Market forces determine the distribution of a leaky function in a simple microbial community

Sarah J. Adkins-Jablonsky[a], Colleen M. Clark[b,c], Spiridon E. Papoulis[b,d], Matthew D. Kuhl[a], and J. Jeffrey Morris[a,b,1]

[a]Department of Biology, University of Alabama at Birmingham, Birmingham, AL 35294; [b]BEACON Center for the Study of Evolution in Action, Michigan State University, East Lansing, MI 48824; [c]Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI 48824; and [d]Department of Microbiology, University of Tennessee, Knoxville, TN 37996

Edited by Joan E. Strassmann, Washington University in St. Louis, St. Louis, MO, and approved August 21, 2021 (received for review June 3, 2021)

Many biological functions are leaky, and organisms that perform them contribute some of their products to a community "marketplace" in which nonperforming individuals may compete for them. Leaky functions are partitioned unequally in microbial communities, and the evolutionary forces determining which species perform them and which become beneficiaries are poorly understood. Here, we demonstrate that the market principle of comparative advantage determines the distribution of a leaky antibiotic resistance gene in an environment occupied by two "species"—strains of *Escherichia coli* growing on mutually exclusive resources and thus occupying separate niches. Communities comprised of antibiotic-resistant cells were rapidly invaded by sensitive cells of both types. While the two phenotypes coexisted stably for 500 generations, in 15/18 replicates, antibiotic sensitivity became fixed in one species. Fixation always occurred in the same species despite both species being genetically identical except for their niche-defining mutation. In the absence of antibiotic, the fitness cost of resistance was identical in both species. However, the intrinsic resistance of the species that ultimately became the sole helper was significantly lower, and thus its reward for expressing the resistance gene was higher. Opportunity cost of resistance, not absolute cost or efficiency of antibiotic removal, determined which species became the helper, consistent with the economic theory of comparative advantage. We present a model that suggests that this market-like dynamic is a general property of Black Queen systems and, in communities dependent on multiple leaky functions, could lead to the spontaneous development of an equitable and efficient division of labor.

Black Queen hypothesis | comparative advantage | ecological species concept

Cooperative interactions are important components of social systems both in the human realm and in a broader natural context. From the perspective of average return, populations that cooperate often enjoy higher average benefits relative to populations that do not [e.g., the "conspiracy of doves" described by Dawkins (1)]. However, selfish cheaters within cooperating populations can reap the benefits of cooperation without paying the costs, and in the absence of mitigating circumstances, these cheaters will typically outperform cooperators and increase their relative share of a population even to the point of driving cooperative behavior to extinction (2).

Despite the threat of cheaters, cooperation is common at all levels in the natural world both within and between species. A major challenge to evolutionary theory is to explain how cooperative behaviors overcome "tragedies of the commons" to establish stable interactions in populations. Inclusive fitness, often manifested as kin selection, is one solution to the problem and is easily invoked in populations that are strongly structured by mating preferences or spatial limitations (3, 4). However, cooperation also evolves in populations without these properties. For instance, microbial communities in the well-mixed surface waters of the open ocean are deeply interdependent (5), and cosmopolitan human populations with low levels of relatedness nevertheless exhibit high levels of social cooperation (6).

Since the 18th century, economic liberalism and market dynamics have been viewed as mechanisms for harnessing selfishness to yield broader social rewards (i.e., Adam Smith's "invisible hand"). Economic and evolutionary theory have influenced each other since Darwin's time (7). For example, the economic law of supply and demand, which regulates the prices of goods and service in marketplaces, has been applied toward understanding the "choosiness" of potential mates under fluctuating sex ratios (8) (e.g., when the "supply" of males increases relative to female demand, their "value" goes down, and females can afford to be pickier). Similarly, the economic concept of comparative advantage (9) has been used to explain why a country may specialize in a single industry and become dependent on foreign exports for other products even if it has an absolute advantage in production of all of the products in question; this same concept has been leveraged in biology to explain the evolution of cooperative exchanges and obligate dependencies in nonhuman organisms at both the macro- and microscopic scales (10–16). However, most biological marketplace analogies have focused on clear instances of two-way trade between organisms (e.g., exchange of fixed nitrogen for photosynthate in plant–rhizobia symbioses). While useful, such analogies fail to capture the abstract power of human markets, in which the actions of arbitrary numbers of buyers and sellers collectively determine the prices of a wide variety of cooperatively produced goods and services.

One way that complex markets might arise in nonhuman systems is through the action of "leaky" biological functions. A function is leaky if the organisms that perform it inevitably lose some of its products or benefits to the environment. According to

Significance

Natural microbial communities are often deeply interdependent despite being locked in competition over a small number of limiting resources. While there are theories about how evolution can generate these dependencies, less is known about how responsibility for different tasks is assigned in a community. Here, we show that even small differences in costs paid by a species for performing a vital but "leaky" function can lead to one species consistently evolving into the sole "helper" for the entire community. We suggest that this process is analogous to the human economic principle of comparative advantage, selecting for efficient divisions of labor in microbial marketplaces.

Author contributions: S.J.A.-J., C.M.C., S.E.P., M.D.K., and J.J.M. performed research; S.J.A.-J., S.E.P., M.D.K., and J.J.M. analyzed data; S.E.P. and J.J.M. designed research; and S.J.A.-J., S.E.P., and J.J.M. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

[1]To whom correspondence may be addressed. Email: evolve@uab.edu.

This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2109813118/-/DCSupplemental.

Published September 21, 2021.

the Black Queen hypothesis (BQH) (17, 18), communities dependent on leaky functions will tend to evolve a partitioning between function-performing helpers and dependent beneficiaries. Integrated across the many goods and services needed to produce living cells, many of which are leaky, Black Queen evolution has the potential to create complex webs of interdependency wherein most organisms are dependent on other members of their community to survive. We have argued previously that microbial Black Queen functions create a marketplace for their leaked products, wherein community members may "decide" whether to manufacture the product themselves or to obtain it from the environment (19). Since energy and resources can either be dedicated to replication and cell division or to the performance of peripheral activities, the goods in such a marketplace can be valued using fitness as a currency.

Previous laboratory experiments have shown that pure bacterial cultures dependent on leaky functions rapidly evolve stable coexistence between function-performing helpers and obligately dependent beneficiaries (20). Attempts have been made to extend the Black Queen concept to multispecies microbial communities using computational models (21, 22), but many open questions remain. For example, are helpers and beneficiaries randomly selected based on mutational appearance, or are more deterministic processes at work that reproducibly assign the roles to the same types of organism? In this work, we demonstrated that, in a simple laboratory community consisting of two ecologically defined species, obligate interspecies interdependency for a leaky function spontaneously evolved, and the one species that paid the lowest opportunity cost to perform the function became the sole provider for the community in the great majority of replicate lineages within 500 generations. This community mirrors the international division of labor by comparative advantage described by Ricardo (9) and provides an example of how macroeconomic principles can be applied to microbial ecology.

