Facultative cheating supports the coexistence of diverse quorum-sensing alleles

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Bacterial quorum sensing enables bacteria to cooperate in a density-dependent manner via the group-wide secretion and detection of specific autoinducer molecules. Many bacterial species show high intraspecific diversity of autoinducer–receptor alleles, called pherotypes. The autoinducer produced by one pherotype activates its coencoded receptor, but not the receptor of another pherotype. It is unclear what selection forces drive the maintenance of pherotype diversity. Here, we use the ComQXPA system of *Bacillus subtilis* as a model system, to show that pherotype diversity can be maintained by facultative cheating—a minority pherotype exploits the majority, but resumes cooperation when its frequency increases. We find that the maintenance of multiple pherotypes by facultative cheating can persist under kin-selection conditions that select against "obligate cheaters" quorum-sensing response null mutants. Our results therefore support a role for facultative cheating and kin selection in the evolution of quorum-sensing diversity.

social evolution | sociomicrobiology | *Bacillus subtilis* | bacteria | quorum sensing

In many bacteria, a cell–cell signaling mechanism, known as quorum sensing, coordinates the response of a bacterial community in a density-dependent manner. Quorum-sensing bacteria secrete a signal molecule known as an autoinducer and express a specific receptor that binds to it with high affinity, resulting in the activation of a specific cellular response (1). Quorum sensing often regulates the secretion of public goods or other cooperative traits that benefit the community, at a cost to the individual responding cell (2).

The regulation of cooperation by a secreted autoinducer allows for the evolution of cheater genotypes that do not produce the autoinducer or do not respond to it (3, 4). Mutants of the latter type were shown to act as cheaters in a variety of different species (3–7). The elimination of these cheater mutants could occur by kin selection, where cooperation is preferentially directed toward other cooperators (3, 7–9).

In contrast to the rarity of quorum-sensing response null alleles in wild populations, many species display a high degree of intraspecific genetic variation in functional quorum-sensing alleles, called pherotypes (Fig. 1*A*). Each allele codes for both receptor and autoinducer genes, where an autoinducer coded by one pherotype will activate its coencoded receptor, but not the receptors encoded by other pherotypes (10–14). Pherotypes differ in their receptor–autoinducer specificity but not in the pathways regulated by the receptor. In addition, many pherotypes show patterns of intraspecific horizontal gene transfer (10, 12) and coexist in the same environment (15, 16).

The mechanisms that lead to the diversification of pherotypes, to the maintenance of their diversity, and to their rapid horizontal gene transfer are not well understood. We have previously proposed, by analyzing a theoretical model, that if quorum sensing regulates cooperation, novel pherotypes can arise adaptively through sequential selection of a receptor mutation followed by selection for a compensating mutation that changes the autoinducer (17). The model also suggested that different pherotypes will coexist by facultative cheating (18)—each pherotype cheats as a minority and returns to cooperation when its frequency increases (Fig. 1*B*). This model can thus explain both the observed diversity and the rapid horizontal gene transfer of quorum-sensing alleles.

The *Bacillus subtilis* ComQXP quorum-sensing system is one of the best-studied systems with multiple characterized pherotypes (Fig. 1*C*) (19). This system is encoded by a single locus that contains a three-gene operon. The ComX autoinducer production genes (*comQ*, *comX*) and the region of *comP* encoding for the extracellular part of the ComP receptor are highly variable and encode for multiple different pherotypes, which coexist in the soil and undergo rapid horizontal gene transfer (10, 15, 16, 20). The interaction between different *comQXP* pherotypes also includes cases of asymmetric cross-activation or cross-inhibition, where a ComX autoinducer of one pherotype would activate the receptor of another pherotype, but not vice versa, or when the autoinducer inhibits the receptor of another pherotype (10, 20). The ComQXP quorum-sensing system activates the ComA transcription factor, which regulates a large array of genes, including the *srfA* operon (19). The *srfA* operon encodes for the structural enzymes necessary for the production of the surfactant Surfactin (21). Importantly, no quorum-sensing response mutants were observed in natural populations of *B. subtilis* (16, 22).

