Supplementary Information for

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

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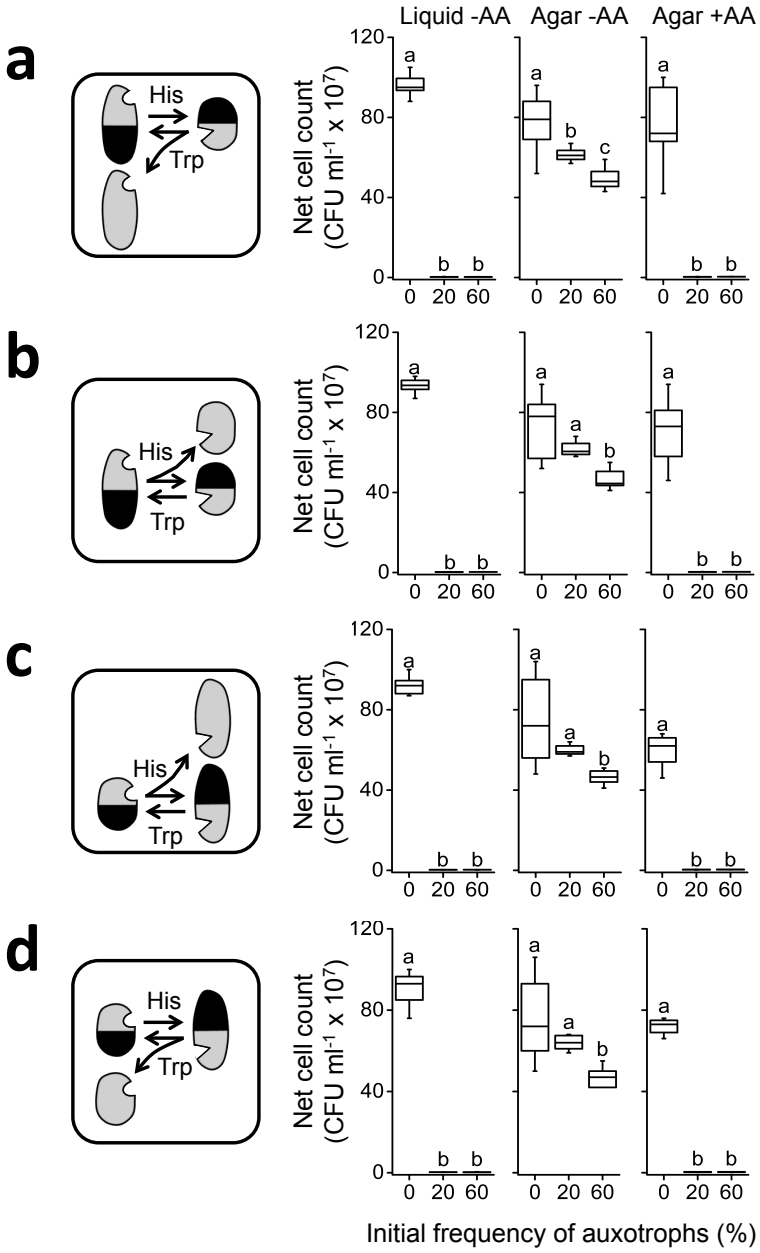
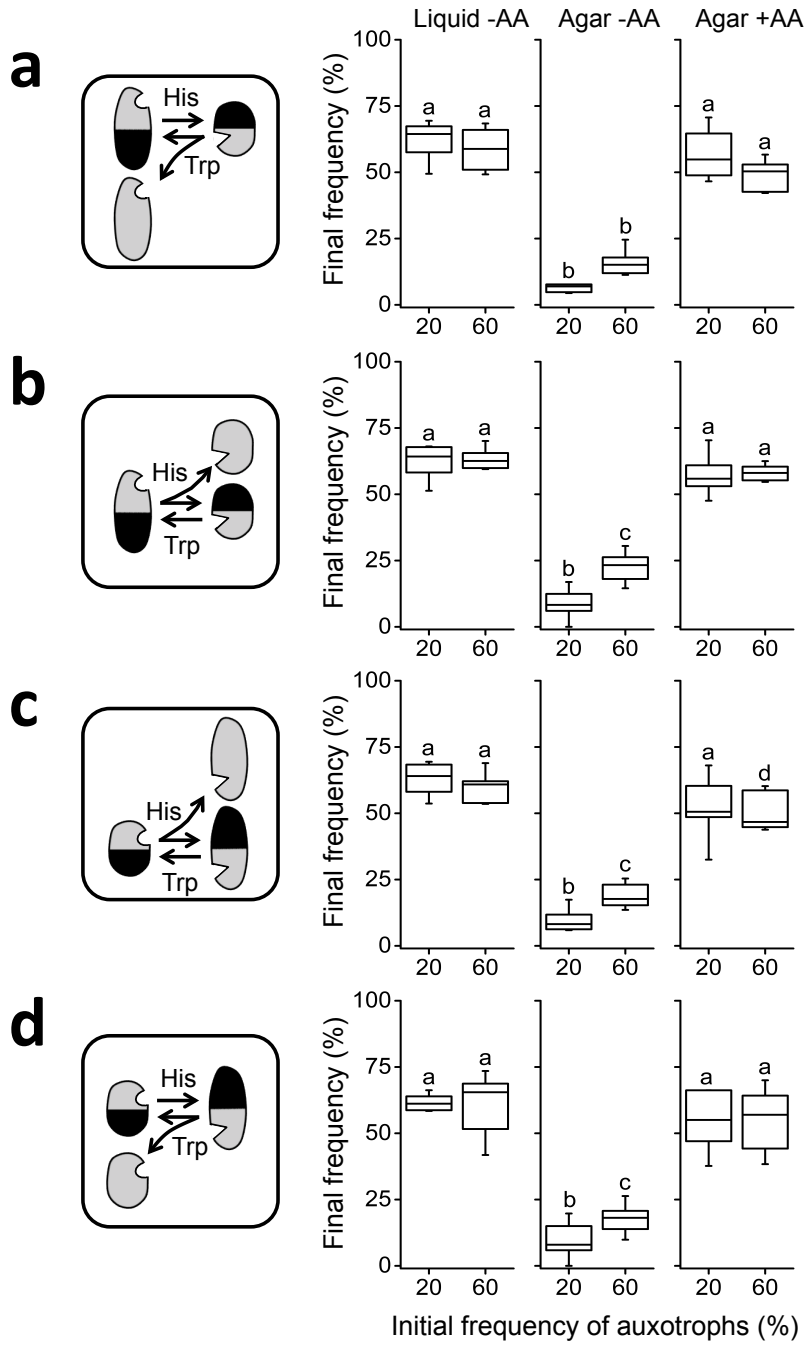
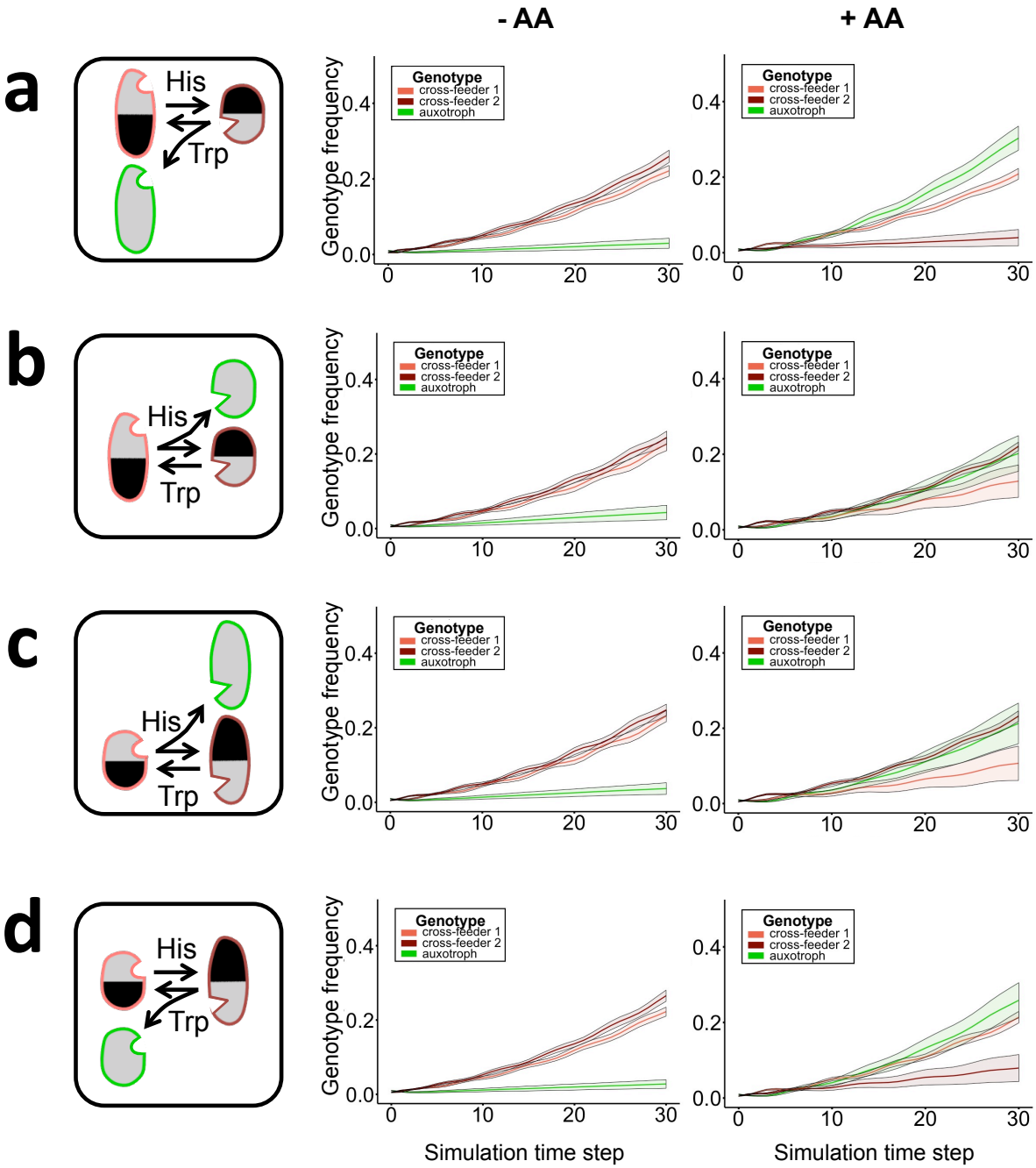
Supplementary Figures:



