the advantage resulting from amino-acid auxotrophies. Moreover, characterising the emergent cross-feeding interactions revealed that negative frequency-dependent selection stabilised pairs of cross-feeders and buffered cross-feeding consortia against the exploitation of non-cooperative mutants.

Distribution of obligate cross-feeding interactions

We have demonstrated that the loss of biosynthetic genes can result in the emergence of ecologically stable metabolic interactions that confer a significant fitness advantage to the strains involved. The enormous adaptive potential of mutations that cause a loss of function or even entire genes to enhance a cell's fitness has only recently been discovered (Koskiniemi *et al.*, 2012; Lee and Marx, 2012; Hottes *et al.*, 2013). Our work extends the possibilities offered by null mutations by generating context-dependent ecological interactions that can stabilise genetic diversity within evolving bacterial populations (Friesen *et al.*, 2004). The larger target size available for this type of mutations combined with the fitness advantage auxotrophs gain in the presence of the required metabolite offers a plausible adaptive argument to account for the rapid evolution of metabolic auxotrophies (Giraud *et al.*, 2001) as well as of cross-feeding interactions within bacterial communities (Harcombe, 2010; Poltak and Cooper, 2011).

Our observation that synergistic growth benefits were prevalent in a broad range of different cross-feeding

mutants tested (Supplementary Figure S2) lends credence to the view that the observed phenomenon is more widespread and likely not limited to the specific set of mutants analysed here. In line with this interpretation is an analysis of 32 *E. coli* strains, in which genomic differences between strains have been identified (Zhang *et al.*, 2007). About 20% of those strains lacked *ppc* as well as essential biosynthetic genes of one or more ($\leqslant$6) amino acids (Supplementary Table 1), suggesting that these strains potentially show an amino-acid cross-feeding phenotype (Figure 1). Of the remaining 26 strains, 14 are most likely auxotroph for 1–5 different amino acids (Supplementary Table 1). This finding suggests that both auxotrophic and cross-feeding phenotypes are likely common in natural populations of *E. coli*. The observation that many different pairs of single-gene deletion mutants of *E. coli* readily formed cooperative interactions (Wintermute and Silver, 2010) supports the view that the same mechanism likely also applies to other classes of metabolites. Moreover, similar phenomena may also be relevant in interspecific interactions. For example, many bacterial species contain only parts of the TCA cycle (Huynen *et al.*, 1999) and show variability in the occurrence of the *mdh* gene (Huynen *et al.*, 1999)—a locus that caused amino-acid overproduction upon deletion in our study.

Another interesting perspective that is opened up by our study is that bacteria may not necessarily require mutational change to enjoy the benefits of a divided metabolic labour. Also regulatory alterations could result in sub-populations of cells that specialise in the uptake and/or production of certain metabolites (Hosoda *et al.*, 2011), thus enhancing metabolic efficiency on a population level. Such a mechanism could explain why in our study certain combinations of overproducers showed an increased fitness over WT (Supplementary Figure S2).

Fitness consequences of obligate cross-feeding

The splitting of two biosynthetic functions between two bacterial cells resulted in fitness benefits due to cross-feeding that significantly exceeded levels of prototrophic cells. One aspect that appears central to explaining this phenomenon is our observation that auxotrophs gained a significant fitness advantage, when the focal amino acid was present in the environment. Interestingly, the extent of benefit gained by auxotrophs was independent of the amino acid analysed and consistently around 20% relative to WT cells (Figure 3; Supplementary Figure S3). This finding suggests that auxotrophs saved production costs by taking up the required metabolites from the environment. However, what caused the increased fitness of cross-feeding consortia relative to WT? Introducing the auxotrophy-causing mutation may have resulted in a reorganisation of the cell's regulatory or metabolic network (Hottes *et al.*, 2013) and thus a reallocation of saved resources into the production of other amino acids and further essential biomass constituents. Consequently, cross-feeding genotypes would specialise into the production of a subset of amino acids while saving the production costs for one particular amino acid when it is provided by the respective partner. This hypothesis could explain the increased amino-acid production rates of cross-feeders relative to both WT and single-gene deletion mutants (Figure 2a) as well as the increased fitness of cross-feeding consortia (Figure 2c). This interpretation is consistent with a division of labour scenario, in which a functional specialisation of cells results in synergistic fitness benefits upon combination of those functions (Wahl, 2002; Rueffler *et al.*, 2012).