Materials and Methods

Media and Culture Conditions. *Escherichia coli* was cultured in Davis Minimal (DM) salts (23) supplemented with mannose (DM-man), galactose (DM-gal), glucose (DM-glu), an equal mix of mannose and galactose (DM-mix), or with a 9:1 ratio of mannose to galactose or vice versa, always to a final total sugar concentration of 0.2%. The molar ratios of C:N and C:P in DM-mix were ∼4:1 and 8:3; given that the C:N and C:P ratios of *E. coli* cells growing in a similar medium have been observed to be ∼5:1 and 50:1, respectively (24), the cultures described here are most likely limited by C, and competition for other nutrients is less likely to occur. Unless otherwise indicated, ampicillin was added to a final concentration of 100 $\mu g \cdot mL^{-1}$ just prior to inoculation. Cultures were prepared in Lenski flasks (i.e., 50-mL Erlenmeyer flasks capped with inverted 25-mL beakers) in 10-mL volume and incubated at 37 °C with 120-rpm orbital shaking. In evolution experiments, cultures were diluted 100-fold into fresh media every 24 h. Cells were quantified by dilution onto appropriate DM media supplemented with 0.02% L-arabinose and solidified with 1.5% agar. Prior to inoculation, plates were spread with 20 μL 2% X-Gal dissolved in dimethylformamide. On these plates, cells containing the pBQ1 plasmid (see *Strain Construction*) formed blue colonies, and cells without the plasmid formed white colonies.

Strain Construction. Knockout mutants of the genes *manA* (mannose 6-phosphate isomerase) and *galK* (galactokinase) from the Keio collection (25) were chosen as our two "species" for evolution experiments. The *manA* strain was unable to grow on DM-man but could grow on DM-gal, and the *galK* strain was unable to grow on DM-gal but could grow on DM-man. Thus, we name these strains by the sugar they can use—*manA* is called Gal and *galK* is called Man.

The ampicillin-resistance plasmid pBQ1 was constructed using Gibson assembly (26) with New England Biolabs Gibson Assembly Master Mix using the manufacturer's recommended procedures. The arabinose-inducible plasmid pBAD24 (27) was used as a backbone, and the entire *lacZ* gene from *E. coli* MG1655 was inserted downstream of the araBAD promoter. Primer sequences and PCR conditions are shown in *SI Appendix*, Table 1. Constructs were transformed into chemically competent *E. coli* DH5-α cells by heat shock and selected on lysogeny broth (LB) agar plates supplemented with 100 $\mu g \cdot mL^{-1}$ ampicillin. The completed pBQ1 was extracted and purified using a Qiagen Miniprep kit and subsequently transformed into Gal and Man strains by heat shock. Henceforth, we refer to strains carrying pBQ1, which confers ampicillin resistance, as Gal+ and Man+ and strains without the plasmid, which were not resistant to ampicillin, as Gal− and Man−. Because the Keio ancestor *E. coli* BW25113 has a deletion in *lacZ* that renders it incapable of utilizing the colorogenic substrate X-Gal, Gal+/Man+ and Gal−/Man− are distinguishable on agar plates containing both arabinose (to induce the *lacZ* gene on pBQ1) and X-Gal, as the former make blue colonies and the latter make white colonies.

Evolution experiments were initiated from separate isolated blue colonies of Gal+ and Man+ strains picked from LB agar supplemented with ampicillin, arabinose, and X-Gal. Gal+ and Man+ were grown separately in DM-mix for one passage prior to being mixed together to initiate coculture evolution experiments. Because of its slower growth rate, Gal would not reach stationary phase in one passage, leading to its being outnumbered by Man during the initial quantification of populations in evolution experiments (e.g., the time 0 points in Fig. 1*B*); however, after 24 h, its abundance stabilized and remained consistent throughout the experiment.

Determination of Cost of Antibiotic Resistance. Direct competitions between ampicillin-resistant (AmpR) and -sensitive (AmpS) strains (e.g., Gal+ versus Gal− and Man+ versus Man−) in media either with or without added ampicillin were used to measure the fitness cost of the pBQ1 plasmid. Strains were first streaked for isolation on LB plates either with or without ampicillin. Each replicate competition was initiated from a single isolated colony of each competitor from these plates. Colonies were grown initially in LB and then transferred to DM-man or DM-gal as appropriate for one passage prior to mixing of resistant and sensitive strains. For competitions performed in antibiotic-free conditions, Gal+ and Man+ were acclimated in ampicillin-free DM media prior to coculturing with Gal− or Man− to avoid interference of residual ampicillin on fitness estimates. Competition cultures were plated at the beginning of the experiment and again at the end (either one or three transfers later), and fitness was calculated as the ratio of the Malthusian parameters for each strain (28). Statistical differences in fitness between strains were determined using linear models in R.

Determination of Essentiality of Antibiotic Resistance. We used two methods to measure the susceptibility of Gal− and Man− to ampicillin (i.e., how essential resistance was for each species). First, we used a modified Kirby–Bauer disk diffusion assay. Overnight cultures of Gal− and Man− grown in LB media at 37 °C were streaked onto six replicate LB agar plates to form confluent lawns, and a standard Kirby–Bauer ampicillin disk was placed in the center of each plate. The following day, the diameter of zone of clearance around the antibiotic disk was measured.

Our second method followed the growth curves of cultures exposed to various concentrations of ampicillin for 48 h in a BioTek Synergy 96-well plate reader. Gal− or Man− cultures were initiated from individual colonies that were first grown overnight in LB media and then diluted 1,000-fold into DM-man, DM-gal, or DM-glu and grown for a further 24 h at 37 °C with 120-rpm shaking. A total of 190 μL of these parent cultures was used to inoculate 48 wells of a prewarmed 96-well plate. Immediately, six wells each were treated with either ampicillin (final concentrations: 20, 10, 5, 2.5, 1.25, 0.63, or 0.31 μg/mL) or sterile water as a control. The plate was then sealed with optically transparent adhesive film. Optical density (OD) measurements at 480 nm were collected every 5 min using a BioTek Synergy H1 plate reader for 48 h at 37 °C, with 5 s of shaking at maximum speed immediately prior to each reading. Each culture was determined to either be alive or dead based on visual inspection of growth curves. Comparisons between species were performed at concentrations of ampicillin at which some but not all cultures failed to grow for one or both species; statistical differences were assessed using Fisher's exact test implemented in R.

Determination of Leakiness of Antibiotic Resistance. As with antibiotic sensitivity, we used two methods to estimate leakiness. First, we grew overnight cultures of Gal+ and Man+ in DM-gal or DM-man, respectively. We then saturated a sterile filter paper disk with ∼10 μL of this culture and placed the disk in the center of an LB agar plate supplemented with 100 μg/mL ampicillin upon which a lawn of *E. coli* MG1655 had been spread. MG1655 is unable to grow on this medium by itself, and therefore leakiness of Gal+ and Man+ was determined by the radius of MG1655 growth facilitated by the presence of the filter disk (*SI Appendix*, Fig. 2*A*).

