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Maintenance of microbial cooperation mediated by public goods in single and multiple traits scenarios

Özhan Özkaya[a,#], Karina B. Xavier[a], Francisco Dionisio[a,b] and Roberto Balbontín[a,#]

[a]Instituto Gulbenkian de Ciência, Oeiras, Portugal

[b]cE3c: Centre for Ecology, Evolution, and Environmental Changes, Faculty of Sciences, University of Lisbon, Edifício C2, 38 piso, 1749-016 Lisboa, Portugal

[#]Address correspondence to Roberto Balbontín (rbalbontin@igc.gulbenkian.pt) and Özhan Özkaya (oozkaya@igc.gulbenkian.pt)

ABSTRACT

Microbes often form densely populated communities, which favor competitive and cooperative interactions. Cooperation among bacteria often occurs through the production of metabolically costly molecules produced by certain individuals that become available to other neighboring individuals, called public goods. This type of cooperation is susceptible to exploitation, since non-producers of a public good can benefit from it while saving the cost of its production (cheating), gaining a fitness advantage over producers (cooperators). Thus, in mixed cultures, cheaters can increase in frequency in the population, relative to cooperators. Sometimes, and as predicted by simple game-theoretic arguments, such increase in the frequency of cheaters causes loss of the cooperative traits by exhaustion of the public goods, eventually leading to a collapse of the entire population. In other cases, however, both cooperators and cheaters remain in coexistence. This raises the question of how cooperation is maintained in microbial populations. Several strategies to prevent cheating have been described involving a single trait and a unique environmental constraint. In this review, we describe current knowledge on the evolutionary stability of microbial cooperation, discussing recent discoveries describing the mechanisms operating in multiple traits and multiple constraints settings. We conclude with a consideration of the consequences of these complex interactions, and we briefly discuss the potential role of social interactions

30 involving multiple traits and multiple environmental constraints in the evolution
31 of specialization and division of labor in microbes.

32 INTRODUCTION

33 Microbes often live in highly dense communities where interactions among
34 individuals are both inevitable and essential (1). In uni-directional interactions
35 between two individuals, the one which engages in an action can be defined as
36 the 'actor' and the one influenced by the effects of the action as the 'recipient'. If
37 a social behavior influences positively the fitness of the recipient regardless of
38 its fitness effect on the actor, it is considered a cooperative behavior (2, 3).
39 Cooperation in microbes often involves the production of public goods by the
40 actor (cooperator) which can benefit both the actor and the recipient. The nature
41 of these public goods and the mechanisms by which they affect the fitness are
42 diverse: materials to generate protective structures (e. g. exopolysaccharides to
43 generate biofilms), toxins to kill competitors, enzymes to digest food,
44 biosurfactants for group motility, proteins to detoxify the environment, molecules
45 to scavenge nutrients, etc. (4, 5).

46 Cooperation, however, can be vulnerable to exploitation, as individuals that are
47 not contributing to the cooperative behavior (cheaters) by not producing public
48 goods can benefit from them. Given that cheaters are not paying the cost but
49 benefit from the cooperative behavior, they gain a relative fitness advantage
50 over cooperators (2, 3). Thus, in mixed populations, cheaters can increase in

frequency, sometimes causing loss of cooperation and a decrease in population size. In other cases, however, there is maintenance of cooperation, even if there is a shift in the proportions of cooperators and cheaters in the population. The study of how cooperation is maintained in populations led to the identification of different mechanisms for protection against possible exploitations by cheaters (recently reviewed in 6).

MECHANISMS TO PREVENT CHEATING ON SINGLE TRAITS

Quorum sensing. Bacteria can produce, secrete and detect signaling molecules, called autoinducers, whose concentration in the medium increases as cells divide. When this concentration reaches a certain threshold, bacteria engage synchronously in a coordinated response. This process is called quorum sensing, since it enables bacteria to make coordinated 'decisions' depending on their '*quorum*' (7, 8, 9).

The expression of genes involved in the production of public goods is often controlled by quorum sensing (10, 11, 12, 13), which causes the public goods to be produced only when cooperative individuals are able to reach the quorum threshold. Thus, in this scenario, public goods production only occurs when cooperators are in greater numbers, precisely when cooperative behavior provides the greatest benefit: at high bacterial density, there is more efficient usage of public goods than at low bacterial density, where accessibility is lower (14). Therefore, via quorum sensing regulation, production of public goods is

restricted to the conditions where it is more productive for the producer cells, thus decreasing the chances for cheaters to outcompete cooperators and contributing to preventing the complete loss of the cooperative behavior (15) (**Fig. 1A**).

Protection of cooperation mediated by traits regulated by quorum sensing has been both inferred theoretically (16, 17) and observed experimentally (18, 19, 20, 21, 22). However, quorum sensing cheaters still arise. For example, it has been shown that mutants of *Pseudomonas aeruginosa* blind to the quorum-sensing signal can avoid the cost of producing the public good regulated by quorum sensing (23, 24, 25).

Bacteria, however, have mechanisms to avoid the spread of these types of mutants. An interesting example involves *Bacillus subtilis* (26). Like other bacterial species, wild populations of *B. subtillis* display a high degree of diversity in functional quorum-sensing alleles, called pherotypes. Each pherotype responds to an autoinducer similar to its own but not to autoinducers produced by other pherotypes. As such, a minority pherotype exploits the public goods produced by the majority of pherotypes (hence having the same fitness as obligatory cheaters) but resumes the production of the public good on its own when its density reaches a high value (26). Moreover, if cheaters take over a population by exploiting a public good regulated by quorum sensing, the decrease in the overall population density can go below the quorum threshold and that would cause the cooperators to seize the production of this public good

due to the quorum sensing regulation. In absence of the public good and with the cooperators saving the cost of its production, the fitness advantage of the cheaters would decrease and the population would stay in a polymorphic state. We recently provided experimental evidences of this; we showed that when cooperators are forced to produce constitutively the quorum sensing-regulated good (by adding exogenous quorum sensing signal), cheaters can invade the population to fixation, thereby causing full loss of cooperators and the subsequent loss of the public good trait (27). Thus, it can be said that the quorum sensing regulation of a public good trait can prevent the extinction of the cooperator phenotype in the population and preserve the genetic architecture of quorum sensing (21, 27).

Partial privatization. In contrast to public goods, private goods are only accessible to producers, making cheating practically impossible. Privatization of public goods is well documented in yeast (28, 29, 30). Invertase is an extracellular enzyme produced by *Saccharomyces cerevisiae* that hydrolyzes sucrose into glucose and fructose, which then can be used by the entire population. Thus, the products of invertase activity mostly function as public goods. However, invertase producers have a certain degree of priority to access the monosaccharides generated by the invertase activity, preventing cheating on this trait (**Fig. 1B**). Although 99% of the monosaccharides are accessible to the entire population and therefore can be used by cheaters, the 1% of

privatized monosaccharides accessible only to cooperators is enough to avoid cheaters to outcompete them. Even though a mutant strain that does not produce invertase is able to invade a population of invertase producers, the latter are also able to increase in frequency if initially at very low frequencies. This leads to a steady state coexistence of invertase producers and cheaters (29). Interestingly, there is also density dependence fitness. The fitness of mutants is lower when the total population density is low because there are not enough cells producing invertase (31). In other words, some goods are public if population density is high, but can only be used privately if density is low. The dependence of the fitness of cheaters on population density seems to simply result from the fact that, at higher densities, cheaters are closer to producers of the public good. This was demonstrated through mathematical models and experimental data obtained from *P. aeruginosa* producing iron-scavenging siderophore molecules as public goods (32). Other systems for which it has been shown that the fitness of cheaters depends on total population density include: the production of siderophore molecule enterochelin by *Escherichia coli* (33), the production of adhesive exopolysaccharides (EPS) in *B. subtilis* biofilms (34) and the synthesis of the enzyme β-lactamase, which degrades β-lactam antibiotics such as ampicillin, by *E. coli* (35). In the latter example, non-producers of β-lactamase may still survive in mixed populations at high cell density (when sensitive bacteria grow close to producers), showing that antibiotic resistance can also display positive density-dependence (35, 36, for a review about degradation and modification of antibiotics, see 37). These

examples, taken from very different systems, show that privatization of extracellular public goods is efficient at low, but not necessarily at high density. As a consequence, in these systems, usually neither producers nor cheaters reach fixation.