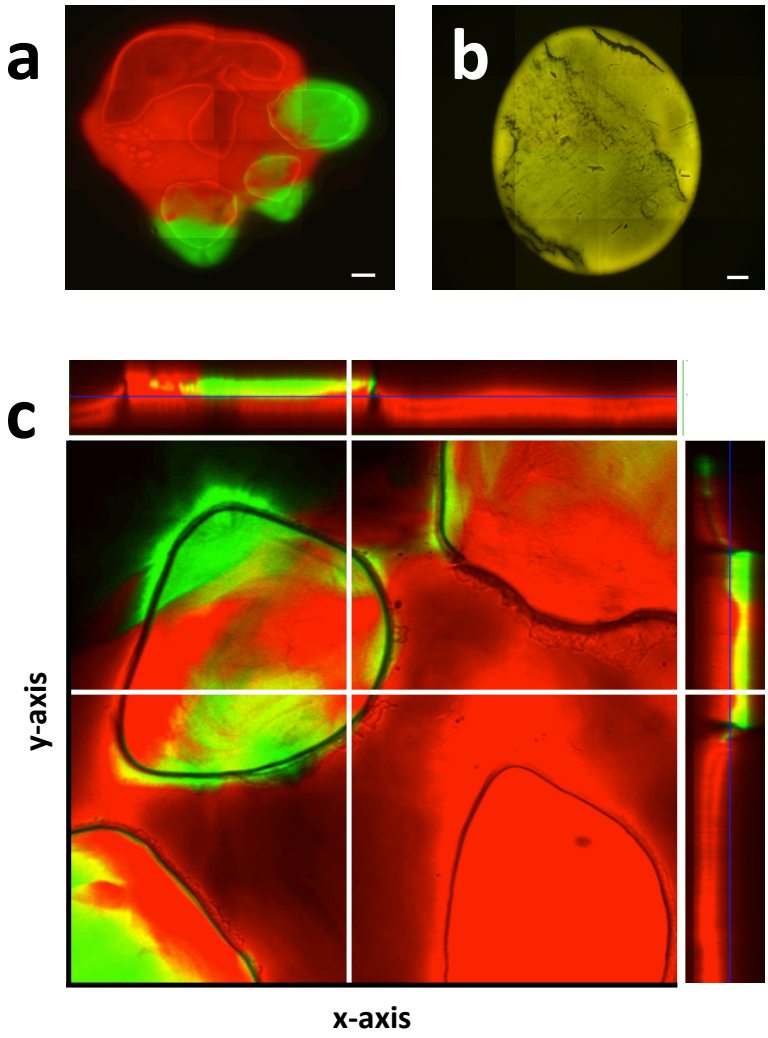
Supplementary Figure 1. Effect of non-cooperating auxotrophs on the productivity of cross-feeding genotypes. Productivity of pairs of cross-feeding genotypes that were co-inoculated with 0%, 20%, or 60% of an initial frequency of auxotrophs as determined by plating. Consortia were cultured in liquid culture or on agar plates containing either unsupplemented minimal medium (-AA) or minimal medium to which both amino acids (100 µM of histidine (His) and tryptophan (Trp)) 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 (i.e. 0 h). Genotype pictograms like in Figure 1B.