Our second method was performed in liquid culture media to measure the ability of Man+ and Gal+ supernatants to restore the growth of MG1655. Man+ and Gal+ were grown overnight on LB agar supplemented with X-Gal, arabinose, and ampicillin, and then four cultures in liquid LB with ampicillin were initiated from isolated blue colonies. After 24 h, each culture was then

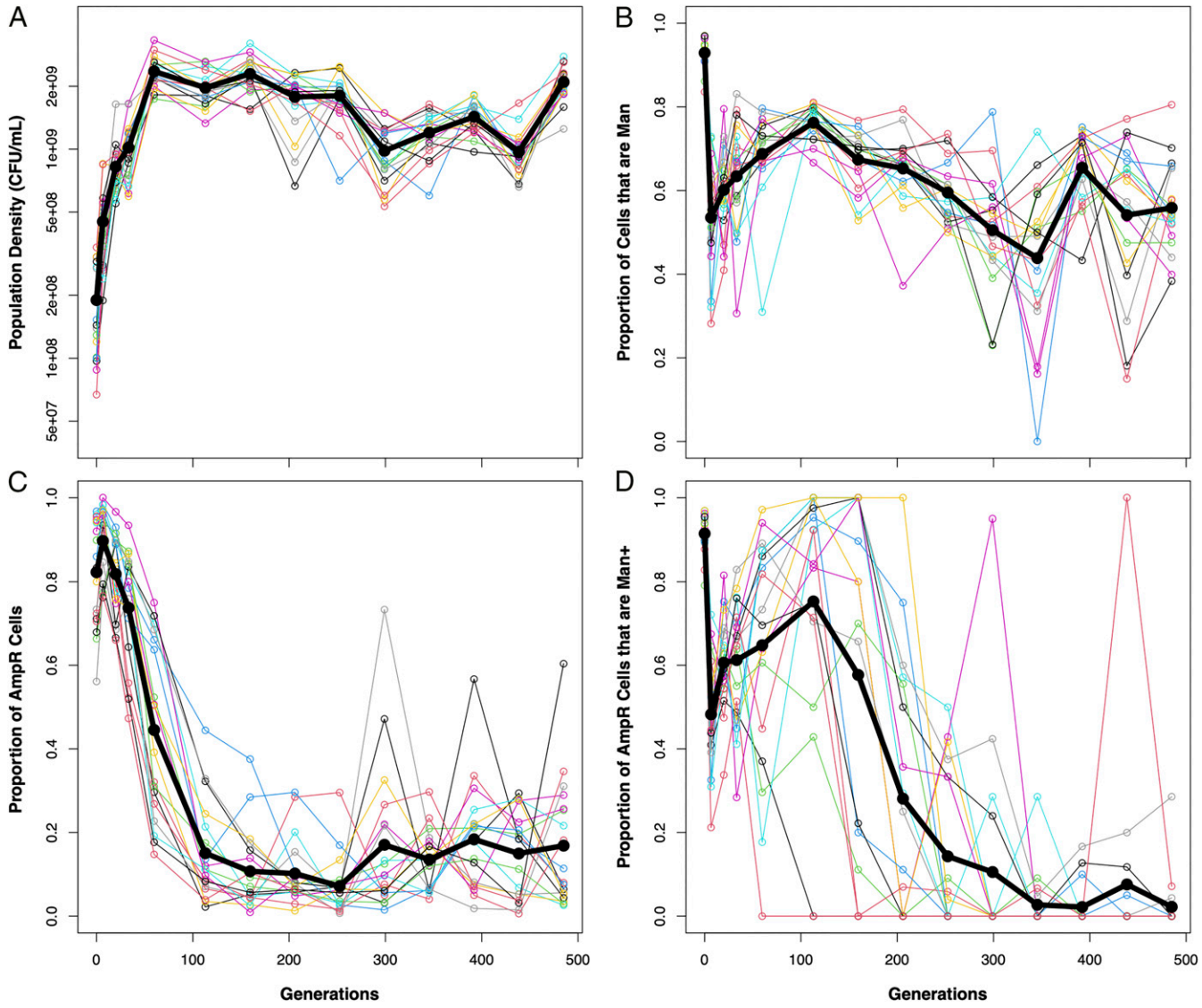

Fig. 1. Experimental evolution of a two-species Black Queen community. Strains of *E. coli* capable of growing on Man or Gal were cocultured in liquid media containing equal amounts of both sugars for ∼500 generations. Culture media also contained 100 μg/mL ampicillin; each culture was seeded with *E. coli* strains carrying AmpR plasmids (Man+ and Gal+). Each colored line represents one of 18 replicate lineages; the thick black line represents the mean value of all 18 lineages at the indicated time point. (*A*) Total population density in CFU/mL. (*B*) Proportion of total populations that were Man. (*C*) Proportion of total populations carrying the AmpR plasmid. (*D*) Proportion of the AmpR cells that were Man+.

diluted 1,000-fold into DM-man or DM-gal treated with ampicillin. MG1655 cultures were initiated similarly but using media without ampicillin or X-Gal. After both Man+/Gal+ had grown another 24 h in DM-man or DM-gal, the cultures were centrifuged at 5,000 × *g* for 2 min to pellet the cells, and the resulting supernatant was sterilized using a 0.2-μm Millex GV syringe filter. The replicate supernatants were passaged through serial twofold dilutions in sterile milli-Q water, and the MG1655 replicates were each diluted 100:1 into fresh DM-man or DM-gal media. The wells of a prewarmed 96-well plate were then filled with 180 μL diluted MG1655 and 20 μL of one of the supernatant dilutions (or sterile milli-Q water as a control). Finally, ampicillin was added to all wells to a final concentration of 100 μg/mL, a dosage that was determined to be 100% lethal to MG1655. The plate was sealed with optically transparent adhesive film, and OD measurements at 480 nm were collected every 5 min using a BioTek Synergy H1 plate reader for 48 h at 37 °C. Area under the curve (AUC) was extracted from the resulting growth curves by simply summing the OD measurements across all time points, and exponential growth rates were determined by linear regression of log (OD) on time over at least 3 h of measurements taken during the exponential phase (determined by visual inspection of plotted growth curves).

Prior to filtration of Man+ and Gal+ cultures, aliquots were serially diluted onto LB agar to determine cell titer. The "strength" *S* of the diluted supernatants used in the growth curve experiment was expressed as colony-forming unit (CFU) · mL^{-1} equivalent, equal to the concentration of Man+ or Gal+ cells that would have been added had they not been removed by filtration. Both AUC and growth rate were normalized by first subtracting the value for the no-supernatant negative control from all samples and then by expressing all values as a proportion of the average value of the undiluted supernatant positive control replicates. The supernatant protective capacity (i.e., leakiness) was then expressed as the half-saturation constant *K* (in units of CFU · mL^{-1} equivalents) obtained by fitting normalized AUC and growth rate data to a rectangular hyperbola in R using the equation

$$AUC \Big/ slope = \frac{S}{K+S}. \quad [1]$$

Statistical differences between strains were determined by comparing the fit of a model with different K values for each strain to one with a single K value using the *anova* function in R.

