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Pleiotropy and the low cost of individual traits promote cooperation

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The evolution of cooperation is thought to be promoted by pleiotropy, whereby cooperative traits are coregulated with traits that are important for personal fitness. However, this hypothesis faces a key challenge: what happens if mutation targets a cooperative trait specifically rather than the pleiotropic regulator? Here, we explore this question with the bacterium *Pseudomonas aeruginosa*, which cooperatively digests complex proteins using elastase. We empirically measure and theoretically model the fate of two mutants—one missing the whole regulatory circuit behind elastase production and the other with only the elastase gene mutated—relative to the wild-type (WT). We first show that, when elastase is needed, neither of the mutants can grow if the WT is absent. And, consistent with previous findings, we show that regulatory gene mutants can grow faster than the WT when there are no pleiotropic costs. However, we find that mutants only lacking elastase production do not outcompete the WT, because the individual cooperative trait has a low cost. We argue that the intrinsic architecture of molecular networks makes pleiotropy an effective way to stabilize cooperative evolution. Although individual cooperative traits experience loss-of-function mutations, these mutations may result in weak benefits, and need not undermine the protection from pleiotropy.

KEY WORDS: **Competition, fitness, pleiotropy.**

The evolution of cooperation is threatened by mutants that lack a cooperative trait but make use of the cooperation of others, often known as "cheater" mutants. Pleiotropy, where a single gene affects multiple traits, is thought to be a key mechanism for stabilizing cooperation against cheater mutants, particularly in cellular systems, including both microorganisms (Foster et al. 2004; Harrison and Buckling 2009; Dandekar et al. 2012; Oslizlo et al. 2014) and within multicellular organisms through the prevention of cancer (Aktipis et al. 2015). This is possible when cooperative traits are linked to others that are necessary for the cell's survival, such that the loss of the cooperative gene reduces a mutant strain's ability to survive. However, this leads to an important conundrum: if loss of function of a number of jointly regulated genes is risky for a cell, why not simply lose the individual cooperative trait, while keeping the remaining circuitry intact?

Here, we explore the role of genetic architecture in the evolution of cooperation and exploitation in the bacterium *Pseudomonas aeruginosa*, which uses the las quorum sensing (QS) system to regulate a whole suite of genes, which include the control of the secretion of "public goods" that are useful for other cells in its environment (Schuster et al. 2003; Galán-Vásquez et al. 2011; Fig. 4A). One of these secreted products is elastase, which degrades a number of otherwise indigestible proteins, and has been tightly associated with virulence (Wretlind and Pavlovskis 1983). Mutations in the QS response gene *lasR* lead to a loss in the ability to produce elastase. These mutants are considered to be "cheats" that free-ride on the elastase produced by others without paying the metabolic costs themselves (Diggle et al. 2007; Sandoz et al. 2007). However, Dandekar et al. (2012) have recently shown that selection for such cheating mutants can be reduced by the pleiotropic structure of the *lasR* circuitry, which regulates a total of 89 genes (Galán-Vásquez et al. 2011), including not only public but also "private goods" (Heurlier et al. 2005; Dandekar et al. 2012). The logic is that while loss of *lasR* saves

EVOLUTION
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the cost of the secreted products such as elastase, cells can no longer produce vital private goods, making them vulnerable in certain environments. This predicts that rather than the risky loss of function of the regulatory *lasR* gene and the associated loss of private goods, selection should favor targeted mutations in the *lasB* gene, which is only responsible for elastase production.

To test this prediction, we conducted competition assays involving three strains: a wild-type strain with the QS circuitry intact (WT), a *lasR* mutant incapable of responding to QS signals and expressing genes downstream of *lasR* ($\Delta lasR$), and a *lasB* mutant, which responds to QS signals by producing all downstream products except for elastase ($\Delta lasB$; Toder et al. 1991).

Methods

BACTERIAL STRAINS

In this study, we used the following strains: *P. aeruginosa* PAO1 WT, $\Delta lasB$ and $\Delta lasR$. The WT strain was kindly provided by Roberto Kolter and the knockout strain constructed by a clean deletion of the *lasB* gene, or the *lasR* gene, respectively. All three strains were labeled with YFP and DsRedExpress tags. Labeling was performed by inserting a miniTn7-Gm/Cm-YFP or a miniTn7-Gm/Cm-DsRedExpress plasmid, respectively, following the protocol described by Suh et al. (2004).