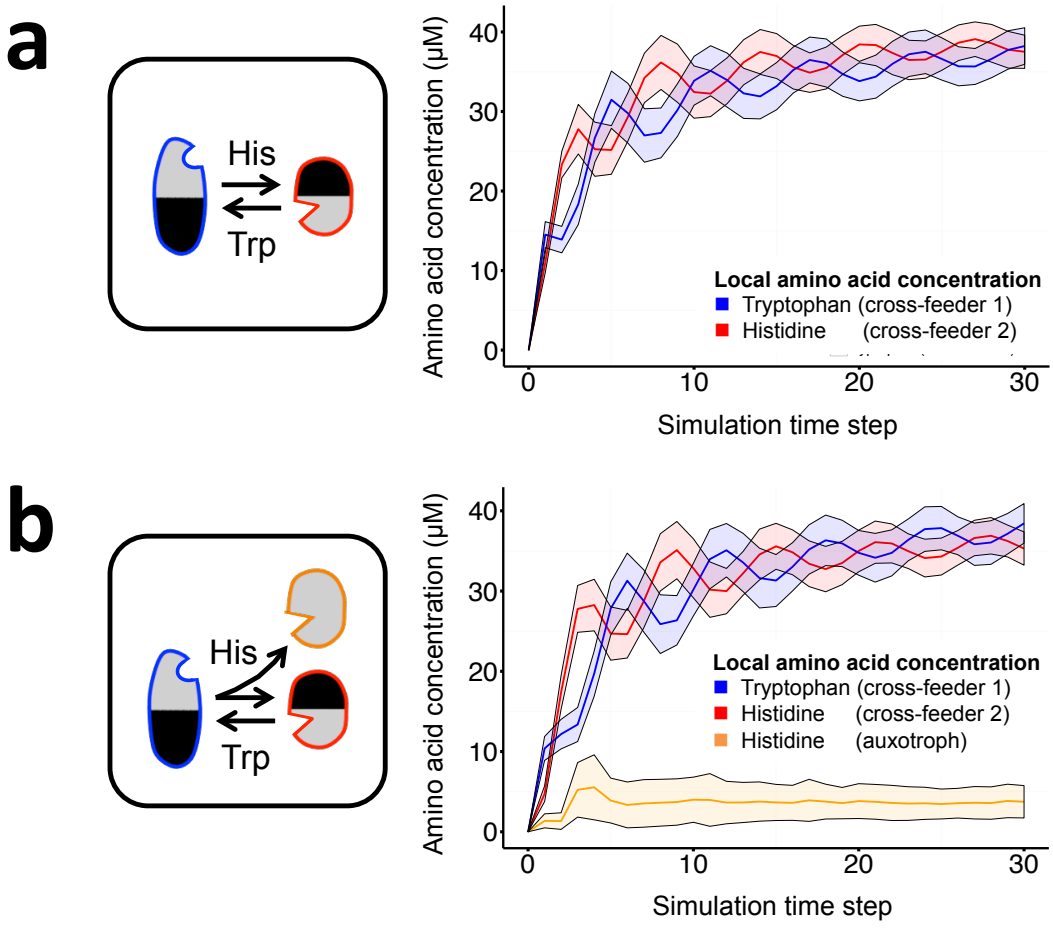
Supplementary Figure 2. Frequency of auxotrophic genotypes after 24 h of coculture when inoculated at an initial proportion of 20% or 60% together with pairs of cross-feeding genotypes. Consortia were grown in liquid culture or on agar plates containing either unsupplemented minimal medium (-AA) or minimal medium to which both amino acids (100 µM of histidine (His) and tryptophan (Trp)) have been added (+AA). Proportions were determined by plating the same cultures a shown in Supplementary Figure 1. Genotype pictograms like in Figure 1B.