Although perhaps not perfect, in nature one finds examples of paths towards privatization. A very interestingly example is the molecular structure of iron-scavenging siderophores (38). Using data from 189 secreted siderophores (considering three different parameters for each enzyme) from 124 bacterial species, Kummerli *et al.* showed that their diffusivity inversely correlate with the structure level of the habitat they occupy. That is, highly diffusible siderophores are preferentially present in species living in structured habitats, such as soil and hosts, while poorly diffusible siderophores are mostly secreted by species from seawaters and other unstructured habitats.

Policing. Policing can be defined as an action that influences negatively the fitness advantage of cheaters, by either limiting the benefit that cheaters obtain from the cooperative behavior or by increasing the advantage that it provides to cooperators (39) (**Fig. 1C**). Several examples of policing against cheaters have been repeatedly observed in many eukaryotes (40, 41, 42, 43) and in bacteria (44, 45, 46). For instance, in *P. aeruginosa*, the production of cyanide and the resistance against it are coupled traits, both regulated by the RhlIR system (47, 48), which is in turn controlled by LasR (49, 11), the master regulator of quorum

161 sensing (50, 51, 52). LasR controls the production of many public goods,
162 including elastase (50, 51), a secreted metalloprotease capable of digesting
163 proteins, such as casein, into amino acids (53, 54). Therefore, in casein
164 medium, *lasR* mutants can cheat (24). However, mutations in *lasR* cause the
165 loss of cyanide production and, importantly, the resistance to it. Thus, LasR+
166 cells (cooperators) are immune to the cyanide they produce, whereas *lasR*
167 mutants (cheaters) are not. In that scenario, the coupling of cyanide immunity
168 with a public good production polices against cheater invasions, preventing the
169 collapse of the population and therefore protecting cooperation (45). Inter-
170 species policing, involving reciprocity as a punishing/rewarding behavior, have
171 been observed in polymicrobial communities (55) and in mutualistic
172 relationships between certain bacteria and their hosts, such as *Rhizobia*-plants
173 (56, 57) and *Vibrio fischeri*-squids (58, 59).

174

175 ***Spatial structure.*** Spatial structures, such as biofilms, favor related cells to
176 stay in close proximity and limit the diffusion of the public goods, increasing the
177 benefit for producers and diminishing the chances for cheating (60, 61) (**Fig.**
178 **1D**). Biofilms are one of the most common ways that bacteria generate spatial
179 structure (62). Lipids, polysaccharides, and nucleic acid molecules secreted by
180 bacteria function as an extracellular matrix (63) where the cells can anchor and
181 switch from motile to static phase (62). The matrix limits the diffusion of the
182 public goods secreted by cooperators (64, 65), making them to be less

accessible to cheaters, reducing their fitness advantage and therefore preventing cheating (66, 61, 64, 67). Interestingly, predictions based on computer simulations of multi-species biofilm formation showed that, when resources abound and therefore competitive effects are less severe, the production of public goods may be maintained in a focal species simply due to the mere presence of cells belonging to other species, which completely surround producers of the exoproduct. This results in the insulation of producers from non-producer cheater mutants of the same species, preventing intraspecific competition between the two social actors, therefore favouring within-species cooperation (68). Interestingly, biofilms often require the production of a costly exoproduct, namely EPS (adhesive exopolysaccharides), that can be a public good itself and, in thosee cases, is susceptible to exploitation (64). In other cases, however, similar but non-costly exoproducts involved in biofilms formation can be social but relatively nonexploitable (69).

A particular effect of spatial segregation can occur, for instance, in a population divided in a set of subgroups composed by mixtures of cooperators and non-cooperators in different proportions. If the cooperative behavior is costly, a decrease in the proportion of cooperators within each subgroup is expected. However, if the proportion of cooperators in each subgroups is sufficiently different, the overall proportion of cooperators in the whole population may increase. This is a statistical effect termed “Simpson’s paradox” after its discoverer, the statistician Edward H. Simpson (70). This effect was later shown by D. S. Wilson to be relevant for the problem of maintenance of cooperation

206 (71, 72). The reason for the increase in the proportion of cooperators in the
207 overall population is that subgroups with more cooperators are also more
208 productive, therefore resulting in higher cell yields. Interestingly, this effect is in
209 fact a direct consequence of kin selection (selection of a costly trait due to
210 increased fitness of individuals genetically related to those possessing that
211 trait), as both kin selection and group selection (selection at the level of group
212 instead of at level of the individual) can be shown to be mathematically
213 equivalent (73). If, from time to time, subpopulations join and then separate
214 again, there should be a mechanism assuring a certain variance of cooperators
215 among subgroups for the proportion of cooperators to continue increasing (71).
216 This phenomenon was experimentally observed in microbial communities (74,
217 75). Griffin *et al.* showed that if competition is local, the higher productivity of
218 groups with more cooperators is (exactly) compensated by a stronger
219 competition between kin individuals (74, 76, 77, 78, 2). Therefore, this effect is
220 mostly observed if competition is global.

221

222 ***Facultative cooperation.*** Another bacterial mechanism to overcome
223 cheating is facultative cooperation, which can operate through metabolic
224 prudence (79). *P. aeruginosa* can produce high levels of rhamnolipids (80), a
225 carbon-rich surface polymer, which enables bacteria swarming to colonize new
226 niches (81, 82). Xavier and colleagues showed that this bacterium only invests
227 in rhamnolipid production under conditions of metabolic unbalance, i. e. when

carbon concentration is higher than nitrogen concentrations. Bacteria require both carbon and nitrogen to divide, whereas the production of rhamnolipids requires carbon but not nitrogen. Thus, by investing in rhamnolipid production only when the C/N ratio does not favor cell division, bacteria minimize the cost of this public good under conditions of strong competition, preventing cooperators to be outcompeted by non-producers (cheaters). Under nitrogen deprivation, the induction of rhamnolipid production allows bacteria to swarm away towards areas that might contain both nitrogen and carbon, where bacteria can start dividing again. Thus, facultative induction of cooperative behaviors reduces the chances of cheaters to invade bacterial populations of producers (79). Xavier *et al.* showed that, if production is constitutive, maintenance of cooperation would be possible only if producers of rhamnolipid were mostly surrounded by other producers of rhamnolipid, a very stringent situation. By facultatively producing rhamnolipid, however, producers strongly decrease the cost of its production, what allows the maintenance of cooperation even if producers and non-producers are mixed (83) (**Fig. 1E**). Another mechanism of facultative cooperation involves the production of a public good molecule (in this case, the siderophore pyoverdine in *P. aeruginosa*) during exponential phase, but not at stationary phase (84). Interestingly, production of this type of reusable public good molecules with long durability only when necessary is also a way of diminishing their production cost (85).

Antagonistic pleiotropy. Antagonistic pleiotropy can also contribute to stabilize cooperation (86). Indeed, some mutations that cause individuals to become cheaters (by ceasing to cooperate with other individuals), have pleiotropic effects and these mutants also become unable to perform a private behavior or to accomplish a fundamental metabolic step (87). Therefore, the pleiotropic effects of these mutations result in a direct fitness cost to these cheaters (**Fig. 1F**). Antagonistic pleiotropy has been observed in different systems, including instances in which cooperation is mediated by public goods. In these cases, the production of public and private goods is coupled (88). For instance, LasR regulates the production of various public and private goods in *P. aeruginosa* (10, 11, 12, 13). As mentioned before, one of the LasR-regulated public goods is elastase (50, 51). Thus, when casein is the sole carbon source, *lasR* mutants can behave as cheaters (24). However, *lasR* also controls the production of the enzyme necessary to digest adenosine (89, 90). In contrast to elastase, adenosine is maintained intracellularly, thus acting as a private good. When casein and adenosine are the only carbon sources available, cooperators (LasR+ cells) can use both while *lasR* mutants (cheaters) can only use the casamino acids generated by elastase activity, and not adenosine (90). The extra carbon source accessible to the cooperators provides them with an advantage over the cheaters, thus protecting the population against cheater expansion (88, 91).