CULTURES AND GROWTH CONDITIONS

Liquid cultures of each strain were incubated at 37°C and constantly shaken (250 rotations/min) overnight in 3 mL of 1 × lysogeny broth (LB). Fresh 3 mL aliquots of 1 × LB were then reseeded with the overnight cultures to an optical density (OD_{600}) = 0.05 and grown for 2 h at 37°C, constantly shaken. Two hundred microliters of fresh cultures at different starting ratios were then prepared in either casamino acids (CAA) medium (1 × M9 + 0.1 mM $CaCl_2$ + 1 mM $MgSO_4 \cdot 7H_2O$ + 0.1% [0.1 g/100 mL] casein acid hydrolysate vitamin free [CAA], Sigma-Aldrich C-7970, Dorset, UK) or bovine serum albumin (BSA) medium (M9 + 0.1mM $CaCl_2$ + 1mM $MgSO_47H_2O$ + 0.1% CAA + 2% [2 g/100 mL] BSA, Sigma-Aldrich B4287, Dorset, UK), with a starting OD_{600} = 0.05. To quantify the starting ratios, a 20 μL sample from the mixture used to start the cultures was subjected to serial dilution, plated onto LB agar and colony forming units (CFUs) counted. OD_{600} was measured hourly in 96-well plates constantly shaken using a Tecan Inifinte 200 PRO microplate reader. To estimate the final ratios, after 48 h, each well was subjected to serial dilution, starting from a 20 μL sample, plated onto LB agar and CFUs counted. All treatments were replicated five times, in both color combinations (e.g., WT-YFP vs. $\Delta lasR$-DsRedExpress as well as WT-DsRedExpress vs. $\Delta lasR$-YFP). Control experiments were conducted, where identical strains with different fluorescent labels and different starting ratios were competed against each other. In these experiments, no significant effect due to labeling could be detected (generalized linear model, $P = 0.55$, Fig. S1). Fitness w was measured for each mutant strain as:

$$w = f(t = 48)\,(1 - f(t = 0))\,/\,(f(t = 0)\,(1 - f(t = 48)))\,,$$

where $f(t = 0)$ is the mean initial proportion of mutants at 0 h from the sample population and $f(t = 48)$ is the final proportion of each sample at 48 h (Ross-Gillespie et al. 2007; Ghoul et al. 2014).

STATISTICAL ANALYSIS

All statistical analyses were conducted using MATLAB 2010b.

MATHEMATICAL MODEL

We used systems of ordinary differential equations describing changes in the biomass x of each strain, and the concentration of nutrients—C represents CAA concentration and B, BSA concentration—and elastase E, over time t. As long as CAA is still available ($C > 0$), changes in biomass and solutes can be described as:

$$\frac{dx_i}{dt} = \mu_i x_i(t)\frac{C(t)}{C(t) + K_C},$$

$$\frac{dC}{dt} = -\frac{1}{Y_C}\sum_{i=1}^{n}\frac{dx_i}{dt},$$

$$\frac{dB}{dt} = 0,$$

$$\frac{dE}{dt} = \sum_{i=1}^{n}\frac{dx_i}{dt}e_i,$$

where e_i represents the rate of elastase secretion of strain i, whose value is equal to 0.02 for the WT, 0.01 for the *lasB* mutant and 0 for the *lasR* mutant strain. x_i represents one of the three strains: WT, $\Delta lasB$, and $\Delta lasR$ with growth rates $\mu_i = 0.5, 0.501$, or 0.55 (see Table 1), respectively, and n represents the number of strains in growth culture ($n = 1$ in monoculture and $n = 2$ in coculture). Y_C was set to 40 for the WT and the *lasB* mutant and 120 for the *lasR* mutant.

Once C has been depleted, changes depend on the availability of BSA B and elastase E:

$$\frac{dx_i}{dt} = \mu_i p x_i(t)\,\frac{B(t)E(t)}{B(t) + K_B},$$

$$\frac{dC}{dt} = 0,$$

$$\frac{dB}{dt} = -\frac{1}{Y_B}\sum_{i=1}^{n}\frac{dx_i}{dt},$$

$$\frac{dE}{dt} = \sum_{i=1}^{n}\frac{dx_i}{dt}e_i,$$

Table 1. In the dimensions column, M_E represents mass of elastase, M_G represents mass of growth substrate, M_X represents cell counts, and T represents time.