In this work, we examine the maintenance of multiple pherotypes by using the *B. subtilis* ComQXP quorum-sensing system as a model system. First, we verify that this system regulates cooperative swarming behavior. Next, we find that cocultured pherotypes undergo negative frequency-dependent selection by mutual facultative cheating during swarming. Finally, we show that kin selection, brought about by repeated population bottlenecks, maintains pherotype coexistence, while selecting against quorum-sensing

Significance

Bacteria cooperate by secretion of public good molecules, which benefit the entire community. Such cooperative behaviors are often regulated by cell–cell signaling mechanisms. In many species, these signaling systems are highly diversified in their signal–receptor specificity, but the causal link between the function of signaling and the maintenance of high genetic diversity was unclear. Here we demonstrate experimentally that signaling diversity is maintained by facultative cheating—a minority strain with one signaling system will exploit the public goods production of a majority strain that possesses a different system, but resumes cooperation on its own. Mutual facultative cheating demonstrates the complexity of social strategies attained by bacteria through the regulation of cooperative behaviors and their impact on population genetics parameters.

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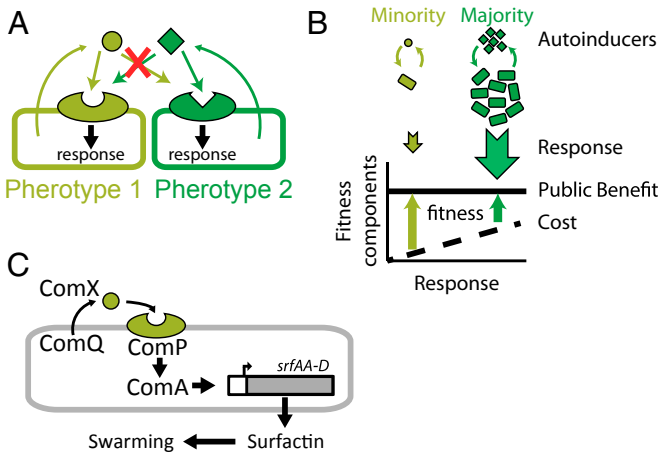

Fig. 1. Quorum-sensing pherotypes and the *Bacillus* Com quorum-sensing system. (*A*) Pherotype variability is defined when two or more homologous receptor and autoinducer alleles are found in the population. Each allele codes for autoinducer production genes and a receptor gene. The autoinducer produced by a cell carrying one pherotype specifically activates the receptor of the same pherotype, but not the receptor of the other pherotype. (*B*) Selection for minority pherotype when quorum sensing controls public goods. A minority pherotype (light green) will produce less signal than the majority pherotype (dark green). Consequently the cost of quorum-sensing response of the minority is lower. The benefits of quorum-sensing response are public and shared by all cells. The fitness of the minority pherotype is therefore larger than the fitness of the majority and it will invade into the population. (*C*) A scheme of the ComQXP pathway. ComQ cleaves and modifies ComX to make the mature secreted autoinducer, which binds the receptor ComP. Bound receptor activates the transcription factor ComA, which regulates production and secretion of surfactin through the *srfA* operon. Surfactin is necessary for swarming.

response null cheaters. Our results therefore support the importance of facultative cheating and kin selection in the evolution of pherotype diversity.

Results

The ComQXPA Pathway Regulates Cooperation During Swarming Motility. According to our model, pherotypes will be maintained by facultative cheating if quorum sensing regulates cooperative behaviors, such as the production of secreted molecules [e.g., extracellular enzymes (4) or surfactants (23)]. In *B. subtilis*, ComA regulates the production of surfactin, which is crucial for swarming motility (24). In addition, exogenous addition of surfactin was shown to rescue the swarming phenotype of strains defective in surfactin production, suggesting that surfactin is a public good (24). Despite the regulation of surfactin by ComA, a quorum-sensing response mutant (Δ*comP*) displayed a moderate swarming phenotype, characterized by slowed swarming, but not a complete lack of it (25). A Δ*comA* deletion mutant displayed a similar phenotype, despite the strong effect this mutation had on the expression of the surfactin production operon, *srfA* (26). We reasoned that previously used swarming conditions led to very high cell densities, allowing the residual surfactin produced by each bacterium to accumulate to high extracellular concentrations and thus maintain the swarming phenotype (25, 27). We therefore studied swarming, using a minimal medium containing a low concentration of glucose (0.005%), compared with the concentration of previously used swarming media. We find that under these conditions the maximal cell density was dramatically reduced, enabling the swarming of the proficient wild type but not of its respective *comQXP* or *comA* mutants (Fig. 1 and Figs. S1 and S2). We therefore used the low-glucose conditions in further experiments, as they probably better reflect the strong dependence of swarming on the Com system in naturally relevant low-nutrient conditions.