Influence of phenotypic heterogeneity on cooperation. In certain cases, the different social roles involved in cooperation can be played by genotypically identical strains that differentiate phenotypically into functionally diverse social actors. Phenotypic differentiation in distinct cell types interacting cooperatively protects the population against invasion by cheaters for any of the functions exerted by the different cell types, since each cell type saves the costs of producing the public goods generated by the other cell types, and this reduces the competitive advantage of cheaters for either of the traits. This type of interactions has been observed in biofilms of *Myxococcus xanthus*, *B. subtilis*, and *P. aeruginosa* (92). A similar example of cooperation mediated by phenotypic heterogeneity was described in *B. subtilis* sliding motility, where the division of labor of two cell types (producers of either surfactin or extracellular matrix) allows a special structural organization that increases the rate of colony expansion, generating a strong ecological advantage and protecting the bacterial population against non-producers of either surfactin or matrix (93). There are also examples of cooperation mediated by phenotypic variation in host-pathogen interactions. For instance, the bistable expression of virulence determinants during infection of the mice gut by *Samonella* Typhimurium protects the bacterial population against the invasion by avirulent mutants, thereby promoting the evolutionary stability of virulence (94).

COOPERATION AND CHEATING IN MULTIPLE TRAITS SCENARIOS

The mechanisms described above allow maintenance of cooperation and prevent cheating in uni-directional interactions, involving a single trait where the actors are either full cooperators or full cheaters for that trait. However, populations are often polymorphic and rely on more than one trait and, typically, diverse environmental constraints simultaneously affect social interactions among individuals. Thus, in natural settings, the most prevalent scenario is possibly that of multi-directional interactions under multiple environmental constraints. In these 'multiple-traits / multiple-constraints' settings, an individual can be "actor" for some traits and "recipient" for others, thus behaving simultaneously as a cooperator and as a cheater for different traits (95). Thus, in nature, interactions among different players can affect their social roles, leading to complex dynamics where the notions of "cooperation" and "cheating" are context dependent. An analogy to illustrate this concept could be a hypothetical study of a hunter/gatherer society by an anthropologist. In such populations, both hunting and gathering vegetables can be considered cooperative behaviors. Hunters are "actors" for meat obtention and "recipients" for vegetable collection, whereas gatherers are "actors" for vegetable obtention and "recipients" for meat acquisition. Thus the population, which requires both meat and vegetables, relies on this mutualistic interaction for its sustainment. If the anthropologist would study exclusively hunting as a cooperative trait, the consequence would be considering hunters as cooperators and gatherers as cheaters, since the latter do not hunt but eat the meat obtained by the hunters. And *vice versa*, if the anthropologist would focus only on gathering, the

consequence would be classifying hunters as cheaters, because they are not gathering but still benefit from it. To understand the social roles of both hunters and gatherers, the anthropologist would need to consider simultaneously the two players and the two traits involved.

Similarly, in order to understand how cheating affects the dynamics of other biological systems involving multiple traits, the complex web of inter-species and inter-strain interactions must be addressed. There has been a growing literature focusing on the inter-strain and inter-species interactions in these communities. Brown and Taylor (95) established a theoretical framework for the evolution of joint social traits. They showed that, if two social traits interact, newly arising behavior not anticipated just by considering each trait alone, can occur. For example, policing mechanisms to refrain rivalry (within-groups competitions) (96) or excludability (through privatization) (97, 98) are mostly selected when relatedness is low. Relatedness is low when the probability that a cheater interacts with a cooperator is almost as high as the probability that a cooperator interacts with a cooperator. If a policing mechanism is effectively decreasing rivalry within groups of individuals, conditions may be favorable even when relatedness is low (95).

Cross-feeding is a relationship wherein one genotype consumes products of another (unidirectional cross-feeding). However, in some cases, cross-feeding can also involve interactions where more than one trait is involved. This can

339 occur when the consumer genotype also produces a metabolite useful for the
340 producer (bidirectional cross-feeding) (99). Therefore, bidirectional cross-
341 feeding is an example of multiple-traits / multiple-constraints interaction and,
342 under certain circumstances, it can influence cooperation and diversity. For
343 instance, a set of mathematical models, computer simulations and experiments
344 involving either a community formed by genetically engineered *Saccharomyces*
345 *cerevisiae* cooperating through metabolite-exchange or biofilms in which the
346 methane-producing archaeon *Methanococcus maripaludis* cooperates with the
347 bacterium *Desulfovibrio vulgaris* showed that, if the interaction between two
348 species confers a strong benefit to both species, they intermix and there is
349 cooperation between them. The intermix occurs by forming alternative layers.
350 This pattern was confirmed using two different synthetic systems composed by
351 two microbial species, but simulations predict the same intermixed pattern if
352 several species strongly interact (100). Interestingly, with such spatial structure,
353 there is maintenance of cooperation despite the appearance of cheater
354 mutants. The reason is that, the less cheater cells contribute to the other
355 partner, the more it were disfavored and eliminated (101). In another work,
356 computer simulations showed that the stronger is the interdependence between
357 interacting species, the higher is the number of mutualisms and community
358 productivity. If, in contrast, interdependence is low, conflict takes over and
359 interacting species tend to separate (102).
360 In these cases of strong interdependence upon the settlement of mutual
361 interchange of metabolites, one of the partners may lose biosynthetic functions,

thereby threatening cooperation. Waite and Shou used engineered *S. cerevisiae* to recreate such scenario and observed that an adaptive race of cooperators and cheaters may give the opportunity to cooperators to defeat cheaters (103). However, using a similar system in *E. coli*, D'Souza and Kost showed that the loss of metabolic functions is strongly selected for when the corresponding metabolites can be extracted from the environment (104, 105, 106), in particular from cross-feeding among coexisting strains (104). Thus, the development of strong interdependences can destabilize cooperation in bidirectional cross-feeding scenarios. However, cooperation mediated by bidirectional cross-feeding can be protected through different mechanisms, as it is well illustrated in two recent papers. Germerodt *et al.* used a cellular automaton to simulate a structured habitat where there were interactions between six experimentally characterized genotypes. These genotypes differ in their ability and propensity to produce amino-acids. Upon varying several ecological parameters, the authors concluded that, in most parameters sets, obligate cross-feeders arise. Moreover, the presence of cross-feeders helped to stabilize genotypic diversity, while supplementing the system with required metabolites significantly reduced it (107). In a second recent study, experiments performed with two bacterial species, *Acinetobacter baylyi* and *E. coli,* able to exchange amino-acids showed that, in a liquid environment, non-cooperating strains were favored. However, in a structured environment, the authors observed that auxotrophs separate from cross-feeders, hence allowing for the stabilization of cooperative cross-feeding (108).

385

386 Recently, there has been an increasing number of studies addressing bacterial
387 interactions in multiple-traits / multiple-constraints scenarios other than
388 bidirectional cross feeding. Ross-Gillespie and colleagues (109) generated
389 general predictions for different settings involving two traits, and tested these
390 predictions by analyzing the evolutionary trajectories of production of two public
391 goods genetically and functionally interlinked (the siderophores pyoverdine and
392 pyochelin) by wild-type *P. aeruginosa* during experimental evolution under
393 different levels of iron limitation (110, 111, 112). Under strong iron deprivation,
394 both non-producers of pyoverdine and pyochelin can act as cheaters (74, 109).
395 Because, under these conditions, pyoverdine-defective mutants have a larger
396 relative fitness, a gradual decrease in pyoverdine production was observed,
397 concomitant with an increase in pyochelin production. This indicates the
398 appearance of pyoverdin non-producing cheaters. In contrast, although
399 production of the two siderophores decreases over time under moderate iron
400 limitation (where only pyochelin mutants are able to cheat), the drop is stronger
401 in the case of pyochelin, revealing the rise of mutants on its production (109).
402 Thus, the work by Ross-Gillespie *et al.* suggests that regulatory cross-link
403 between two traits may help to stabilize cooperation as pyoverdine-defective
404 cheaters become pyochelin-producing cooperators, which reduces their relative
405 advantage (Fig. 2A).