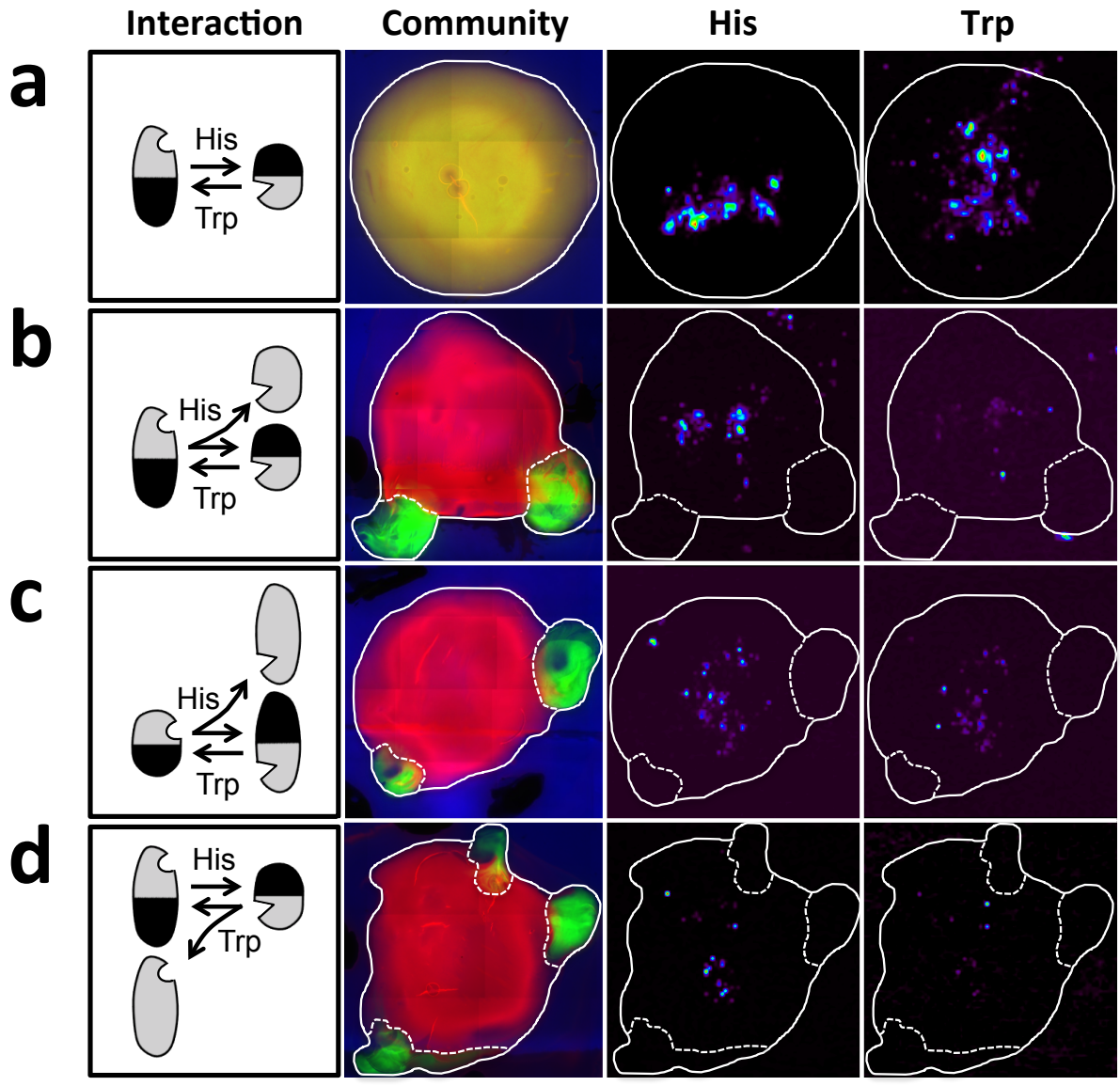
Supplementary Figure 3. Effect of amino acid availability on the growth of non-cooperating auxotrophs in consortia of two cross-feeding genotypes. Simulation results over thirty time steps show the frequency of a non-cooperating auxotroph (*A. baylyi ΔtrpB*, green) and two cross-feeding genotypes (*A. baylyi ΔtrpBΔhisL* and *E. coli ΔhisDΔtdcC,* shadings of red) in the absence (- AA) or presence (+ AA) of the focal amino acids (His and Trp). Mean values (line) ± standard deviation (coloured areas) are displayed. Genotype pictograms like in Figure 1B.