It was previously shown that exogenously added surfactin can rescue the swarming phenotype of surfactin production mutants, implying that ComQXPA quorum-sensing response mutants will be able to exploit surfactin-producing strains during coculture. To examine this hypothesis, we cocultured a swarming-proficient derivative of the laboratory strain [$swrA^{+}$;sfp^{+} strain (25)] with an isogenic Δ*comA* quorum-sensing response mutant at varying initial frequencies of the two strains and monitored their growth (Fig. 2 and Fig. S1). The average growth rate of the culture was determined by measuring the total cell number after 65 h of growth. The relative fitness of the two strains was measured by determining the change in frequency of the two strains during swarming cocultures. Relative fitness of genotype 1 over genotype 2 is defined as the ratio of the frequencies of each strain at the end of the experiment to their ratio at the beginning of the experiment (28). Relative fitness measurement was made possible by introducing different constitutive fluorescent reporters, which allowed us to monitor genotype frequencies before and after swarming (*Methods*). We find that in contrast to slower, motility-independent, forms of growth, swarming does not lead to significant segregation of genotypes (Fig. S3) (29). Swarming in *B. subtilis* can therefore be regarded as an unstructured environment to a good approximation (23).

We find that a quorum-sensing reception mutant (either a Δ*comA* mutant or a Δ*comQXP* mutant) displayed the signatures of "cheating" behavior (Fig. 2*A* and Fig. S2). First, the cell yield of the culture was reduced as the frequency of the Δ*comA* mutant increased (Fig. 2*A*). Second, the Δ*comA* mutant had a pronounced fitness advantage, which led to its invasion into the population (Fig. 2*B*). At a low initial frequency, the mutant had a >50-fold growth advantage over the wild type. This growth advantage was reduced, but remained positive, as the initial frequency of the Δ*comA* mutant increased (Fig. 2*B*, *t* test, *P* value = 1.6*e*-07, $n = 23$). The reduction in the relative fitness of the Δ*comA* strain can be attributed to the large reduction in the average growth of the coculture.

To further explore the observed cheating behavior, we used a P_{srfA}-YFP transcriptional reporter to monitor the quorum-sensing response of the wild type and the Δ*comA* mutant during swarming coculture (*Methods*). We find that the average per cell *srfA* expression level of the wild type was significantly higher than that of the Δ*comA* mutant (Fig. 2*C*, two-sample *t* test, *P* value = 4.2*e*-08, $n = 12$). The expression level of the wild type was constant irrespective of the initial frequency of the two strains [Fig. 2*C*, linear regression, $F(1,10) = 0.237$, $n = 12$, *P* value = 0.637 for constant model null hypothesis]. This finding supports the obligatory cheating strategy of the Δ*comA* mutant. In contrast, when the wild type was cocultured with a Δ*comQXP* mutant, the wild-type average per cell quorum-sensing response decreased with the mutant frequency, but was always higher than that of the mutant (Fig. S2, *t* test, *P* value = 0.0026, $n = 9$). This probably reflects the reduction in the total signal level with the increase of the Δ*comQXP* mutant frequency (30).

These results were obtained from a swarming-proficient derivative of the domesticated laboratory strain (24, 27). The domestication of the laboratory strain has been accompanied by a multitude of mutations and the loss of additional traits and regulatory circuits in addition to those related to swarming, such as the ability to form biofilms (31). To verify that these additional mutations do not significantly affect the social interactions between ComQXPA variants, we repeated some of the experiments in a biofilm-forming isolate (32). We find that in this genetic background, the Δ*comA* and Δ*comQXP* mutants exploited the wild type, as was observed in the laboratory strain background, albeit with a lower relative fitness (Fig. S4). The reduced relative fitness most likely reflects the lower levels of cooperative investment observed in this genetic background (32). Together, our results suggest that the quorum-sensing response, and specifically the production of surfactin during swarming, is a costly cooperative behavior and