406 Inglis and colleagues (113) used mathematical models and experimental
407 communities of *P. aeruginosa* to demonstrate that the presence of a loner strain
408 (non-producing the main public good but producing a functionally linked one
409 which is less efficient) can lead to rock-paper-scissor dynamics in which
410 cooperators outcompete loners, cheaters outcompete cooperators and loners
411 outcompete cheaters (**Fig. 2B**). In this scenario, the presence of the loner
412 protects cooperation in environments that would otherwise favor cheating and
413 reduction in diversity, such as well-mixed communities (113).

414 Recently, Popat and colleagues (114) explored the interaction between two
415 traits not linked at the regulatory level but connected functionally: production of
416 the quorum sensing molecule *Pseudomonas* Quinolone Signal (PQS) (115) and
417 production of siderophores in *P. aeruginosa*. PQS is a powerful iron chelator
418 that can act as an iron trap (116), decreasing iron availability and therefore
419 increasing the relative fitness of siderophore-defective cheaters. Thus, Popat *et*
420 *al.* showed that production of one social trait may indirectly affect the costs and
421 benefits of another social trait, influencing the social behavior of cheaters for the
422 latter (**Fig. 2C**). The authors speculate that similar mechanisms might have the
423 potential to contribute to preserving cooperation (114).

424 Using a novel tri-partite co-culture system involving three social actors (wild-
425 type *P. aeruginosa* as full cooperator, and *lasR* and *pvdS* mutants, unable to
426 produce elastase and the siderophore pyoverdine, respectively, as
427 cheaters/partial cooperators) in single- or double-constraint environments

(presence of casein as sole carbon source and/or iron limitation), we studied the consequences of the interactions between two regulatorily and functionally independent social traits (27). When the two constraints are imposed (presence of casein as sole carbon source and iron limitation), the *lasR* mutant is a cheater for elastase but a cooperator for pyoverdine, whereas the *pvdS* mutant cooperates for elastase production and is a cheater for pyoverdine production. We observed that, under these conditions, the advantage of the *pvdS* mutant for not producing pyoverdine is higher than that of *lasR* for not producing elastase. As a consequence of the different costs associated with the different traits, in 3-way competitions, *pvdS* causes the cessation of the cheating advantage by *lasR* mutants and dominates the population. This domination of *pvdS* prevents the drastic population collapse otherwise caused by the invasion of *lasR* mutants, which occurs if *pvdS* is not present or in a single-constraint (casein as a sole carbon source) environment. Thus, this two-traits / two-constraints system allowed us to unveil the existence of strong context dependent ecological interactions between cheaters for orthogonal (i. e. independent) social traits, which can induce or prevent a drastic collapse in population fitness (helping to stabilize/destabilize cooperation) (**Fig. 2D**). We also developed a mathematical model to determine the universal factors governing these social interactions. The model showed that social dynamics in multiple-traits / multiple-constraints systems are determined mainly by the differences between the costs of the social traits involved, whereas their benefits only affect population yields. Therefore, the degree of the population

collapse induced upon cheaters invasion depends on the benefit provided by the most costly trait. This work highlights the importance of the relative costs and benefits of the different cooperative traits in predicting the outcome for populations in complex environments where multiple traits are required (27).

CHEATING ON CHEATERS: A STEP TOWARDS THE DIVISION OF LABOR?

Cooperative interactions in multiple-traits / multiple-constraints scenarios tend to generate polymorphism and potentially lead to the evolution of functional dependency, as formulated in the Black Queen Hypothesis (117, 118). Moreover, the simultaneous role of different social actors as cooperators and cheaters, which generate the establishment of these inter-dependent interactions, could be considered as a germinal stage in the path towards functional specialization (119). Thus, social interactions in multiple-traits / multiple-constraints microbial communities can function as an intermediate phase towards the division of labor (120, 121, 122). Interestingly, Hammerschmidt *et al.* showed that, in spatially structured microcosms inhabited by “wrinkly spreader” cooperators and “smooth” cheaters of *Pseudomonas fluorescens,* the latter can function as a germ line that facilitates the reproduction of bacterial populations as collectives (123), describing a mechanism for the role of cheaters in the evolution of multicellularity.

472 However, Oliveira and colleagues (124) proposed the existence of certain
473 limitations for cooperation for multiple traits. Through mathematical modeling,
474 they inferred that this type of cooperation evolves under conditions of
475 intermediate genetic mixing. They inferred that the evolution of inter-dependent
476 cooperative exchanges of multiple public goods reduces the overall productivity
477 of the community with respect to genotypes able to produce all the public goods
478 autonomously (124). Thus, there seem to be constraints to the evolution of
479 inter-dependent cooperation for multiple traits in bacterial communities.
480 Interestingly, this model does not consider situations where production of public
481 goods vary in the cost and benefits and, as discussed above, these factors can
482 change dramatically the outcome of social interactions involving multiple traits
483 (27). Additional experimental and theoretical research will help to clarify the
484 extension and limits of social interactions involving multiple traits and multiple
485 constraints in microbial communities.

486 The exploration of these thrilling research avenues will significantly contribute to
487 revealing the complex eco-evolutionary implications of social relationships in the
488 bacterial world. However, the information obtained from the study of these
489 interactions can be considered in a broader perspective. For instance, certain
490 theories in economics, such as ”comparative advantage”, which describes how
491 individuals, companies, or countries can save time and energy by specializing in
492 the production of one trait while obtaining others by exchanging with different
493 partners (125), are currently being tested in microbial systems (126, 127). Thus,
494 sociomicrobiology not only might help to address biomedical threats (128) but,

495 through these interdisciplinary approaches, might also contribute to further
496 understand socioeconomic challenges.

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512 **REFERENCES**

1. Reissig JL. 1977. Microbial Interactions. Springer USA.

2. West SA, Pen I, Griffin AS. 2002. Cooperation and competition between relatives. *Science* 296:72–75.

3. Nowak MA. 2006. Five rules for the evolution of cooperation. *Science* 314:1560–1563.

4. Crespi BJ. 2001. The evolution of social behavior in microorganisms. *Trends Ecol Evol* 16:178–183.

5. Velicer GJ. 2003. Social strife in the microbial world. *Trends Microbiol* 11:330–337.

6. Bruger E, Waters C. 2015. Sharing the sandbox: Evolutionary mechanisms that maintain bacterial cooperation. *F1000Research* 4 (F1000 Faculty Rev):1504 (doi: 10.12688/f1000research.7363.1).

7. Fuqua WC, Winans SC, Greenberg EP. 1994. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J Bacteriol* 176:269–275.

8. Bassler BL. 2002. Small talk. Cell-to-cell communication in bacteria. *Cell* 109:421–424.

9. Jimenez PN, Koch G, Thompson JA, Xavier KB, Cool RH, Quax WJ. 2012. The multiple signaling systems regulating virulence in *Pseudomonas aeruginosa. Microbiol Mol Biol Rev* 76:46–65.

10. Whiteley M, Lee KM, Greenberg EP. 1999. Identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa. Proc Natl Acad Sci U S A* 96:13904–13909.

11. Schuster M, Lostroh CP, Ogi T, Greenberg EP. 2003. Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. *J Bacteriol* 185:2066–2079.

12. Wagner VE, Bushnell D, Passador L, Brooks AI, Iglewski BH. 2003. Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. *J Bacteriol* 185:2080–2095.

13. Arevalo-Ferro C, Hentzer M, Reil G, Görg A, Kjelleberg S, Givskov M, Riedel K, Eberl L. 2003. Identification of quorum-sensing regulated

proteins in the opportunistic pathogen *Pseudomonas aeruginosa* by proteomics. *Environ Microbiol* 5:1350–1369.

14. Darch SE, West SA, Winzer K, Diggle SP. 2012. Density-dependent fitness benefits in quorum-sensing bacterial populations. *Proc Natl Acad Sci U S A* 109:8259–8263.

15. Asfahl KL, Schuster M. 2017. Social interactions in bacterial cell-cell signaling. *FEMS Microbiol Rev* 41:92–107.

16. Czárán T, Hoekstra RF. 2009. Microbial communication, cooperation and cheating: quorum sensing drives the evolution of cooperation in bacteria. *PloS One* 4:e6655.

17. Cornforth DM, Sumpter DJT, Brown SP, Brännström Å. 2012. Synergy and group size in microbial cooperation. *Am Nat* 180:296–305.