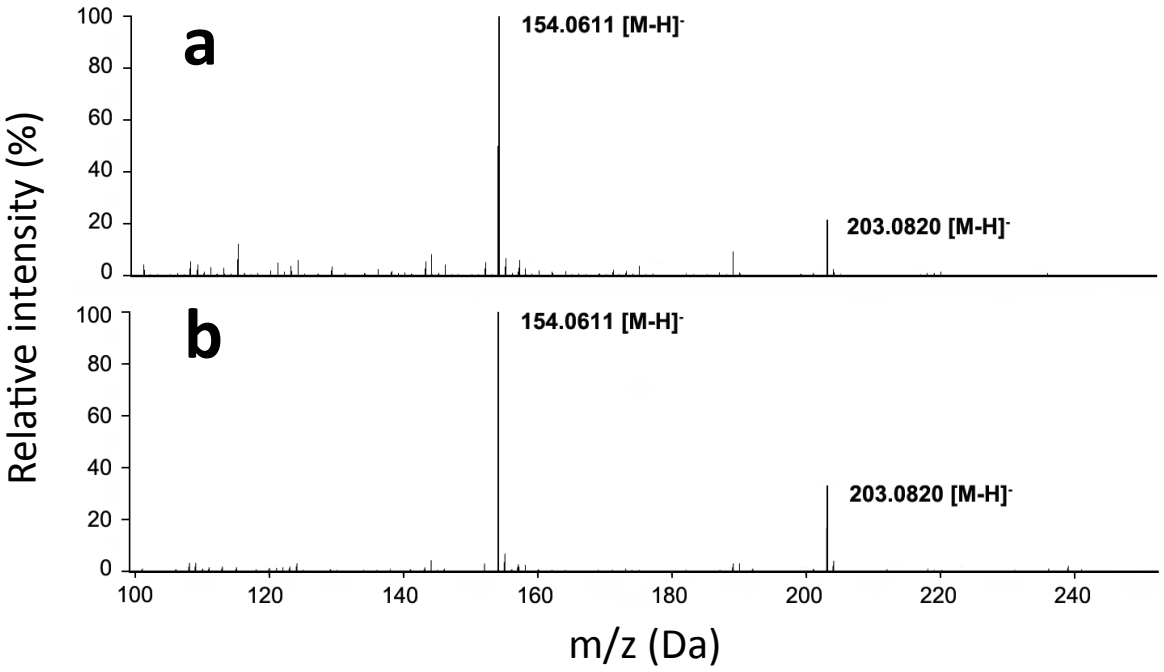
Supplementary Figure 4. Segregation of cooperative cross-feeders and non-cooperating auxotrophs in spatially structured environments. Pair of cross-feeding genotypes (*A. baylyi ΔhisDΔtrpB* and *E. coli ΔtrpBΔhisL*) initially inoculated in the presence (60%) of an auxotrophic genotype (*A. baylyi ΔhisD*). Cross-feeding strains were labelled with plasmids expressing mCherry (red colour) and auxotrophs were marked with an EGFP-expressing plasmid (green colour). Images show a top view of a colony after 24 h on solid MMAB minimal medium in (**a**) the absence or (**b**) presence of amino acids (100 µM of histidine and tryptophan). (**c**) Top view of the colony shown in (a) imaged with a laser scanning confocal microscope. White lines through the main panel highlight a vertical (x-axis) or horizontal (y-axis) cross-section through the colony that is shown in Z-direction at the top and the right side of the main panel, respectively.



Supplementary Figure 5. Time course of amino acid availability to pairs of cross-feeding and auxotrophic cells as predicted in simulations of an individual-based model. Depicted is the amino acid concentration in (µM) available to (**a**) pairs of cross-feeding genotypes or (**b**) cross-feeders and auxotrophs over time. Mean values (line) ± standard deviation (coloured areas) are displayed. Genotype pictograms like in Figure 1B.