Mathematical Modeling. To explore the economics of our two-resource, one-toxin experimental system and the degree to which it might be generalized to other similar systems, we modeled the effects of changing key Black

Queen parameters on the beneficiary:helper ratios of two species (29). Much like our experimental system, we define each "species" as having a single and mutually exclusive limiting resource, and each species also has both a helper and beneficiary population. We model the change in resource as

$$\frac{dR_i}{dt} = S_r - \mu_i(1 - c_i)\frac{R_i}{(k_i + R_i)}H_i - \mu_i\frac{R_i}{(k_i + R_i)}B_i, \quad [2]$$

where R_i is the concentration of the i^{th} resource at time t. For notational simplicity, we quantify the resource concentration as "cellular equivalents · mL^{-1}," which assumes fixed resource quotas for all competing populations and implies each unit of resource is sufficient to produce one cell given 100% resource utilization efficiency (see Eq. **3**). B_i and H_i are the concentration of species i beneficiary and helper populations, respectively, that consume resource i at time t and are in units of cells · mL^{-1}. S_r is the supply rate of all resources into the system (cellular equivalents · mL^{-1} · d^{-1}). μ_i is the resource utilization (mL · $cell^{-1}$ · d^{-1}) of species i on resource i, while c_i is the fractional cost to the helper for performing the detoxification function. k_i is the half-saturation constant of species i for resource i and was assumed to be identical for beneficiaries and helpers. We define the change in each species' beneficiary population as the following:

$$\frac{dB_i}{dt} = \varepsilon_i\mu_i\frac{R_i}{(k_i + R_i)}B_i - \frac{T}{(\varphi_i + T)}B_i - \delta B_i. \quad [3]$$

T is the concentration of toxin at time t, and φ_i is the intrinsic resistance (i.e., essentiality) parameter of species i, describing the concentration of T capable of killing half of the beneficiaries of species i in unit time. ε_i is the resource efficiency coefficient, in units of cell · cellular $equivalents^{-1}$, that modifies the biomass transfer from R_i to B_i, essentially scaling the "cellular equivalents" unit of resource i to yield less than one cell, functionally equivalent to the loss of some resource as metabolic waste. δ is background loss (e.g., death or washout, in units of $time^{-1}$). Similarly, we define the change in each species' helper population as the following:

$$\frac{dH_i}{dt} = \varepsilon_i\mu_i(1 - c_i)\frac{R_i}{(k_i + R_i)}H_i - \delta H_i. \quad [4]$$

We assume that ε_i is the same for both helper and beneficiary populations of species i, but the helper population pays cost of resistance c_i and thus attains complete resistance to the toxin. Finally, we define the change in the toxin concentration as

$$\frac{dT}{dt} = S_t - \sum_{i=1}^{n}\frac{H_i}{(\lambda_i + H_i)}T, \quad [5]$$

where S_t is the supply rate of toxin (concentration · d^{-1}), λ_i is the contribution of H_i toward public resistance against toxin T (e.g., leakiness, in units of cells · mL^{-1} · d^{-1}, reflecting the number of cells of species i necessary to remove half of T in unit time), and the total public detoxification is a summation of the contributions of all helper species removing toxin T from the system.

Eqs. **2**–**5** could be extended in many ways, including multiple limiting resources, explicitly modeling the extracellular concentration of β-lactamase, and considering the seasonal character of batch culture (30, 31). We opted for the current model form, as it represents a simple and parsimonious way to explore the economics of Black Queen dynamics when there are multiple species coexisting on unique resources.

Our model was implemented in ODElib, a python library aimed at analyzing ordinary differential equation ecosystem models. All numerical simulations were allowed to reach steady state, and model plots were generated in R with ggplot2. Modeling results were executed in jupyter notebooks and can be found at (https://github.com/SEpapoulis/MicrobialBlackQueenMarket) (32). ODElib can be downloaded at (https://github.com/SEpapoulis/ODElib) (33).

Results

We performed a 500-generation evolution experiment in which two ecological species (Man and Gal, defined by their growth on, respectively, the sugars mannose and galactose) were grown in 18 replicate cocultures in media containing equal concentrations of both sugars as well as the bactericidal antibiotic ampicillin. Each replicate culture was inoculated with Man+ and Gal+ varieties carrying the pBQ1 plasmid expressing the enzyme β-lactamase, an extracellular (and therefore leaky) enzyme that destroys ampicillin and thus detoxifies the culture media. Because the species grew on mutually exclusive sugar substrates and other nutrient requirements were in excess, they were unlikely to be in direct competition over resources but were potentially in indirect Black Queen competition over which would shoulder the burden of removing the antibiotic. Based on preliminary results, we knew that a relatively small proportion of the total population (~10%) of either species was sufficient to detoxify the medium, and therefore we predicted, based on the BQH, that over time, plasmid-free varieties would invade cultures and eventually reach an equilibrium between the AmpR helper (Man+ /Gal+) and AmpS beneficiary (Man− /Gal−) cells.

Initially, we hypothesized that one species in each of the 18 replicate cultures would come to be an obligately AmpS beneficiary due to extinction of the plasmid, leaving the other species to carry the burden of detoxifying the medium alone. Because Man and Gal were assumed to be identical except for the deletion of a single gene conferring their sugar utilization phenotype, we expected that 50% of the replicate cultures would come to be protected exclusively by Man+ and the other 50% by Gal+. However, our results were quite different (Fig. 1). As expected, the proportion of AmpR cells decreased rapidly in all cultures, approaching a relatively stable equilibrium point of 10 to 20% of the total community (Fig. 1*C*). However, despite a stable total population size (Fig. 1*A*) that was consistently greater than 50% Man (Fig. 1*B*), Man+ dropped below our limit of detection in 15 of 18 cultures by 500 generations (Fig. 1*D*), whereas Gal+ remained abundant in all 18 populations throughout the experiment. The probability of all 15 extinction events occurring in the same species if there was a 50% probability of fixation for each species is ~0.006% (binomial test), definitively rejecting our initial hypothesis.

We next investigated possible causes for the striking reproducibility of Man+ extinction and evolution of obligate dependency of Man on Gal+. One simple possibility was that Man's small numerical advantage during the experiment (Fig. 1*B*) gave it an edge in discovering the beneficial mutations that allowed it to become a beneficiary. To address this possibility, we ran a second evolution experiment, in which we grew six replicate populations each in media with either a 9:1 or 1:9 mannose-to-galactose ratio. Under these conditions, total population density was approximately the same as in the initial experiment (*SI Appendix*, Fig. 1*A*), but the proportion of the community composed of Man cells was very different between the two media types (*SI Appendix*, Fig. 1*B*). The dynamics of AmpR decrease were initially similar to the first experiment (*SI Appendix*, Fig. 1*C*), although in later stages, the culture-to-culture variability in AmpR proportion increased dramatically, perhaps due to more pronounced population bottlenecks in the minority species during daily transfers. Nevertheless, Man+ cells remained rarer than expected in the high-mannose medium in which Man cells outnumbered Gal cells by an order of magnitude (*SI Appendix*, Fig. 1*D*). Considering all the time points in both experiments, Man+ was significantly less abundant than expected based on the current Man share of the population (paired Wilcoxon signed-rank tests comparing the Man proportion of the total population to the Man+ share of the AmpR population; $P < 0.001$ for both evolution experiments). Moreover, after 500 generations in high-galactose media in which Gal+ outnumbered Man+ by an order of magnitude, Man+ was undetectable in one replicate, but Gal+ remained present in all six. On the other hand, in high-mannose media, Man+ and Gal+ were below the limit of detection in two of six replicates each. If the results of our first experiment were due to Man's numerical overrepresentation, we would expect Man+ to go extinct more often in high-mannose medium than in DM-mix, which is the opposite of what was observed. We thus concluded that numerical advantage was not sufficient to explain the reproducible extinction of Man+ in our original experiment.