18. Nadell CD, Bassler BL. 2011. A fitness trade-off between local competition and dispersal in *Vibrio cholerae* biofilms. *Proc Natl Acad Sci U S A* 108:14181–14185.

19. Köhler T, Perron GG, Buckling A, van Delden C. 2010. Quorum sensing inhibition selects for virulence and cooperation in *Pseudomonas aeruginosa. PLoS Pathog* 6:e1000883.

20. Gupta R, Schuster M. 2013. Negative regulation of bacterial quorum sensing tunes public goods cooperation. *ISME J* 7:2159–2168.

21. Allen RC, McNally L, Popat R, Brown SP. 2016. Quorum sensing protects bacterial co-operation from exploitation by cheats. *ISME J* 10:1706–1716.

22. Bruger EL, Waters CM. 2016. Bacterial Quorum Sensing Stabilizes Cooperation by Optimizing Growth Strategies. *Appl Environ Microbiol* 82:6498–6506.

23. Diggle SP, Griffin AS, Campbell GS, West SA. 2007. Cooperation and conflict in quorum-sensing bacterial populations. *Nature* 450:411–414.

24. Sandoz KM, Mitzimberg SM, Schuster M. 2007. Social cheating in *Pseudomonas aeruginosa* quorum sensing. *Proc Natl Acad Sci U S A* 104:15876–15881.

25. Wilder CN, Diggle SP, Schuster M. 2011. Cooperation and cheating in *Pseudomonas aeruginosa*: the roles of the *las*, *rhl* and *pqs* quorum-sensing systems. *ISME J* 5:1332–1343.

26. Pollak S, Omer-Bendori S, Even-Tov E, Lipsman V, Bareia T, Ben-Zion I, Eldar A. 2016. Facultative cheating supports the coexistence of diverse quorum-sensing alleles. *Proc Natl Acad Sci U S A* 113:2152–2157.

27. Özhan Özkaya, Roberto Balbontín, Isabel Gordo and Karina B. Xavier. Cheating on orthogonal social traits prevents the tragedy of the commons in *Pseudomonas aeruginosa*. *bioRxiv*. http://dx.doi.org/10.1101/118240.

28. Craig Maclean R, Brandon C. 2008. Stable public goods cooperation and dynamic social interactions in yeast. *J Evol Biol* 21:1836–1843.

29. Gore J, Youk H, van Oudenaarden A. 2009. Snowdrift game dynamics and facultative cheating in yeast. *Nature* 459:253–256.

30. Koschwanez JH, Foster KR, Murray AW. 2013. Improved use of a public good selects for the evolution of undifferentiated multicellularity. *eLife* 2:e00367.

31. Greig D, Travisano M. 2004. The Prisoner's Dilemma and polymorphism in yeast *SUC* genes. *Proc Biol Sci* 271 Suppl 3:S25-26.

32. Ross-Gillespie A, Gardner A, Buckling A, West SA, Griffin AS. 2009. Density dependence and cooperation: theory and a test with bacteria. *Evol Int J Org Evol* 63:2315–2325.

33. Scholz RL, Greenberg EP. 2015. Sociality in *Escherichia coli*: Enterochelin Is a Private Good at Low Cell Density and Can Be Shared at High Cell Density. *J Bacteriol* 197:2122–2128.

34. van Gestel J, Weissing FJ, Kuipers OP, Kovács AT. 2014. Density of founder cells affects spatial pattern formation and cooperation in *Bacillus subtilis* biofilms. *ISME J* 8:2069–2079.

35. Domingues IL, Gama JA, Carvalho LM, Dionisio F. 2017. Social behaviour involving drug resistance: the role of initial density, initial frequency and population structure in shaping the effect of antibiotic resistance as a public good. Heredity advance online publication 21 June 2017; doi: 10.1038/hdy.2017.33.

36. Meredith HR, Srimani JK, Lee AJ, Lopatkin AJ, You L. 2015. Collective antibiotic tolerance: mechanisms, dynamics and intervention. *Nat Chem Biol* 11:182–188.

37. Wright GD. 2005. Bacterial resistance to antibiotics: enzymatic degradation and modification. *Adv Drug Deliv Rev* 57:1451–1470.

38. Kümmerli R, Schiessl KT, Waldvogel T, McNeill K, Ackermann M. 2014. Habitat structure and the evolution of diffusible siderophores in bacteria. *Ecol Lett* 17:1536–1544.

39. Travisano M, Velicer GJ. 2004. Strategies of microbial cheater control. *Trends Microbiol* 12:72–78.

40. Clutton-Brock TH, Parker GA. 1995. Punishment in animal societies. *Nature* 373:209–216.

41. Foster KR, Ratnieks FL. 2000. Facultative worker policing in a wasp. *Nature* 407:692–693.

42. Foster KR, Ratnieks FL. 2001. Convergent evolution of worker policing by egg eating in the honeybee and common wasp. *Proc Biol Sci* 268:169–174.

43. Kümmerli R. 2011. A test of evolutionary policing theory with data from human societies. *PloS One* 6:e24350.

44. Manhes P, Velicer GJ. 2011. Experimental evolution of selfish policing in social bacteria. *Proc Natl Acad Sci U S A* 108:8357–8362.

45. Wang M, Schaefer AL, Dandekar AA, Greenberg EP. 2015. Quorum sensing and policing of *Pseudomonas aeruginosa* social cheaters. *Proc Natl Acad Sci U S A* 112:2187–2191.

46. Tannières M, Lang J, Barnier C, Shykoff JA, Faure D. 2017. Quorum-quenching limits quorum-sensing exploitation by signal-negative invaders. *Sci Rep* 7:40126.

47. Pearson JP, Passador L, Iglewski BH, Greenberg EP. 1995. A second N-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* 92:1490–1494.

48. Brint JM, Ohman DE. 1995. Synthesis of multiple exoproducts in *Pseudomonas aeruginosa* is under the control of RhlR-RhlI, another set of regulators in strain PAO1 with homology to the autoinducer-responsive LuxR-LuxI family. *J Bacteriol* 177:7155–7163.

49. Pesci EC, Pearson JP, Seed PC, Iglewski BH. 1997. Regulation of *las* and *rhl* quorum sensing in *Pseudomonas aeruginosa. J Bacteriol* 179:3127–3132.

50. Gambello MJ, Iglewski BH. 1991. Cloning and characterization of the Pseudomonas aeruginosa lasR gene, a transcriptional activator of elastase expression. *J Bacteriol* 173:3000–3009.

51. Passador L, Cook JM, Gambello MJ, Rust L, Iglewski BH. 1993. Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. *Science* 260:1127–1130.

52. Pearson JP, Gray KM, Passador L, Tucker KD, Eberhard A, Iglewski BH, Greenberg EP. 1994. Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. *Proc Natl Acad Sci U S A* 91:197–201.

53. Morihara K. 1964. Production of elastase and proteinase by *Pseudomonas aeruginosa*. *J Bacteriol* 88:745–757.

54. Schad PA, Bever RA, Nicas TI, Leduc F, Hanne LF, Iglewski BH. 1987. Cloning and characterization of elastase genes from *Pseudomonas aeruginosa. J Bacteriol* 169:2691–2696.

55. Estrela S, Trisos CH, Brown SP. 2012. From metabolism to ecology: cross-feeding interactions shape the balance between polymicrobial conflict and mutualism. *Am Nat* 180:566–576.

56. West SA, Kiers ET, Simms EL, Denison RF. 2002. Sanctions and mutualism stability: why do rhizobia fix nitrogen? *Proc Biol Sci* 269:685–694.

57. Kiers ET, Rousseau RA, West SA, Denison RF. 2003. Host sanctions and the legume-rhizobium mutualism. *Nature* 425:78–81.

58. Lee KH, Ruby EG. 1994. Effect of the Squid Host on the Abundance and Distribution of Symbiotic *Vibrio fischeri* in Nature. *Appl Environ Microbiol* 60:1565–1571.

59. Koch EJ, Miyashiro T, McFall-Ngai MJ, Ruby EG. 2014. Features governing symbiont persistence in the squid-vibrio association. *Mol Ecol* 23:1624–1634.