Symbol	Description	Value	Dimension
$C(0)$	Initial CAA concentration	5×10^6	M_G
$B(0)$	Iinitial BSA concentration	2×10^8	M_G
$x_i(0)$	Initial cell count	5×10^6	M_X
K_C	Half-saturation constant for CAA	1	M_G
K_B	Half-saturation constant for BSA	1	M_G
Y_{C_WT}	Yield of WT cell count on CAA	40	M_X/M_G
$Y_{C\_\Delta lasB}$	Yield of ΔlasB cell count on CAA	40	M_X/M_G
$Y_{C\_\Delta lasR}$	Yield of ΔlasR cell count on CAA	120	M_X/M_G
Y_B	Yield of cell count on BSA	10	M_X/M_G
μ_{WT}	Maximum growth rate of WT	0.5	$1/T$
$\mu_{\Delta lasB}$	Maximum growth rate of ΔlasB	0.501	$1/T$
$\mu_{\Delta lasR}$	Maximum growth rate of ΔlasR	0.55	$1/T$
p	Factor change in growth rate due to elastase concentration in BSA	1.09×10^{-5}	Dimensionless
e_{WT}	Elastase production rate of WT	0.02	$M_E/M_X/T$
$e_{\Delta lasB}$	Elastase production rate of ΔlasB	0.01	$M_E/M_X/T$
$e_{\Delta asR}$	Elastase production rate of ΔlasR	0	$M_E/M_X/T$

where p is a factor that adjusts the growth rate of all strains based on elastase availability in BSA. All model parameters were chosen such that growth curves in monoculture resembled the experiments, and are listed in Table 1. The model was implemented in *R*, and differential equations were solved using the backward differentiation method (method "bdf" in the "deSolve" library).

Results

COMPETITION WHEN ELASTASE IS NOT NEEDED

We first evaluated the fitness of each of the three strains alone in a defined minimal liquid medium supplemented with a small amount of predigested CAA, which all strains should be able to take up. Indeed, all three strains grew exponentially for 6 h and then stopped, with the *lasB* mutants reaching a similar population size compared to the wild-type (WT mutants CFU/mL: 2.16×10^8, *lasB* CFU/mL: 1.91×10^8, Mann–Whitney test: $P = 0.06$, degrees of freedom [df] = 13), and the final *lasR* optical density exceeding both significantly (*lasR* CFU/mL: 8.11×10^8, $P < 0.001$, df = 13; Figs. 1A and 3A). This suggests a negligible cost for the *lasB* gene and a greater cost for expressing *lasR*. However, because it is unclear whether these costs will translate into an evolutionary advantage for the mutants, we next conducted competition assays between the WT and each of the mutants. In coculture under a range of starting frequencies, the fitness of *lasB* mutants did not differ significantly from the WT (mean ± SD of log relative fitness: 0.02 ± 0.19, sign test $P = 0.58$, df = 29), whereas *lasR* mutants consistently outcompeted the WT (0.4 ± 0.19, $P < 0.001$, df = 29; Fig. 1B). Despite the loss in elastase production, then, in contrast to *lasR* mutants, *lasB* mutants do not appear to be under positive selection when growth does not depend on elastase availability.

COMPETITION WHEN ELASTASE IS REQUIRED

We next ask how these costs affect competition with the WT in a growth medium that requires elastase to be digested. To this end, we repeated the experiment with BSA—which contains proteins that are broken down by elastase—added to the growth medium. In monoculture, all strains appeared to grow similarly during the first 6 h. Once all the CAA had been used up, the *lasB* and *lasR* mutants hardly grew any further (Fig. 2A). The limited amount of growth in the *lasB* mutant is likely because cells still produce some elastase due to the presence of other genes and regulators, such as *lasA* (Toder et al. 1991; Fig. 2A). Nevertheless, this shows that by simply mutating *lasB*, we can largely mimic the growth of *lasR* mutants in BSA. In contrast, by the end of the 48-h experiment, the WT had grown to 9.32 times its population size in CAA alone (Figs. 2A and 3).