We next considered factors other than numerical discrepancies that may explain the reproducibility of Man+ extinction.

Previous modeling efforts (21) suggested that the dynamics of helper/beneficiary evolution in an idealized Black Queen system were controlled by three parameters related to the leaky function—cost, leakiness, and essentiality—and we experimentally investigated each of these for both Man and Gal.

Cost. We measured the cost of ampicillin detoxification by competing AmpR and AmpS varieties of Man and Gal against each other in media without ampicillin. Under these conditions, expression of β-lactamase provided no benefit, and its cost was represented in terms of decreased relative fitness of both Man+ and Gal+ of ∼20%, with no significant difference in cost between the two species (Fig. 2*A*). In contrast, when AmpR and AmpS varieties were competed against each other in media with ampicillin, the relative fitness of AmpR was frequency dependent, with AmpR having a fitness deficit when initially common and a fitness advantage when initially rare, reflecting the Black Queen interaction occurring under these conditions (Fig. 2*B*). While the predicted equilibrium AmpR frequency was very similar for both species (∼30 to 40%, Fig. 2*B*), the slope of negative frequency dependence was much steeper for Man+ than Gal+, suggesting that stronger selective forces were acting on Man+ than Gal+ in the presence of ampicillin (Fig. 2*C*). We reasoned that the different strengths of negative frequency dependence for the two species in the presence of ampicillin were likely the result of differences in leakiness and/or essentiality, and therefore we measured both of these next.

Leakiness. We next measured leakiness using two approaches. There was no significant difference between the two species in the ability of AmpR cells embedded in filter paper discs to facilitate the growth of a lawn of AmpS *E. coli* MG1655 on ampicillin-treated agar plates (*SI Appendix*, Fig. 2). However, Gal+ supernatants exerted a significantly stronger protective effect than Man+ supernatants on MG1655 growing in ampicillin-containing liquid cultures (*SI Appendix*, Fig. 3): significantly fewer Gal+ cells than Man+ cells were required to produce sufficient extracellular β-lactamase to restore the growth of MG1655 in the presence of lethal ampicillin (Fig. 3*A*).

Essentiality. We also assayed the essentiality of the AmpR trait by measuring the innate resistance of Man− and Gal− cells to ampicillin. As with leakiness, there was no difference between the two species in innate resistance when determined by zone of inhibition in an agar plate disk-diffusion assay (*SI Appendix*, Fig. 4). However, in liquid media, Man− had significantly higher innate resistance than Gal, regardless of whether resistance was compared in media containing the species' defining sugars (e.g., mannose or galactose) or in a common medium with glucose as the substrate (Fig. 3*B*). This differential resistance was not caused by a difference in cell density; both species were inoculated at the same initial density (*SI Appendix*, Fig. 5*A*). On the other hand, Man− grew significantly faster than Gal− in both glucose and mannose/galactose media (*SI Appendix*, Fig. 5*B*), which may have contributed to its superior resistance through an unknown mechanism.

In order to disentangle the relative impact of leakiness and essentiality for determining which species' AmpR variety would prevail, we designed a dynamical model similar to that of Estrela et al. (21) that included multiple ecologically defined species not in direct competition with each other but mutually dependent on a leaky function to survive in a toxic environment. We first calibrated the model on a one-species, one-resource system, confirming that it recapitulated the expected dynamics of coexistence predicted by the BQH. As in Estrela et al. (21), leakier (lower λ) and more intrinsically resistant (higher φ) populations (*SI Appendix*, Fig. 6*A*), as well as populations paying higher costs of resistance (*SI Appendix*, Fig. 6*D*), support larger beneficiary:helper ratios (*SI Appendix*, Fig. 6). Parameters that affect equilibrium population density (efficiency of resource conversion and loss rate, *SI Appendix*, Fig. 6 *B* and *F*) also influence the beneficiary:helper ratio, but parameters affecting the growth rate (resource utilization and half saturation constants, *SI Appendix*, Fig. 6 *C* and *E*) do not.

When we introduce a second species to the model, the same parameters control the global helper:beneficiary ratio (e.g., as in Fig. 1*C*) but have different impacts on the ratio of the two species' contributions to toxin removal. When the two species have unequal costs of resistance *c* or differential levels of intrinsic resistance φ, deeply skewed ratios can arise (Fig. 4 *A* and *B*). Leakiness λ has no such effect, however, and even variation between the species of five orders of magnitude does not lead to a dominance of the helper population by one species (Fig. 4*C*). As in the one-species trials, if one species has greater efficiency of resource utilization than the other, this can lead to a small skew in helper ratios, but the gradients are much shallower than those observed for *c* and φ (*SI Appendix*, Fig. 7*C*). Differences in growth rate and resource-use efficiency, like λ, have no effect on which species dominates the helper population (*SI Appendix*, Fig. 7 *A* and *E*).

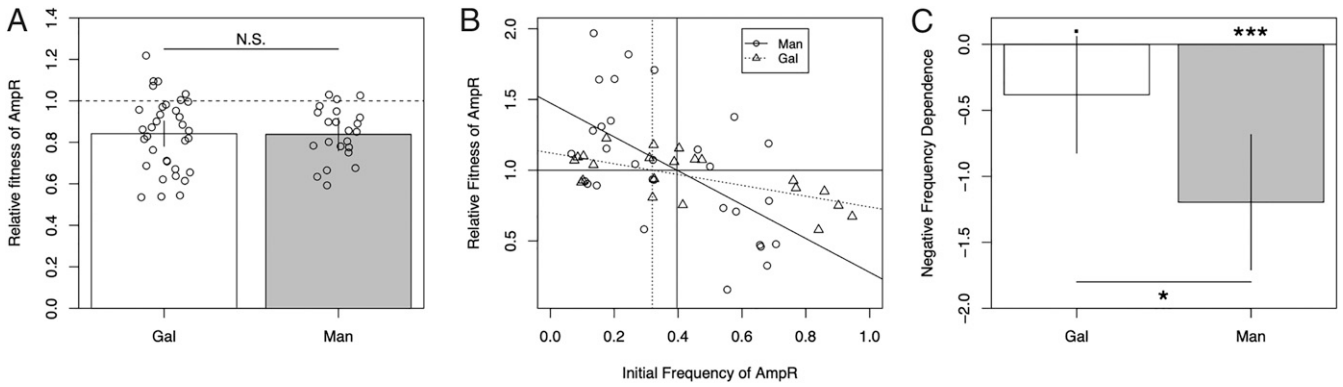

Fig. 2. Competitive fitness of AmpR *E. coli* strains. Cocultures were inoculated with AmpR and AmpS varieties of either Gal or Man, and competitive fitness of AmpR was measured as the change in ratios of AmpR versus AmpS over time. (*A*) Competition in media without ampicillin. (*B*) Competition in media with ampicillin. Diagonal lines indicate the regression of fitness on initial frequency of the AmpR variety; vertical lines indicate the predicted equilibrium frequency in which AmpR and AmpS have equal fitness. (*C*) The slope of frequency dependence of AmpR's relative fitness. Asterisks between bars indicate a significant difference in predicted means of the two treatments; asterisks above bars indicate the slope is significantly different from zero. ·, $P < 0.1$; *$P < 0.05$; ***$P <$ 0.001; N.S., not significant, $P > 0.05$.