60. Lion S, Baalen M van. 2008. Self-structuring in spatial evolutionary ecology. *Ecol Lett* 11:277–295.

61. Kümmerli R, Griffin AS, West SA, Buckling A, Harrison F. 2009. Viscous medium promotes cooperation in the pathogenic bacterium *Pseudomonas aeruginosa. Proc Biol Sci* 276:3531–3538.

62. López D, Vlamakis H, Kolter R. 2010. Biofilms. *Cold Spring Harb Perspect Biol* 2:a000398.

63. Dragoš A, Kovács ÁT. 2017. The Peculiar Functions of the Bacterial Extracellular Matrix. *Trends Microbiol* 25:257–266.

64. Drescher K, Nadell CD, Stone HA, Wingreen NS, Bassler BL. 2014. Solutions to the public goods dilemma in bacterial biofilms. *Curr Biol* 24:50–55.

65. Persat A, Nadell CD, Kim MK, Ingremeau F, Siryaporn A, Drescher K, Wingreen NS, Bassler BL, Gitai Z, Stone HA. 2015. The mechanical world of bacteria. *Cell* 161:988–997.

66. Kreft J-U. 2004. Biofilms promote altruism. *Microbiol Read Engl* 150:2751–2760.

67. Schluter J, Nadell CD, Bassler BL, Foster KR. 2015. Adhesion as a weapon in microbial competition. *ISME J* 9:139–149.

68. Mitri S, Xavier JB, Foster KR. 2011. Social evolution in multispecies biofilms. *Proc Natl Acad Sci U S A* 108 Suppl 2:10839–10846.

69. Irie Y, Roberts AEL, Kragh KN, Gordon VD, Hutchison J, Allen RJ, Melaugh G, Bjarnsholt T, West SA, Diggle SP. 2017. The *Pseudomonas*

aeruginosa PSL Polysaccharide Is a Social but Noncheatable Trait in Biofilms. m*Bio* vol. 8. no. 3 e00374-17.

70. Simpson EH. 1951. The Interpretation of Interaction in Contingency Tables. *J R Stat Soc Ser B Methodol* 13:238–241.

71. Wilson DS. 1975. A theory of group selection. *Proc Natl Acad Sci U S A* 72:143–146.

72. Price GR. 1970. Selection and covariance. *Nature* 227:520–521.

73. Lehmann L, Keller L, West S, Roze D. 2007. Group selection and kin selection: two concepts but one process. *Proc Natl Acad Sci U S A* 104:6736–6739.

74. Griffin AS, West SA, Buckling A. 2004. Cooperation and competition in pathogenic bacteria. *Nature* 430:1024–1027.

75. Chuang JS, Rivoire O, Leibler S. 2009. Simpson's paradox in a synthetic microbial system. *Science* 323:272–275.

76. Wilson DS, Pollock GB, Dugatkin LA. 1992. Can altruism evolve in purely viscous populations? *Evol Ecol* 6:331–341.

77. Taylor PD. 1992. Inclusive Fitness in a Homogeneous Environment. *Proc R Soc Lond B Biol Sci* 249:299–302.

78. Queller DC. 1994. Genetic relatedness in viscous populations. *Evol Ecol* 8:70–73.

79. Xavier JB, Kim W, Foster KR. 2011. A molecular mechanism that stabilizes cooperative secretions in *Pseudomonas aeruginosa. Mol Microbiol* 79:166–179.

80. Johnson MK, Boese-Marrazzo D. 1980. Production and properties of heat-stable extracellular hemolysin from *Pseudomonas aeruginosa. Infect Immun* 29:1028–1033.

81. Déziel E, Lépine F, Milot S, Villemur R. 2003. *rhlA* is required for the production of a novel biosurfactant promoting swarming motility in *Pseudomonas aeruginosa*: 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs), the precursors of rhamnolipids. *Microbiol Read Engl* 149:2005–2013.

82. Caiazza NC, Shanks RMQ, O'Toole GA. 2005. Rhamnolipids modulate swarming motility patterns of *Pseudomonas aeruginosa. J Bacteriol* 187:7351–7361.

83. de Vargas Roditi L, Boyle KE, Xavier JB. 2013. Multilevel selection analysis of a microbial social trait. *Mol Syst Biol* 9:684.

84. Ghoul M, West SA, McCorkell FA, Lee Z-B, Bruce JB, Griffin AS. 2016. Pyoverdin cheats fail to invade bacterial populations in stationary phase. *J Evol Biol* 29:1728–1736.

85. Kümmerli R, Brown SP. 2010. Molecular and regulatory properties of a public good shape the evolution of cooperation. *Proc Natl Acad Sci U S A* 107:18921–18926.

86. Foster KR, Shaulsky G, Strassmann JE, Queller DC, Thompson CRL. 2004. Pleiotropy as a mechanism to stabilize cooperation. *Nature* 431:693–696.

87. Mitri S, Foster KR. 2013. The genotypic view of social interactions in microbial communities. *Annu Rev Genet* 47:247–273.

88. Dandekar AA, Chugani S, Greenberg EP. 2012. Bacterial quorum sensing and metabolic incentives to cooperate. *Science* 338:264–266.

89. Schuster M, Hawkins AC, Harwood CS, Greenberg EP. 2004. The *Pseudomonas aeruginosa* RpoS regulon and its relationship to quorum sensing. *Mol Microbiol* 51:973–985.

90. Heurlier K, Dénervaud V, Haenni M, Guy L, Krishnapillai V, Haas D. 2005. Quorum-sensing-negative (*lasR*) mutants of *Pseudomonas aeruginosa* avoid cell lysis and death. *J Bacteriol* 187:4875–4883.

91. Sanchez A, Gore J. 2013. feedback between population and evolutionary dynamics determines the fate of social microbial populations. *PLoS Biol* 11:e1001547.

92. van Gestel J, Vlamakis H, Kolter R. 2015. Division of Labor in Biofilms: the Ecology of Cell Differentiation. *Microbiol Spectr* 3:MB-0002-2014.

93. van Gestel J, Vlamakis H, Kolter R. 2015. From cell differentiation to cell collectives: Bacillus subtilis uses division of labor to migrate. *PLoS Biol* 13:e1002141.

94. Diard M, Garcia V, Maier L, Remus-Emsermann MNP, Regoes RR, Ackermann M, Hardt W-D. 2013. Stabilization of cooperative virulence by the expression of an avirulent phenotype. *Nature* 494:353–356.

95. Brown SP, Taylor PD. 2010. Joint evolution of multiple social traits: a kin selection analysis. *Proc Biol Sci* 277:415–422.

96. Dionisio F, Gordo I. 2006. The tragedy of the commons, the public goods dilemma, and the meaning of rivalry and excludability in evolutionary biology. *Evol Ecol Res* 8:321–332.

97. Frank SA. 1995. Mutual policing and repression of competition in the evolution of cooperative groups. *Nature* 377:520–522.

98. Dionisio F, Gordo I. 2007. Controlling excludability in the evolution of cooperation. E*vol Ecol Res* 9:365–373.

99. Pande S, Kost C. 2017. Bacterial Unculturability and the Formation of Intercellular Metabolic Networks. *Trends Microbiol* 25:349–361.

100. Momeni B, Brileya KA, Fields MW, Shou W. 2013. Strong inter-population cooperation leads to partner intermixing in microbial communities. *eLife* 2:e00230.

101. Momeni B, Waite AJ, Shou W. 2013. Spatial self-organization favors heterotypic cooperation over cheating. *eLife* 2:e00960.

102. Estrela S, Brown SP. 2013. Metabolic and demographic feedbacks shape the emergent spatial structure and function of microbial communities. *PLoS Comput Biol* 9:e1003398.

103. Waite AJ, Shou W. 2012. Adaptation to a new environment allows cooperators to purge cheaters stochastically. *Proc Natl Acad Sci U S A* 109:19079–19086.

104. D'Souza G, Kost C. 2016. Experimental Evolution of Metabolic Dependency in Bacteria. *PLoS Genet* 12:e1006364.

105. Giraud A, Matic I, Tenaillon O, Clara A, Radman M, Fons M, Taddei F. 2001. Costs and benefits of high mutation rates: adaptive evolution of bacteria in the mouse gut. *Science* 291:2606–2608.