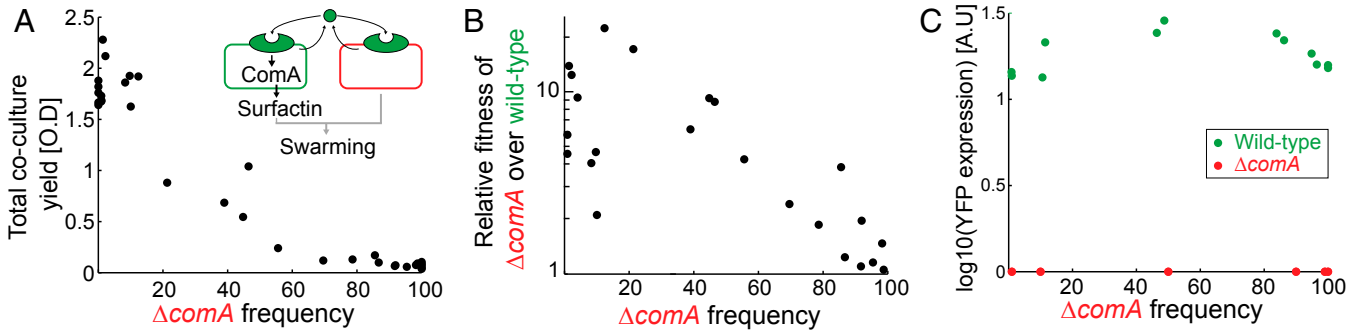

Fig. 2. A *ΔcomA* mutant is an obligate cheater of the wild type during swarming. (*A* and *B*) Final cell yield (*A*) and relative fitness of the *ΔcomA* strain (*B*) were measured for different cocultures of *ΔcomA* (strain AES3001) and wild type (strain AES2137), as a function of the initial frequency of the *ΔcomA* strain in each coculture (*Methods*). (*C*) Average per cell quorum-sensing-dependent gene expression in a swarming coculture, as a function of the initial frequency of the *ΔcomA* strain in the coculture. Per cell average response was measured by flow cytometry, using a P_{srfA}-YFP construct inserted either into the wild type [green, in coculture between strains AES2075 (wild type with reporter) and AES3007 (*ΔcomA*)] or into the *ΔcomA* [red, in coculture between strains AES2033 (wild type) and AES3008 (*ΔcomA* with reporter)] strains. Further details of the strains used for *A*–*C* are given in Table S1. Each data point in *A*–*C* represents a measurement from a different swarming plate. Experiments were done on multiple days.

that the wild type and the *ΔcomA* strain behave as a cooperator and an obligate cheater, respectively.

Different Pherotypes Are Facultative Cheaters of Each Other During Swarming. Our model predicts that if quorum sensing regulates cooperative behavior, different pherotypes will perform mutual facultative cheating—a minority pherotype will have reduced cooperative investment compared with the majority, by virtue of its lower density in the population and thus its lower autoinducer concentration. This will lead to exploitation of the majority by the minority pherotype and invasion into the population. In contrast to an obligate cheater, the minority pherotype will resume cooperation as its frequency increases, owing to the increasing autoinducer concentration (17) (Fig. 1*B*). To test this key prediction in *B. subtilis*, we studied the interaction between several different pherotypes under swarming conditions. We deleted the endogenous *comQXP* allele and introduced a *comQXP* locus of one of four different strains with different pherotypes (168, RO-H-1, NAF4, and RO-FF-1) into an ectopic location (20). We cocultured all possible pairs of strains. For each pair we performed two competitions, where one or the other pherotype is a minority with an initial frequency of ∼1% (Fig. 3*A*). In almost all cases, a strain had a fitness advantage as a minority and a fitness disadvantage as a majority. We found two exceptions to this trend when either pherotype RO-FF-1 or NAF-4 were cocultured as a minority with pherotype 168. The former case most likely arises from the asymmetric signaling interactions between strains 168 and RO-FF-1, where the former activates the latter, but not vice versa (20).

To better understand selection dynamics between strains, we further studied the swarming behavior of strains NAF4 and RO-H-1, as these systems are both orthogonal and exogenous to the 168 background. We cocultured the strains in varying initial frequencies under swarming conditions. We find that the two strains exhibited negative frequency-dependent selection—as a small minority, each strain had a fitness advantage over its cocultured majority pherotype (Fig. 3*B*). Selection between pherotypes was not symmetrical—the two strains had no relative fitness advantage at a RO-H-1:NAF4 ratio of ∼1:5 (Fig. 3*B*, *x* axis intercept at RO-H-1:NAF4 = 0.18, *x* axis intercept 95% confidence interval [0.11, 0.26], linear slope of line = −0.44725 [linear regression, $F(1,26) = 148$, $n = 28$, P value = 3.0644*e*-12 to a no-selection null hypothesis]). The two strains therefore coinvade each other and coexist at an intermediate frequency.