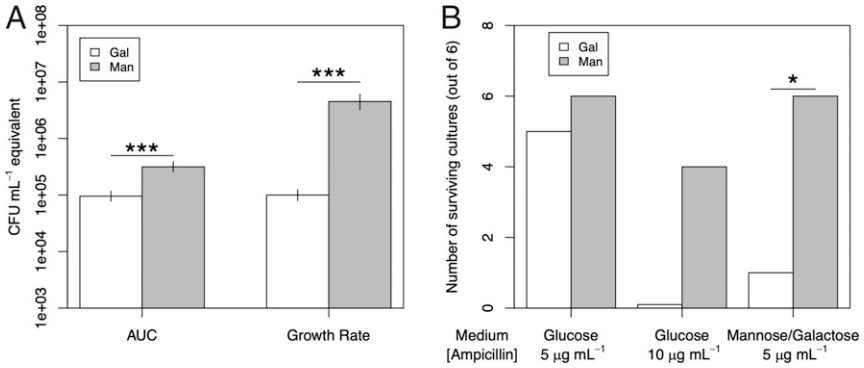

Fig. 3. Leakiness and essentiality of ampicillin resistance for the *E. coli* ancestors. (*A*) Half-saturation constants for the protective capacity of supernatants from AmpR cultures to restore AUC and exponential growth rate of an AmpS strain to antibiotic-free levels. Error bars are the 95% CIs of the parameter estimates. (*B*) Number of cultures out of six that showed visible growth after 24 h of exposure to the indicated ampicillin concentration in the indicated growth medium. In both panels, asterisks over bars indicate significant differences between Gal and Man. *$P < 0.05$; ***$P < 0.001$.

Discussion

The highly reproducible extinction of Man+ and not Gal+ in our initial evolution experiment shows that, in a community initially protected by two species equally contributing to toxin removal, one species predictably evolved to be the sole helper (Fig. 1). We demonstrated that the two species paid an identical direct cost to express β-lactamase (Fig. 2*A*) but potentially paid significantly different opportunity costs due to different levels of leakiness and essentiality (Fig. 3) that affect the value Man+ and Gal+ receive in return for their investment in β-lactamase. These differences may explain the different slopes of negative frequency dependence we observed (Fig. 2*B*). Because Man is less sensitive to ampicillin (Fig. 3*B*), selection is stronger against Man+ than Gal+ when resistance is initially abundant. However, because Man is also less leaky than Gal (Fig. 3*A*), selection is stronger in favor of Man+ when it is initially rare, as each cell secretes fewer β-lactamase molecules into the medium than Gal+. However, this result alone cannot explain why Gal+ evolved to be the sole helper, as the two parameters support different outcomes: lower leakiness suggests Man+ receives

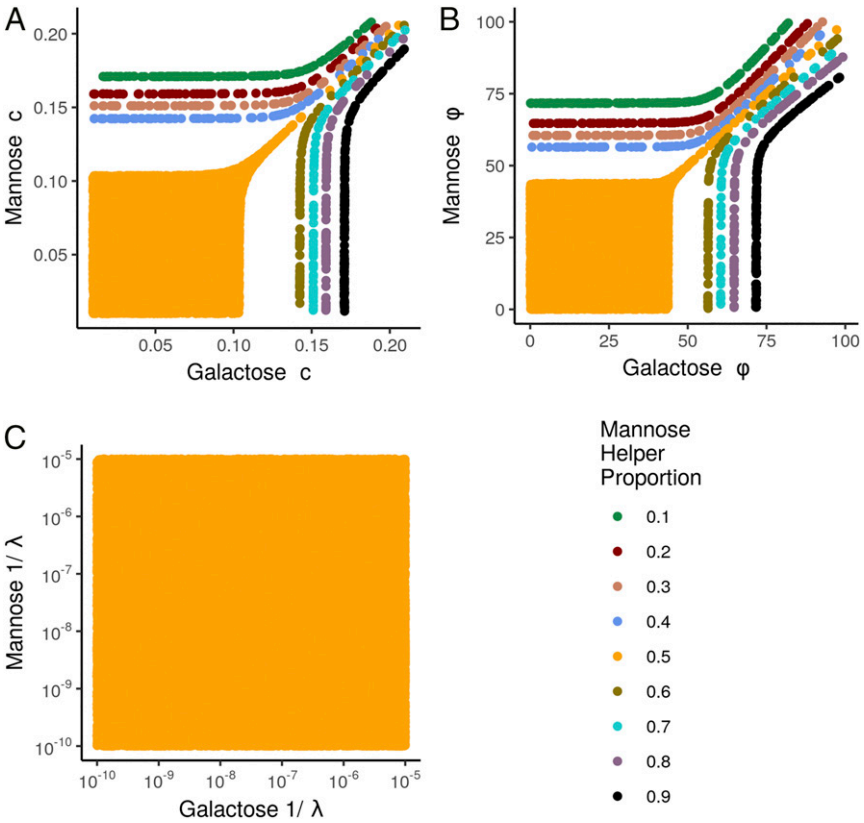

Fig. 4. Black Queen parameter effects on the helper species proportion landscape. Differences in cost (*A*) and essentiality (*B*) between the two species alter mannose helper proportions, while leakiness (*C*) does not. Data points represent the parameter selection for each species in each subplot, while the data point color shows the proportion of helpers that consume only mannose. Except when being varied in each subplot, all other parameters remain static (μ = 1.0, $\varepsilon = 0.4$, $f = 60$, $\lambda = 1 \times 10^7$, $\delta = 0.1$, $c = 0.15$, $k = 10$, $S_r = 5 \times 10^7$, $S_t = 1$).

more return on its AmpR investment, whereas lower essentiality suggests the opposite. Our modeling results suggest that we can eliminate leakiness from consideration; while it affects the total abundance of helpers, differential leakiness does not influence the ratio of helpers from different species within the model (Fig. 4). Only the cost of resistance and intrinsic resistance affect the relative abundance of helpers in each species in the model (Fig. 4), and since we demonstrated that cost of resistance is the same in Man+ and Gal+, we conclude that the difference between Man– and Gal– in terms of their intrinsic resistance is the most likely cause of the unexpected results from our evolution experiment.