106. Funchain P, Yeung A, Stewart JL, Lin R, Slupska MM, Miller JH. 2000. The consequences of growth of a mutator strain of *Escherichia coli* as measured by loss of function among multiple gene targets and loss of fitness. *Genetics* 154:959–970.

107. Germerodt S, Bohl K, Lück A, Pande S, Schröter A, Kaleta C, Schuster S, Kost C. 2016. Pervasive Selection for Cooperative Cross-Feeding in Bacterial Communities. *PLoS Comput Biol* 12:e1004986.

108. Pande S, Kaftan F, Lang S, Svatoš A, Germerodt S, Kost C. 2016. Privatization of cooperative benefits stabilizes mutualistic cross-feeding interactions in spatially structured environments. I*SME J* 10:1413–1423.

109. Ross-Gillespie A, Dumas Z, Kümmerli R. 2015. Evolutionary dynamics of interlinked public goods traits: an experimental study of siderophore production in *Pseudomonas aeruginosa. J Evol Biol* 28:29–39.

110. Cox CD, Graham R. 1979. Isolation of an iron-binding compound from *Pseudomonas aeruginosa. J Bacteriol* 137:357–364.

111. Cox CD, Rinehart KL, Moore ML, Cook JC. 1981. Pyochelin: novel structure of an iron-chelating growth promoter for *Pseudomonas aeruginosa. Proc Natl Acad Sci U S A* 78:4256–4260.

112. Cox CD, Adams P. 1985. Siderophore activity of pyoverdin for *Pseudomonas aeruginosa. Infect Immun* 48:130–138.

113. Inglis RF, Biernaskie JM, Gardner A, Kümmerli R. 2016. Presence of a loner strain maintains cooperation and diversity in well-mixed bacterial communities. *Proc Biol Sci* 283, issue 1822.

114. Popat R, Harrison F, da Silva AC, Easton SAS, McNally L, Williams P, Diggle SP. 2017. Environmental modification via a quorum sensing molecule influences the social landscape of siderophore production. *Proc Biol Sci* 284, issue 1852.

115. Pesci EC, Milbank JB, Pearson JP, McKnight S, Kende AS, Greenberg EP, Iglewski BH. 1999. Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* 96:11229–11234.

116. Bredenbruch F, Geffers R, Nimtz M, Buer J, Häussler S. 2006. The *Pseudomonas aeruginosa* quinolone signal (PQS) has an iron-chelating activity. *Environ Microbiol* 8:1318–1329.

117. Morris JJ, Lenski RE, Zinser ER. 2012. The Black Queen Hypothesis: evolution of dependencies through adaptive gene loss. *mBio* vol. 3 no. 2 e00036-12.

118. Morris JJ, Papoulis SE, Lenski RE. 2014. Coexistence of evolving bacteria stabilized by a shared Black Queen function. *Evol Int J Org Evol* 68:2960–2971.

119. Mas A, Jamshidi S, Lagadeuc Y, Eveillard D, Vandenkoornhuyse P. 2016. Beyond the Black Queen Hypothesis. I*SME J* 10:2085–2091.

120. Michod RE. 2007. Evolution of individuality during the transition from unicellular to multicellular life. *Proc Natl Acad Sci U S A* 104 Suppl 1:8613–8618.

121. Grosberg RK, Strathmann RR. 2007. The Evolution of Multicellularity: A Minor Major Transition?http://dx.doi.org/101146/annurev.ecolsys36102403114735.

122. Herron MD, Rashidi A, Shelton DE, Driscoll WW. 2013. Cellular differentiation and individuality in the “minor” multicellular taxa. *Biol Rev Camb Philos Soc* 88:844–861.

123. Hammerschmidt K, Rose CJ, Kerr B, Rainey PB. 2014. Life cycles, fitness decoupling and the evolution of multicellularity. *Nature* 515:75–79.

124. Oliveira NM, Niehus R, Foster KR. 2014. Evolutionary limits to cooperation in microbial communities. *Proc Natl Acad Sci U S A* 111:17941–17946.

125. Torrens R. 1815. An essay on the external corn trade, containing an inquiry into the general principles of that important branch of traffic, an examination of the exceptions to which these principles are liable and a comparative statement of the effects which restrictions on importation and

free intercourse are calculated to produce upon subsistence, agriculture, commerce and revenue. J. Hatchard, London.

126. Enyeart PJ, Simpson ZB, Ellington AD. 2015. A microbial model of economic trading and comparative advantage. *J Theor Biol* 364:326–343.

127. Tasoff J, Mee MT, Wang HH. 2015. An Economic Framework of Microbial Trade. *PloS One* 10:e0132907.

128. Brown SP, West SA, Diggle SP, Griffin AS. 2009. Social evolution in micro-organisms and a Trojan horse approach to medical intervention strategies. *Philos Trans R Soc Lond B Biol Sci* 364:3157–3168.

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FIGURE LEGENDS

Figure 1. Mechanisms that prevent cheater invasions in single-trait scenarios.

A. Quorum sensing. Bacteria minimize the exploitation by cheaters via collectively producing public goods only when cells reach a given cell density (quorum), which is determined by sensing the concentration of signaling molecules **B. Partial privatization.** Producers of a given public good having prioritized access (represented by thicker green arrows) to it can prevent exploitation by cheaters. **C. I. Policing via direct harm.** By coupling production of a public good with production of a deleterious factor and its corresponding resistance system, cooperators harm cheaters while being immune. **C. II. Policing via reciprocity.** If, in a mutualistic public good exchange relationship, one of the actors stops their production, the other party can downregulate their own production to reciprocate the non-producer. **D. Facultative cooperation.**

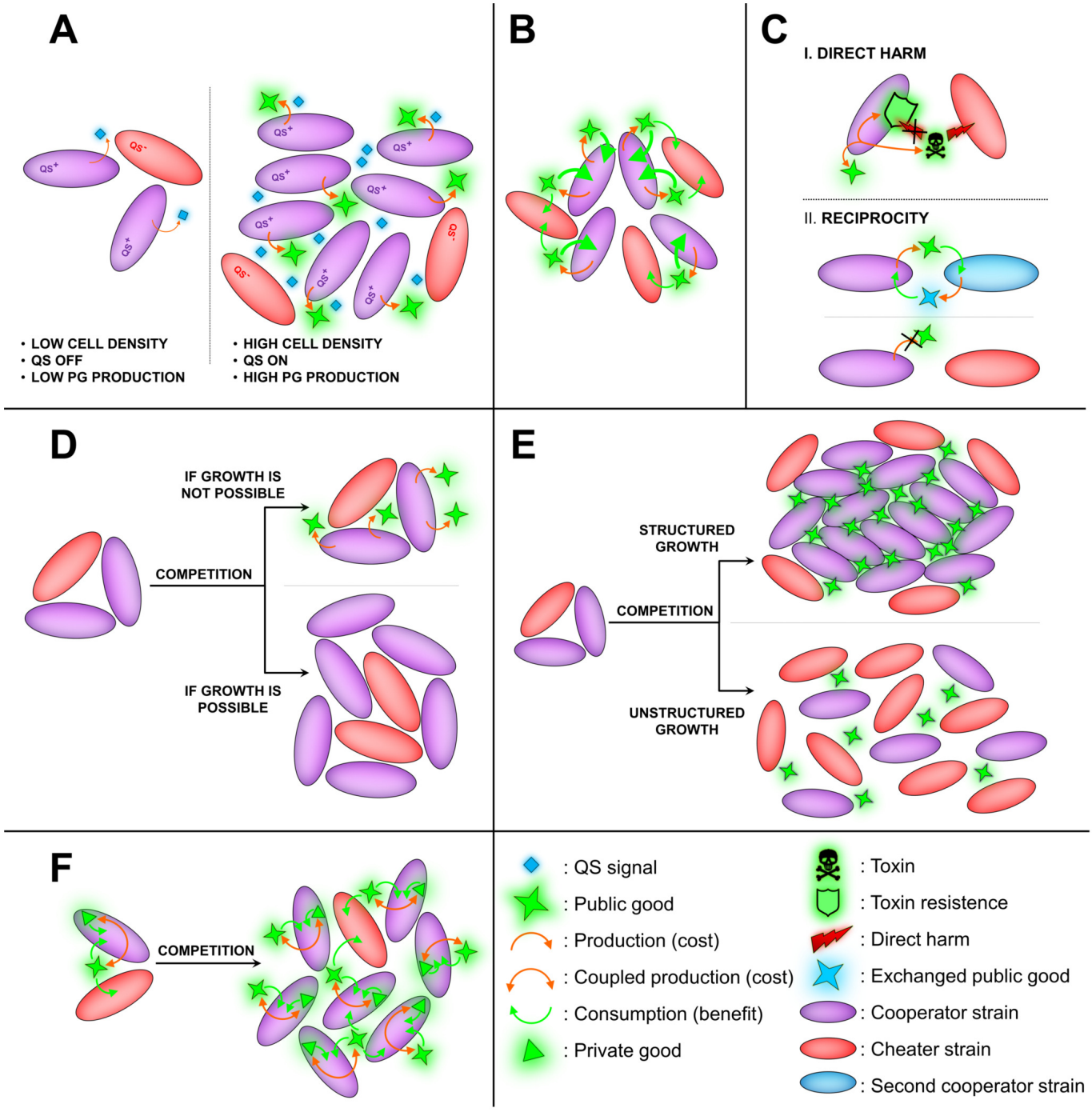
545 In the case of metabolic prudence, bacteria produce a public good only when
546 the cell division is not possible; the public good can alter the environment or
547 allow bacteria to move to a new environment where growth is possible, and
548 then the public good production stops and bacteria start dividing. **E. Spatial**
549 **structure.** When sister cells can stick together, producer cells share the public
550 good mostly among other producers, minimizing the exploitation by cheaters. **F.**
551 **Antagonistic pleiotropy.** By coupling production of a public and a private
552 good, cooperators can access both, whereas the cheaters can only have
553 access to the public good. This process diminishes the relative advantage of the
554 cheaters and minimizes exploitation by cheaters.