Although the mutant strains could not digest much BSA on their own, we expect them to grow better in the presence of the WT because the elastase secreted by the WT will digest BSA external to the cells. Indeed, in competition assays against the WT in the BSA-supplemented medium, both mutant strains grew better than they did alone, with a frequency-dependent increase (for starting frequency of 0.1, 0.5, or 0.9, *lasB* mutant increase factor: 1.94×, 1.81×, 0.67×; *lasR* mutant increase factor: 2.9×, 1.36×, and 1× at 48 h). However, compared to their growth in the CAA medium, the relative fitness of both mutants did not differ significantly: *lasB* mutants maintained a fitness that could not be distinguished from that of the WT (sign test $P = 0.36$, df = 29),

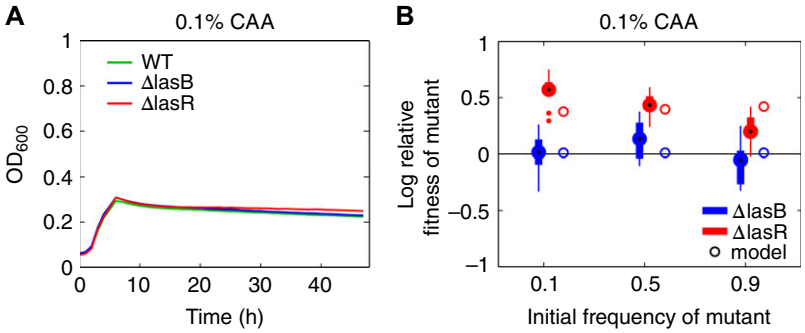

Figure 1. Growth and competition in CAA. (A) OD_{600} of monocultures of the three strains in M9 supplemented with 0.1% CAA medium. Lines show the mean over 15 replicates, and the transparent band around them the standard deviation (SD). (B) Log-relative fitness of each of the mutants at different starting frequencies when grown together with the WT in CAA medium. Filled symbols represent boxplots of the experimental data (large filled circles are the median, thick lines show 25th–75th quartile, lines show the extent of the data in the absence of outliers, which are represented by dots), while empty circles represent model predictions.

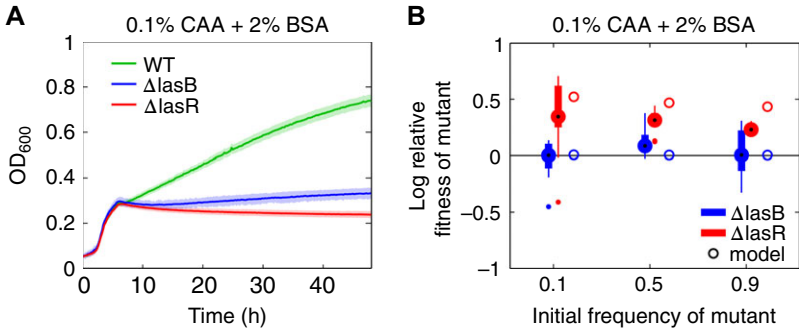

Figure 2. Growth and competition in BSA. (A) OD_{600} of monocultures of the three strains in M9 supplemented with 0.1% CAA medium and 2% BSA medium. Lines show the mean over 15 replicates, and the transparent band around them the SD. (B) Log-relative fitness of each of the mutants at different starting frequencies when grown together with the WT in BSA medium. Filled symbols represent boxplots of the experimental data (large-filled circles are the median, thick lines show 25th–75th quartile, lines show the extent of the data in the absence of outliers, which are represented by dots), while empty circles represent model predictions.

whereas the *lasR* mutant strain outgrew the WT consistently, with a greater relative fitness at lower frequencies ($P < 0.001$, df = 29, see Fig. 2B). In sum, both mutant strains could make use of the elastase secreted by the WT and continue to grow in BSA, but their competitive advantage over the WT was simply a function of the cost of the genes they had lost.

MATHEMATICAL MODEL

Our data suggest that the competitive fate of the different genotypes we are studying can be explained by two factors. First, individual differences within a population are driven by differences in growth rate and yield, which result from the metabolic costs saved by the different mutations. Second, the productivity of the whole population is determined by the ability to produce elastase when it is needed for growth. To evaluate whether these two factors were sufficient to explain our data, we built a system of ordinary differential equations using standard Monod kinetics to describe the changes in nutrient abundances as well as cellular growth. We selected parameters by fitting the minimal model to the monoculture growth curves in the experiments. This led to fitting of the two aforementioned factors as follows: (1) the growth rates of the WT, *lasB* mutants and the *lasR* mutants were set to 0.5, 0.501, and 0.55, respectively; (2) the yield of biomass on CAA to 40, 40, and 120, respectively; and (3) elastase production to 0.02, 0.01, and 0, respectively. With these parameters representing the only differences between the three strains, we could then test whether our model recapitulates our experimental observations when different strains were put together in coculture (see Figs. 1B, 2B, and 3, methods, Figs. S2–S3). We find that our simple model and the behavior of the strains in monoculture are a good predictor of the coculture data. We conclude therefore that strain growth rate and the effects of the public goods are key factors explaining the outcomes of the competition experiments.