The BQH describes an evolutionary dynamic wherein stable dependencies naturally arise for leaky functions (17, 18). Most modeling and experimental investigations of the BQH have focused on simple communities sustained on a single limiting resource, in which the negative frequency dependence conferred by the Black Queen function allows coexistence between helpers and beneficiaries in contradiction of the competitive exclusion principle (20, 21). However, one of the more interesting predictions of the BQH is that these same dynamics, integrated across a multispecies community, may result in species-level specialization on various leaky tasks, effectively providing an engine for the creation and maintenance of diversity and interdependence and providing one explanation for the classic "paradox of the plankton." Our earliest thinking about this dynamic suggested that the distribution of leaky functions would be largely random and determined by the chance loss of function in various taxa during a kinetic "race to the bottom," leaving a small number of unfortunate helpers behind to carry the burden alone. Under that hypothesis, one would predict large variations between spatially separated communities in terms of which taxa performed which functions. The experiments presented here suggest a very different possibility: that the roles played by taxa are much more deterministic, fixed by relatively subtle differences in physiology that impact the cost–benefit balance of expressing a leaky function.

The origin of the differences between the two species in our experiment is unclear. We designed the experiment to minimize the differences between the species; both were derived from the same *E. coli* strain with their sugar utilization phenotypes dictated by a single clean deletion of a gene at roughly the same point in their respective catabolic pathway (25). Nevertheless, there was a clear difference in both their intrinsic resistance to ampicillin and the leakiness of the β-lactamase enzyme. One possible explanation lies in the fact that the two sugars are utilized by fundamentally different pathways in *E. coli*; mannose is acquired via the phosphotransferase system (PTS) (specifically the mannose–fructose–sorbose family PTS) (34), whereas galactose uptake uses a singular permease. We know that while the mannose PTS is indeed a target for bacteriocins (34, 35) and is involved in catabolite control (36, 37), there is no evidence in the literature that it is involved in β-lactam susceptibility. On the other hand, a nucleotide substitution in another PTS transporter enzyme was shown to confer β-lactam tolerance in *Streptococcus gordonii* (38), and it is possible that a similar unselected mutation in the PTS (or indeed elsewhere in the genome) may underlie the differences between our strains. One major argument against this possibility, however, is that the sugar phenotypes were conferred by deletion of genes encoding cytoplasmic enzymes, and both Man and Gal likely expressed mannose and galactose transporter enzymes throughout the experiment.

Another possible explanation lies in the different growth rates of the two species (*SI Appendix*, Fig. 5*B*), although the exact mechanism by which growth rate could affect antibiotic resistance is not clear. Because ampicillin prevents the synthesis of new cell wall (39), it is expected to have increased lethality on faster-growing cells (40), but the faster-growing species, Man, was also the more resistant. In any case, it is not clear why the two species should have different growth rates even when growing in glucose media, in which their deletion mutations in galactose or mannose catabolism pathways should not be relevant. As mentioned in the previous paragraph, one likely explanation is that there are one or more unselected, unintended mutations in Gal that reduce its growth rate and/or produce increased ampicillin sensitivity. Indeed, a wide variety of mutations in genes with no obvious relationship to antibiotics can impact antibiotic sensitivity (41). The Keio knockout strains we utilized have not been resequenced, so such mutations would be unnoticed outside of their putative effect on the organism's phenotype.

Regardless of the explanation for these differences between Man and Gal, the fact that these subtle physiological differences between nearly identical species had such a strong impact on the outcome of our evolution experiment is striking. Our modeling results suggest that even small differences between species in terms of cost and resistance can greatly change the intraspecies competition between helper and beneficiary in a deterministic way and may ultimately contribute to the reproducible competitive exclusion of the helper phenotype in one species. If this dynamic accurately describes the evolution of natural microbial communities dependent on Black Queen functions, then it suggests that the distribution of Black Queen functions is both predictable and indeed equitable, as the taxa that gain the most individual benefit from expressing the function will tend to evolve to become providers for the entire community.

We have previously argued that leaky functions create a sort of metabolic marketplace in which organismal lineages can "decide" whether to perform leaky functions themselves or, rather, "purchase" their products from the environment (19). The currency employed in such a marketplace is, essentially, fitness, since resources expended performing functions create an opportunity cost because those same resources cannot also be used for growth and reproduction. Most theory to date exploring the BQH has focused on how this marketplace manifests in communities with a single limiting resource for which all individuals compete. To the extent that we have previously extrapolated Black Queen evolution to more complex communities, we have described it as a "race to the bottom," in which helper status merely reflects which taxa failed to acquire random loss-of-function mutations in the leaky genes before the community reached its equilibrium point (18). Our current results suggest that this perspective was incorrect and that, instead, helper status will be determined by which taxa have the greatest comparative advantage for performing a leaky function. In this way, the Black Queen marketplace resembles the world of international trade described by Ricardo (9), in which nations are encouraged to specialize in products for which they have a comparative advantage. According to Ricardo, these comparative advantages arise due to peculiarities of resource access and labor pools specific to different countries, not necessarily because of their specific skill in the industry in question. By analogy, the repeatable evolution of Man into an obligate beneficiary and Gal into a helper appears to have been caused by peculiarities of the two species' physiology leading to differential leakiness and antibiotic susceptibility. Importantly, this result was not caused by one of the species being a better helper, and indeed, our modeling results suggest that the degree of help provided (i.e., the leakiness, which is analogous to domestic industrial skill in Ricardo's example) was the one parameter that had no effect on determining which species became fixed as the helper.

In conclusion, we have shown that a simple multispecies community reproducibly evolved an obligate interspecies dependency, with the roles played by each species controlled by forces very similar to market forces regulating human economic behavior. It reveals a mechanism by which Black Queen evolution can generate and maintain complex interactions in microbial communities. Moreover, it shows how marketplace interactions between species can decisively impact intraspecies competition and highlights the importance of understanding community

context when analyzing microbial physiology and evolution. Finally, our experiment suggests that the logic of markets may be a universal structuring force across the tree of life and a primary way by which ordered social interactions may arise by the "invisible hand" of self-interest. Future work should not only investigate the impact of leaky functions on the structure of natural microbial communities but also whether leaky "positive externalities" of human activity exert a similar stabilizing force on cooperation and trade in our own species.

Data Availability. Raw experimental data have been deposited in Dryad (DOI: 10.5061/dryad.000000036) (42). Code used to generate the modeling results in Fig. 4 can be accessed at GitHub (https://github.com/SEpapoulis/MicrobialBlackQueenMarket) (32).

ACKNOWLEDGMENTS. We thank Robert Fillinger, Alex Durrant, and Neerja Hajela for laboratory assistance. This work was partially supported by a Simons Foundation Early Career Fellowship to J.J.M., two grants from the NSF BEACON Center (DBI-0939454) to J.J.M., and an NSF Graduate Research Fellowship (Grant No. 1450078) to S.J.A.-J.