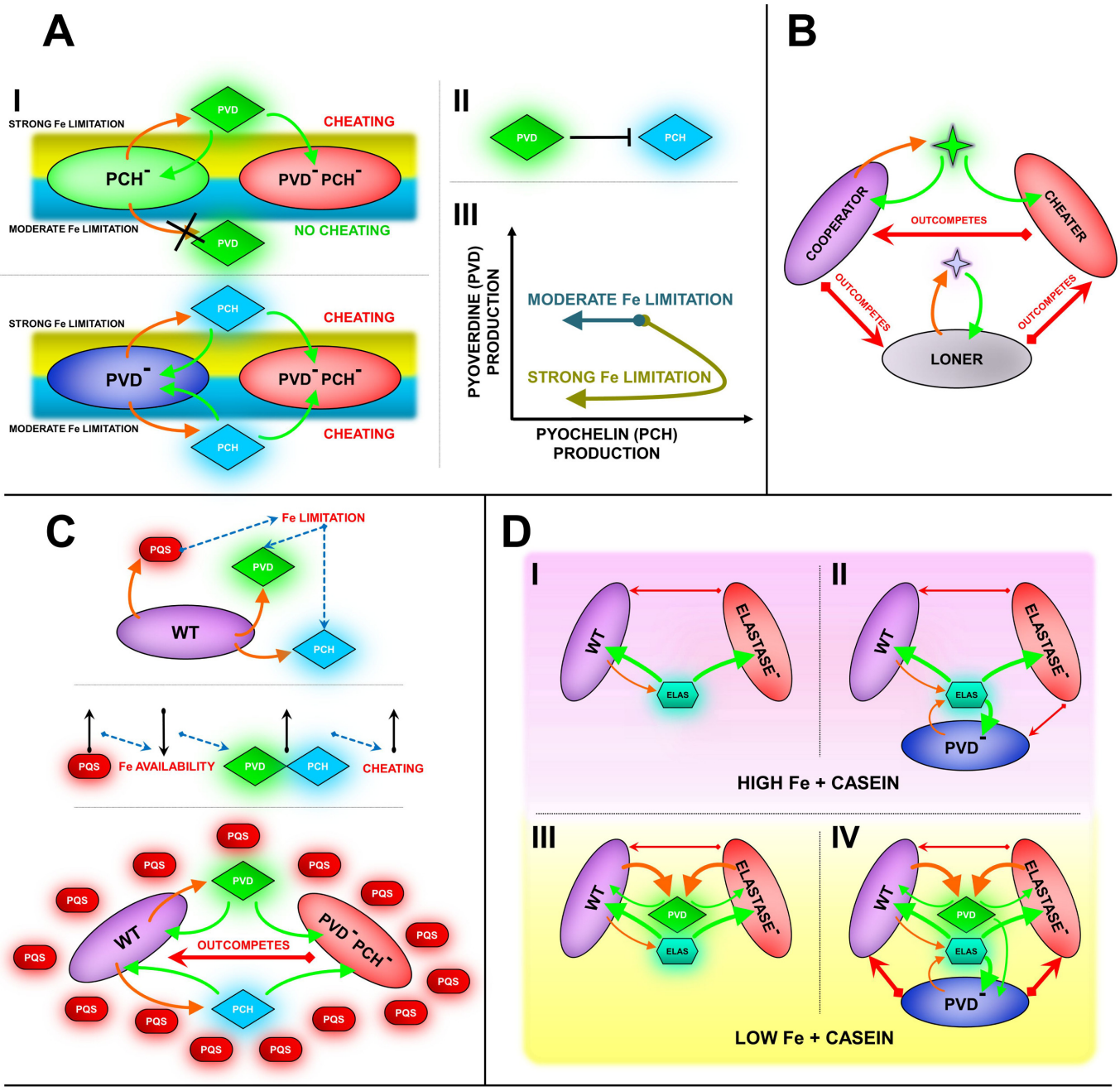
555 **Figure 2. Cheating and cooperation in multiple traits scenarios**

556 **A. Interlinked public good traits.** Regulatory and functional linkage of public
557 good production affects the course of adaptation depending on the
558 environment. Expression of pyoverdine and pyochelin differ in high and
559 moderate iron limitation environments (**I**); uni-directional inhibition of pyochelin
560 by pyoverdine (**II**) affects the evolution of these traits under different iron
561 limitation (**III**) (109) (the direction of the arrows in (**III**) indicate the changes in
562 siderophore production during propagations of cultures along the experimental
563 evolution). **B. Rock-paper-scissors.** Cheaters can outcompete cooperators,
564 loners can outcompete cheaters, and cooperators can outcompete loners. In
565 triple mixtures, loners reduce the relative advantage of cheaters and thus allow
566 the maintenance of cooperation (113). **C. Effects of one public good trait on**

another via environmental modification. Changes in the environment caused by a public good trait can affect the production of another, unrelated public good, and eventually affecting the relative fitness of its cheaters (114). **D. Cheating on cheaters.** A drastic decrease in the population size due to the invasion of a cheater (elastase-deficient *P. aeruginosa*) occurs in mixed populations in the following scenarios: (**I**) in environments where only that public good (elastase) is necessary for growth; (**II**) when a mutant of a second public good trait (pyoverdine-deficient *P. aeruginosa*) is introduced but only the first public good (elastase) is produced; (**III**) when both public goods (elastase and pyoverdine) are necessary but the population lacks the mutant of the second public good. Invasion of the first cheater and the drastic population collapse due to its invasion is only avoided in the following scenario: (**IV**) when the both public goods are necessary and both cheaters are present, the cheater that avoids producing the most costly public good (pyoverdine-deficient *P. aeruginosa*) outcompetes the other (elastase-deficient *P. aeruginosa*) (27).

The thicknesses of the arrows indicate relative differences in cost (orange arrows) and benefits (green arrows). Symbols that are the same as in Figure 1 are described in the legend of Figure 1 to avoid repetition.





: Pyoverdine
: Pyochelin
: Wild-type strain
: Pyochelin mutant strain
: Pyoverdine mutant strain
: Pyoverdine-pyochelin double mutant strain
: High iron limitation
: Moderate iron limitation
: Loner strain
: Alternative public good that the loner strain produces
: Indicates that one strain (back of the arrow) outcompetes another (tip of the arrow)
: Pseudomonas Quinolone Signal
: Elastase
: Iron-supplemented casein media
: Iron-depleted casein media