Discussion

Taken together, we find that the cost of the *lasB* gene is not sufficiently high in our experiments for natural selection to act against it, even in environments where elastase production can

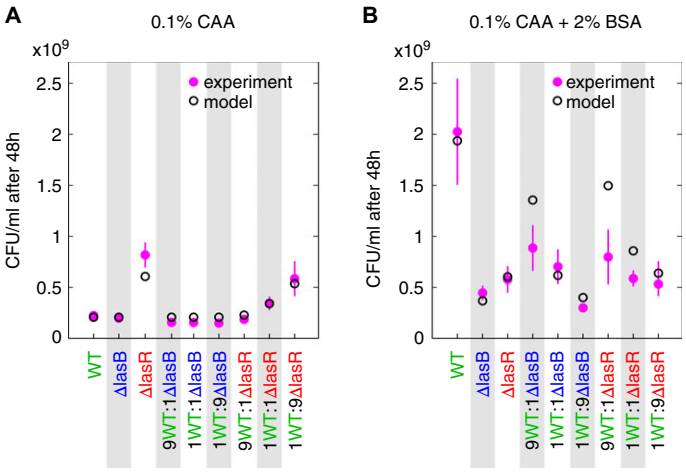

Figure 3. Final CFU/mL after 48 h of each of the monocultures and the cocultures at different starting ratios in CAA (A) and BSA (B). Filled magenta circles show the mean over either 15 (monocultures) or 10 (cocultures) replicates, and lines represent the SD. Empty black circles show model predictions. Gray bars are drawn for visual clarity.

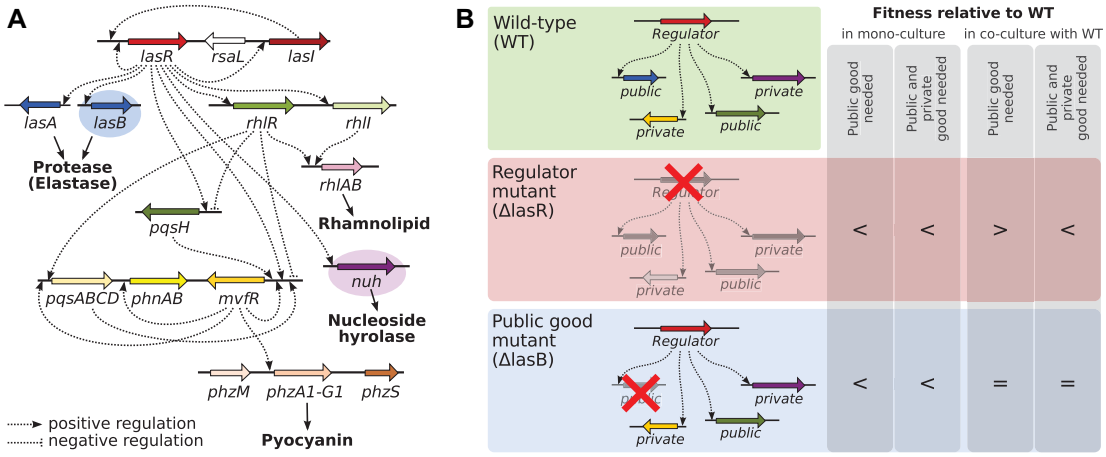

Figure 4. (A) Subset of *lasR* gene regulatory network, showing examples of different public and private goods directly or indirectly regulated by *lasR*. In total, LasR regulates 89 genes (Galán-Vásquez et al. 2011). The gene responsible for elastase production *lasB*, as well as *nuh*, which participates in the uptake of adenosine and is thus a private good (Heurlier et al. 2005) are highlighted. This diagram is based on Figure 5 in Kim et al. (2015). (B) Pleiotropy and the lost cost of individual traits protects public good cooperation. Compared to the WT, a regulator mutant loses function in all downstream genes, making it grow poorly whenever private goods are needed (pleiotropic cost; Foster et al. 2004; Dandekar et al. 2012). However, when public goods are needed, if the WT—which produces the public good—is present, the mutant can grow faster by saving a significant metabolic burden (Diggle et al. 2007; Sandoz et al. 2007). In contrast, a public good mutant can grow well in the presence of the WT when public or private goods are needed, but here we show that because the cost saved by not producing the public good is insignificant, it does not out-compete the WT.