If negative frequency dependence is due to facultative cheating, the fitness advantage of the invading strain should result from its reduced investment in the quorum-sensing response and in particular in *srfA* expression. Using the P_{srfA}-YFP reporter, we measured the average per cell gene expression of each of the strains in a swarming coculture (*Methods* and Fig. 3*C*). We find that gene expression patterns correspond well with selection. The minority strain has a lower per cell *srfA* expression level than the majority strain and the frequency of equal expression corresponded well with the frequency of coexistence (Fig. 3*C*, compare with Fig. 3*B*, 95% confidence intervals [0.1, 0.2], P value > 0.05). The gene expression patterns agree with the asymmetry of selection strength between the two pherotypes. We find that the RO-H-1 expression levels as a majority are higher than those of NAF-4 as a majority (Fig. 3*C*). This asymmetry can stem from higher gene expression of the $comQXP_{RO\text{-}H\text{-}1}$ system or a higher affinity between the $ComP_{RO\text{-}H\text{-}1}$ receptor and its $ComX_{RO\text{-}H\text{-}1}$ autoinducer, compared with the affinity of the NAF4 receptor–autoinducer pair.

We expect that facultative cheating will have only a weak effect on average population fitness, as most cells strongly invest in cooperative activity. We measured the total yield of a swarming coculture containing the two pherotypes (Fig. 3*D*). In agreement with our expectation, we find the yield to be high and independent of the initial frequency of the two strains [Fig. 3*D*, linear regression, $F(1,47) = 0.0161$, $n = 49$, P value = 0.89971 to a zero slope null hypothesis]. Together, our results suggest that a minority pherotype will invade a majority pherotype to a stable coexistence by facultative cheating, without significantly altering the average fitness of the population.

Kin Selection Maintains Pherotype Diversity While Eliminating Obligate Cheating. Whereas swarming conditions select for coexistence of pherotypes (Fig. 3), they also select for the invasion and the eventual fixation of quorum-sensing response mutants such as *ΔcomA* (Fig. 2). Kin-selection theory predicts that cooperation can be maintained if the relatedness between cooperating bacteria is sufficiently high. In bacteria, relatedness can be established if the population goes through growth bottlenecks, and it has been shown that this mechanism is effective in eliminating cheater mutants from the population (3, 33–35). To test for the effect of bottlenecks on pherotype variability and the elimination of quorum-sensing response mutants, we performed three-way competitions between the two pherotypes and the *ΔcomA* strain in a simple experimental assay, where the population is undergoing repeated cycles of growth and propagation with varying frequencies of growth bottlenecks. At the beginning of each cycle, 96 isolates are randomly chosen from cells of the previous growth cycle and are then propagated either in coculture or in multiple