Supplementary Figure 6. Spatial distribution of genotypes and amino acids in bacterial colonies. Distribution of cross-feeding genotypes (red colour) and auxotrophs (green colour) as visualized by fluorescence microscopy (left panel) as well as the spatial distribution of histidine (His, central panel) and tryptophan (Trp, right panel) as determined by MALDI-TOF MS imaging in the same colony. White lines represent the boundary of the respective colony and the dashed lines delimit the spatial distribution of non-cooperating auxotrophs. Genotype pictograms like in Figure 1B.



Supplementary Figure 7. Crystals observed on the surface of colonies consisting of cross-feeding genotypes correspond to the two amino acids histidine and tryptophan. HESI-FT-MS spectra have been measured on a FT-Orbitrap MS (Q-Exactive Plus, Thermo Fisher Scientific, Bremen, Germany) in negative ion mode with FT resolution of 70,000. (**a**) Representative FT-MS spectrum of crystals obtained from the surface of colonies consisting of the two cross-feeding genotypes (*A. baylyi ΔhisDΔtrpB* and *E. coli ΔtrpBΔhisL*). (**b**) FT-MS spectrum of amino acid standards (1:1 weight ratio mixture of histidine and tryptophan). Histidine: *m/z* 154.0611 [(M-H)$^-$]. Tryptophan: *m/z* 203.0820 [(M-H)$^-$].

Supplementary Methods

Construction of A. baylyi ADP1 *deletion mutants*

Linear constructs of the kanamycin cassette with 5'-overhangs homologous to the insertion site were generated by PCR. To amplify the kanamycin resistance cassette, plasmid pKD4 (Datsenko & Wanner 2000) was used as a template. 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. 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 linear fragments into the WT strain. Transformation was done by diluting 20 µl of a 16 h-old culture grown in liquid lysogeny broth (LB) medium (Carl Roth GmbH and Co. KG, Germany) into 500 µl fresh LB medium. This diluted culture was again incubated at 30 °C with shaking. 50 µl PCR mix containing the deletion cassette was added to this culture and again incubated at 30 °C with shaking for 2 h. Finally, the whole culture volume was concentrated to 100 µl and plated on LB agar plates containing kanamycin (50 µg ml^{-1}) 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 was complementary to each other. The resulting products were combined by PCR to obtain the cassette with the upstream and downstream homologous overhangs of respective genes. The PCR product was transformed into the respective kanamycin resistant mutants as explained above.

Matrix-assisted laser desorption/ ionization mass spectrometry imaging (MALDI-MSI)

The amino acid distribution within coculture colonies was determined by co-inoculating both cross-feeders (1:1 ratio, ~10^5 CFU each) and one auxotroph (60% of total population) in liquid medium to reach a final volume of 10 µl. This suspension was then inoculated on a filter membrane placed on top of a MMAB agar plate. After 24 h, colonies were analysed using fluorescence microscopy and then gently dried using blotting paper. Next ,the filter was attached to the metal MALDI plate by double-sided tape (Scotch®, www.scotchbrand.com, Minneapolis, MN, USA) and the MALDI target containing the bacterial samples was placed into a desiccator containing potassium hydroxide. Samples were dried for two hours under ambient temperature and pressure. After that, vacuum was applied for another 10 hours. The 9-aminoacridine matrix was applied on completely dried bacterial colonies by sublimation. For this, the sublimation chamber (Gebr. Rettberg GmbH, www.rettberg.biz, Germany) was evacuated (0.001 mbar) and the temperature of the oil bath was set to 170 ºC. The sublimation step was 1.5 min to ensure a sufficiently thick and reproducible layer of matrix on the samples.

For MALDI MSI experiments, a MALDI Micro MX mass spectrometer (Waters®, www.waters.com, Milford, MA, USA) was operated in a reflectron negative mode with acceleration and plate voltages at +12 kV and -5 kV, respectively. Delayed extraction time was set to 500 ns. Desorption and ionization was realized by nitrogen (337 nm) UV laser MNL 103-LD (LTB Lasertechnik Berlin GmbH, www.ltb-berlin.de, Germany) and matrix ions were suppressed with a low mass cut-off set at 110 Da.