1. R. Dawkins, *The Selfish Gene* (Oxford University Press, Oxford, 1976).
2. D. J. Rankin, K. Bargum, H. Kokko, The tragedy of the commons in evolutionary biology. *Trends Ecol. Evol.* **22**, 643–651 (2007).
3. S. A. West, A. S. Griffin, A. Gardner, S. P. Diggle, Social evolution theory for microorganisms. *Nat. Rev. Microbiol.* **4**, 597–607 (2006).
4. W. D. Hamilton, The genetical evolution of social behaviour. I. *J. Theor. Biol.* **7**, 1–16 (1964).
5. J. A. Fuhrman, J. A. Cram, D. M. Needham, Marine microbial community dynamics and their ecological interpretation. *Nat. Rev. Microbiol.* **13**, 133–146 (2015).
6. A. P. Melis, D. Semmann, How is human cooperation different? *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **365**, 2663–2674 (2010).
7. D. S. Wilson, J. M. Gowdy, Evolution as a general theoretical framework for economics and public policy. *J. Econ. Behav. Organ.* **90** (suppl.), S3–S10 (2013).
8. R. Noë, P. Hammerstein, Biological markets: Supply and demand determine the effect of partner choice in cooperation, mutualism and mating. *Behav. Ecol. Sociobiol.* **35**, 1–11 (1994).
9. D. Ricardo, *On the Principles of Political Economy and Taxation* (Batoche Books, Kitchener, Ontario, ed. 3, 1817).
10. M. W. Schwartz, J. D. Hoeksema, Specialization and resource trade: Biological markets as a model of mutualisms. *Ecology* **79**, 1029–1038 (1998).
11. P. Hammerstein, R. Noë, Biological trade and markets. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **371**, 20150101 (2016).
12. J. D. Hoeksema, M. W. Schwartz, Expanding comparative-advantage biological market models: Contingency of mutualism on partners' resource requirements and acquisition trade-offs. *Proc. Biol. Sci.* **270**, 913–919 (2003).
13. B. McGill, A mechanistic model of a mutualism and its ecological and evolutionary dynamics. *Ecol. Modell.* **187**, 413–425 (2005).
14. R. Noë, P. Hammerstein, Biological markets. *Trends Ecol. Evol.* **10**, 336–339 (1995).
15. G. D. A. Werner *et al*., Evolution of microbial markets. *Proc. Natl. Acad. Sci. U.S.A.* **111**, 1237–1244 (2014).
16. M. Archetti *et al*., Economic game theory for mutualism and cooperation. *Ecol. Lett.* **14**, 1300–1312 (2011).
17. J. J. Morris, R. E. Lenski, E. R. Zinser, The Black Queen Hypothesis: Evolution of dependencies through adaptive gene loss. *mBio* **3**, e00036-12 (2012).
18. J. J. Morris, Black Queen evolution: The role of leakiness in structuring microbial communities. *Trends Genet.* **31**, 475–482 (2015).
19. J. J. Morris, E. Schniter, Black Queen markets: Commensalism, dependency, and the evolution of cooperative specialization in human society. *J. Bioecon.* **20**, 69–105 (2017).
20. J. J. Morris, S. E. Papoulis, R. E. Lenski, Coexistence of evolving bacteria stabilized by a shared Black Queen function. *Evolution* **68**, 2960–2971 (2014).
21. S. Estrela, J. J. Morris, B. Kerr, Private benefits and metabolic conflicts shape the emergence of microbial interdependencies. *Environ. Microbiol.* **18**, 1415–1427. (2016).
22. A. Mas, S. Jamshidi, Y. Lagadeuc, D. Eveillard, P. Vandenkoornhuyse, Beyond the Black Queen hypothesis. *ISME J.* **10**, 2085–2091 (2016).
23. B. C. Carlton, B. J. Brown, "Gene mutation" in *Manual of Methods for General Bacteriology*, P. Gerhardt, Ed. (American Society for Microbiology, Washington, D.C., 1981), pp. 222–242.
24. C. B. Turner, B. D. Wade, J. R. Meyer, B. A. Sommerfeld, R. E. Lenski, Evolution of organismal stoichiometry in a long-term experiment with *Escherichia coli*. *R. Soc. Open Sci.* **4**, 170497 (2017).
25. T. Baba *et al*., Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: The Keio collection. *Mol. Syst. Biol.* **2**, 2006.0008 (2006).
26. D. G. Gibson *et al*., Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods* **6**, 343–345 (2009).
27. L. M. Guzman, D. Belin, M. J. Carson, J. Beckwith, Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. *J. Bacteriol.* **177**, 4121–4130 (1995).
28. R. E. Lenski, M. R. Rose, S. C. Simpson, S. C. Tadler, Long-term experimental evolution in *Escherichia coli*. I. Adaptation and divergence during 2,000 generations. *Am. Nat.* **138**, 1315–1341 (1991).
29. R. E. Lenski, S. E. Hattingh, Coexistence of two competitors on one resource and one inhibitor: A chemostat model based on bacteria and antibiotics. *J. Theor. Biol.* **122**, 83–93 (1986).
30. F. Vasi, M. Travisano, R. E. Lenski, Long-term experimental evolution in *Escherichia coli*. II. Changes in life-history traits during adaptation to a seasonal environment. *Am. Nat.* **144**, 432–456 (1994).
31. E. A. Yurtsev, A. Conwill, J. Gore, Oscillatory dynamics in a bacterial cross-protection mutualism. *Proc. Natl. Acad. Sci. U.S.A.* **113**, 6236–6241 (2016).
32. S. E. Papoulis, J. Morris, Market forces determine the distribution of a leaky function in a simple microbial community. GitHub. https://github.com/SEpapoulis/MicrobialBlackQueenMarket. Deposited 8 September 2021.
33. S. E. Papoulis, D. Talmy, ODElib. GitHub. https://github.com/SEpapoulis/ODElib. Deposited 8 June 2021.
34. X. Liu, J. Zeng, K. Huang, J. Wang, Structure of the mannose transporter of the bacterial phosphotransferase system. *Cell Res.* **29**, 680–682 (2019).
35. M. Kjos, I. F. Nes, D. B. Diep, Mechanisms of resistance to bacteriocins targeting the mannose phosphotransferase system. *Appl. Environ. Microbiol.* **77**, 3335–3342 (2011).
36. P. Kotrba, M. Inui, H. Yukawa, Bacterial phosphotransferase system (PTS) in carbohydrate uptake and control of carbon metabolism. *J. Biosci. Bioeng.* **92**, 502–517 (2001).
37. M. Opsata, I. F. Nes, H. Holo, Class IIa bacteriocin resistance in *Enterococcus faecalis* V583: The mannose PTS operon mediates global transcriptional responses. *BMC Microbiol.* **10**, 224 (2010).
38. A. Bizzini *et al*., A single mutation in enzyme I of the sugar phosphotransferase system confers penicillin tolerance to *Streptococcus gordonii*. *Antimicrob. Agents Chemother.* **54**, 259–266 (2010).
39. E. Tuomanen, A. Tomasz, Mechanism of phenotypic tolerance of nongrowing pneumococci to beta-lactam antibiotics. *Scand. J. Infect. Dis. Suppl.* **74**, 102–112 (1990).
40. E. Tuomanen, Phenotypic tolerance: The search for β-lactam antibiotics that kill nongrowing bacteria. *Rev. Infect. Dis.* **8** (suppl. 3), S279–S291 (1986).
41. O. Lamrabet, M. Martin, R. E. Lenski, D. Schneider, Changes in intrinsic antibiotic susceptibility during a long-term evolution experiment with *Escherichia coli*. *mBio* **10**, e00189-19 (2019).
42. J. Morris, S. Adkins-Jablonsky, C. Clark, M. Kuhl, S. E. Papoulis, Market forces determine the distribution of a leaky function in a simple microbial community. *Dryad*. https://doi.org/10.5061/dryad.000000036. Deposited 9 September 2021.