be exploited. The genetic architecture of the QS circuitry in *P. aeruginosa* appears then to doubly protect elastase production from loss of function (Fig. 4). On the one hand, the phenotype can be lost by knocking out the whole *lasR* circuitry. Although this saves a significant metabolic burden, it also exposes the cell to the risk of losing important metabolic functions that cannot be "borrowed" from others (Dandekar et al. 2012). On the other hand, we show that targeted loss of elastase production presented no advantage to the cells over the WT: on its own, the cost of elastase production is too low for cheaters to be strongly selected (Figs. 1B and 2B). In addition, in environments where elastase is necessary, but no other cells are producing it, *lasB* mutants can hardly grow (Fig. 2A).

In agreement with our data, *lasB* mutants have so far rarely been found in natural isolates, whereas *lasR* mutants are common (Schaber et al. 2004; Heurlier et al. 2006; Smith et al. 2006). The prevalence of *lasR* mutants in isolates shows that pleiotropic constraints do not offer perfect protection against loss-of-function mutation. *lasR* mutants can thrive either when (1) none of the genes downstream of *lasR* are required for resource uptake

(Fig. 1B), or (2) when only the extracellular products of *lasR* are required, and are provided by other cells (Fig. 2B). Consistent with the first case, natural strains with loss of function mutations in *lasR* have been isolated from the lungs of chronically ill cystic fibrosis patients (Smith et al. 2006; D'Argenio et al. 2007; Köhler et al. 2009), which is thought to represent an environment in which the *lasR*-regulated genes are not required for growth (D'Argenio et al. 2007; Bjarnsholt et al. 2013; Harrison et al. 2014; Turner et al. 2015). The second case appears to occur in some acute infections where *lasR*-regulated virulence factors are needed and secreted by the WT, which can then be exploited by arising *lasR* mutants (Köhler et al. 2009; Rumbaugh et al. 2009). The potential for pleiotropic constraints in *lasR* then depends strongly upon environmental conditions.

The combination of pleiotropy and low cost traits may affect selection in other pleiotropic genes in *Pseudomonas*. For example, loss-of-function mutations of global regulators *gacA* and *gacS* arise at high rates in the laboratory and in natural populations (Duffy and Défago 2000; van den Broek et al. 2005; Driscoll et al. 2011; Seaton et al. 2013). These mutations can confer significant fitness benefits (Duffy and Défago 2000; Driscoll et al. 2011), and lead to loss of public goods, including extracellular enzymes, antibiotics, and biofilm formation (Bull et al. 2001; Seaton et al. 2013), but also private goods involved in primary metabolism (Wei et al. 2013). Similarly, mutations in a LysR-type regulator in *Pseudomonas aureofaciens* lead to an inability to produce antifungal compounds (Silby et al. 2005). Mutations in the regulator confer large benefits in the absence of fungal threat, while other mutations altering the antifungal phenotype result in only small fitness benefits (Silby et al. 2005).

We find then that the regulatory architecture of elastase production in *P. aeruginosa* is highly amenable to stabilizing cooperation. Previous work has shown that pleiotropic constraints may limit the loss of the regulator LasR and we have shown here that this is combined with weak benefits to losing the cooperative trait itself. There is a general logic to these findings: losing a regulator has the potential to be extremely beneficial when this leads to the loss of a suite of cooperative traits. However, loss of regulators can be effectively controlled by pleiotropic links to non-cooperative traits. By contrast, while pleiotropy may not prevent the loss of an individual cooperative trait, losing an individual trait will confer fewer benefits than losing a suite of traits. Such weak benefits mean that this route also need not be a major threat to cooperation. This intrinsic architecture of molecular networks makes pleiotropy an effective way to promote cooperative evolution.

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DATA ARCHIVING

The doi for our data is doi:10.5061/dryad.h5fg1.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1. Control experiments with differently labeled but otherwise identical strains grown in cocultures.

Fig. S2. Mathematical model's predictions of growth curves in mono- and coculture over time, as well as final population sizes, ratios, and fitnesses of mutants relative to the WT in CAA medium.

Fig. S3. Mathematical model's predictions of growth curves in mono- and coculture over time, as well as final population sizes, ratios, and fitnesses of mutants relative to the WT in BSA medium.