All samples were imaged using a step size of 50 µm. The number of laser shots per spot was optimized and set to 100 (50 µJ shot^{-1}) in negative mode. Masses between 100 Da and 1 kDa were measured. Data were collected with MassLynx 4.0 software (Waters®, www.waters.com, Milford, MA, USA) and processed with the custom-made software MALDI Image Convertor (Waters®, www.waters.com, Milford, MA, USA) to obtain spatially resolved data. These data were exported to the BioMap software (Novartis®, www.novartis.com, Basel, Switzerland) and converted to 2-D ion intensity maps.

High Resolution Fourier Transform Mass Spectrometry (FT-MS)

FT-MS experiments were performed on a FT-Orbitrap MS (Q-Exactive Plus, Thermo Fisher Scientific, Bremen, Germany) operated in negative ion mode with FT resolution of 70,000. Samples of crystals that gathered on the surface of dehydrated colonies were diluted in MilliQ water and ionized in a HESI ion source (Thermo Fisher Scientific, Bremen, Germany). His and Trp were purchased as standard (Sigma-Aldrich®, Germany), mixed together (1:1 weight ratio), diluted in MilliQ water, and analyzed using the same method as has been applied to the crystal samples from the surface of a colony. The range of measured masses was set from 100 Da to 250 Da.

Individual-based model

A theoretical model was devised to identify the ecological mechanism that stabilizes cooperative cross-feeding interactions despite the presence of non-cooperating auxotrophs. For this, a cellular automaton was used, where each patch of the discrete, two-dimensional environment can either contain one cell of a certain genotype (i.e. two auxotrophic genotypes for His (aux_{His}) and Trp (aux_{Trp}) as well as two cross-feeding strains (cf_{His} i.e. auxotroph for His and cf_{Trp} i.e. auxotroph for Trp) or alternatively be empty (i.e. state of the patch). Thus, every patch can have 5 states including the empty state. To mimic experiments as close as possible, the environment was 'inoculated' by randomly distributing a certain number of each genotype (N^{i}_{init}) within a limited radius (r_{init}) at the centre of the grid. Each patch of the grid was characterized by three major properties: (i) a state, (ii) the concentrations of His and Trp, and (iii) a fitness value. Depending on its current state, the cell within a given patch can produce a certain amount of His or Trp. Fitness of cells was recalculated after diffusion took place and cells consumed the amino acids necessary to achieve this fitness value. The fitness of a patch in a certain state thus corresponds to the cells' growth rate and depends on the current amino acid concentration, while it is zero for empty patches. Growth rates of the different strains were experimentally determined for concentrations of 0 µM, 20 µM, 50 µM, 100 µM, 150 µM, and 200 µM of either His or Trp and interpolated using a Michealis-Menten kinetics. In order to estimate the amino acid production levels of the different strains, biosensor experiments were performed for each type of donor cell as described above and growth rates of the acceptor strains were mapped to amino acid concentrations using the inverse of the amino acid-dependent growth kinetics. For simplification, it was assumed that all amino acids a strain could consume were used up in one time-step to limit the history in amino acid accumulation. During simulations, patches can change their state, according to the fittest patch in their Moore neighbourhood. Patches with a fitness value of zero (i.e. essential amino acid is absent) become empty. The update of the states takes place synchronously. Additional amino acids were supplied with a constant rate (k_{feed}) every time-

step, thereby resulting in an equal distribution of amino acids among patches. Five different scenarios were tested with the automaton model (Supplementary Table 1).

Supplementary Table 1. Parameter settings for the different simulations.

		Scenario				
Parameter	**Description**	**CF only**	**CF, Aux**		**CF, Aux, AA**	
Grid size	Dimensions of the plate	100 x 100	100 x 100		100 x 100	
r_{init}	Inoculation radius (grid cells)	8	8	8	8	8
	Initial frequency of histidine auxotrophs (%)	0	0	50	0	50
	Initial frequency of tryptophan auxotrophs (%)	0	50	0	50	0
	Initial frequency of histidine auxotroph cross-feeders (%)	50	25	25	25	25
	Initial frequency of tryptophan auxotroph cross-feeders (%)	50	25	25	25	25
k_{Feed}	Rate of amino acids fed per time-step (μM ts^{-1})	-	-	-	10	10
T_{Stop}	Time at which simulation is stopped (ts)	30	30	30	30	30

CF = cross-feeders, Aux = auxotrophic genotype, AA = amino acid supplementation (histidine and tryptophan).

References

Datsenko K, Wanner B. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* **97:** 6640-6645.