Another potential benefit that can result from the sharing of metabolic functions between two bacterial strains is that this behaviour significantly extends the spectrum of biochemical and physiological capabilities of each individual partner. As a consequence, the resulting consortia can exploit new ecological niches that were inaccessible to the individual genotypes before entering into the synthrophic interaction (Morris *et al.*, 2013), thus allowing them to escape competition with conspecifics.

Stability of obligate cross-feeding interactions

One key finding of this study is the unexpected ecological stability of cross-feeding consortia despite the presence of non-cooperating cells (that is, both prototrophic and non-amino-acid producing auxotrophs). As amino-acid overproduction incurred significant fitness costs (Figure 2b), genotypes that take advantage of the released 'public good' without reciprocating are selectively favoured and thus expected to exploit the resource until the ecological interaction collapses (Axelrod and Hamilton, 1981; Bull and Rice, 1991; Sachs *et al.*, 2004). Nevertheless, obligate mutualisms are widespread in nature among both micro- (Schink, 2002; Morris *et al.*, 2013) and macroorganisms (Boucher, 1988; Douglas, 1994) and, in some of these cases, non-cooperating types have been described to coexist with mutualists for extended evolutionary periods (Sachs and Simms, 2006). It is generally believed that either derived 'partner choice' mechanisms such as the punishment of non-cooperating individuals (Bull and Rice, 1991; Yu, 2001) or spatially structured environments (Doebeli and Knowlton, 1998; Wilson *et al.*, 2003) are required to protect mutually beneficial interactions from being exploited by non-cooperators. By engineering and analysing obligate cross-feeding interactions, however, we could demonstrate experimentally for the first time that even in the absence of a shared evolutionary history, obligate two-way cooperative interactions can be ecologically stable in a well-mixed environment.

This finding is in line with previously published theoretical models that predict mutualistically cooperating asexual organisms can stably coexist when (a) the rate of commodity provisioning is intermediate and (b) competition for the exchanged commodity is asymmetric (Ferriere *et al.*, 2002; Ferriere *et al.*, 2007). The obligate dependency on different amino acids implemented in our study system was also characterised by differential competitive abilities of the genotypes tested (Bertels *et al.*, 2012) (Figure 6), which could thus explain the observed result.

Conclusion

The picture that emerges from our findings implicates the mutational loss of biosynthetic genes as a potential source for generating metabolic diversity within bacterial populations. Whenever local metabolite concentrations exceed certain threshold levels, selection should favour the loss of biosynthetic genes and thus the evolution of obligate cross-feeding. The interactions that emerge in this way confer a significant fitness advantage to the genotypes involved and facilitate stable coexistence of cross-feeding mutants. Together, our results suggest that obligate metabolic cross-feeding is a powerful ecological mechanism that can lead to stable coexistence of different genotypes within a bacterial community and thus help to maintain genetic diversity.

Conflict of Interest

The Authors declare no conflict of interest.

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Author contributions

Conceived the project: SP and C Kost. Designed the experiments: SP, C Kaleta and C Kost. Performed all experiments: SP. Developed the AA measurement protocol: HM and MR. Independently replicated some experiments (data not shown): HM. Performed AA measurements: HM, SP, and MR. Analysed the data: SP and C Kost. Developed and applied the CASOP-GS method: C Kaleta, KB, LFdF, and SS. Wrote the paper: SP and C Kost. Amended the manuscript: all authors.

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