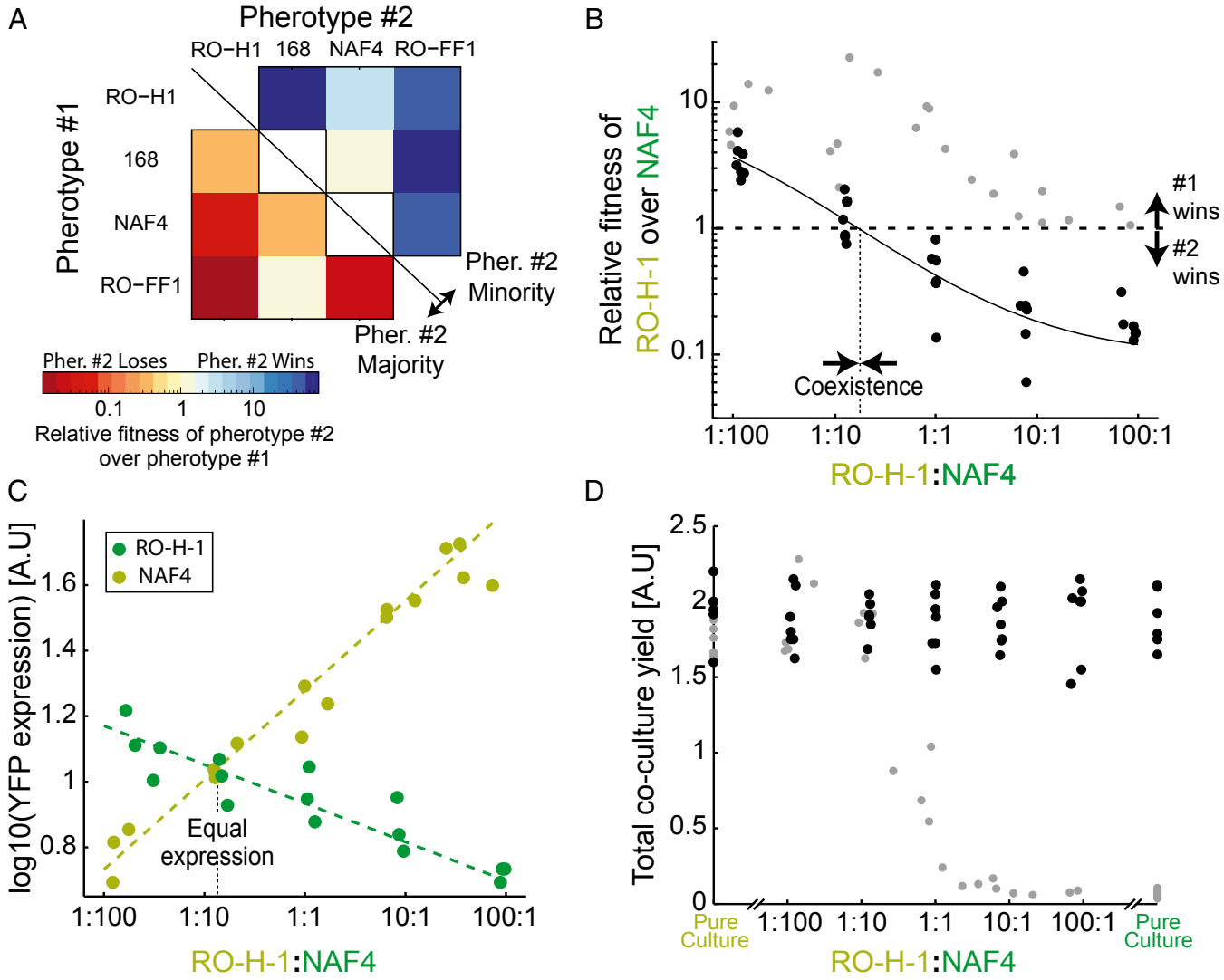

Fig. 3. Pherotypes display negative frequency-dependent selection due to facultative cheating under swarming conditions. (*A*) An interaction matrix between all pairs of nonidentical pherotypes. For each pherotype pair, either pherotype 1 (bottom left) or pherotype 2 (top right) was inoculated as a minority. Each colored square marks the relative fitness of pherotype 2 over pherotype 1, according to the color bar. Strains used are AES3005 (pherotype RO-H-1), AES2355 (pherotype 168), AES3003 (pherotype NAF4), and AES3530 (pherotype RO-FF-1). (*B*) Shown in black circles are the relative fitness values of the RO-H-1 pherotype over the NAF4 pherotype for varying initial ratios of RO-H-1:NAF4 (strains AES3005 and AES3004). The solid black line serves as a guide to the eye. For comparison, the data of Fig. 2*B* are redrawn on the same scale and shown as gray circles (note that for these data points, the *x* axis is the *ΔcomA*:wild-type frequency ratio). (*C*) Average per cell YFP gene expression of the P_{srfA}-YFP reporter inserted into the chromosomes of RO-H-1 (light green, coculture of AES3012 and ASE3009) or NAF4 (dark green, coculture of AES3011 and AES3010) strains. Expression was measured at the end of swarming for cocultures with varying initial ratios of the RO-H-1 and NAF4 strains. (*D*) Final cell yield of swarming cocultures with varying initial ratios of the RO-H-1 and NAF4 pherotype strains (black circles). Yields were also measured for pure cultures of the two strains. As in *B*, gray circles are a representation of the data of Fig. 2*B* on the relative scale and serve for comparison. Each data point in *B*–*D* represents a measurement from a different swarming plate. Experiments were done on multiple days.

clonal cultures, in a medium requiring quorum-sensing-dependent cooperation for growth (see Fig. 4*A* legend and *Methods* for further details). The level of relatedness between cooperating cells is determined by the frequency of cycles that initiates in a growth bottleneck. To facilitate our ability to perform a large number of growth experiments simultaneously, we engineered a synthetic quorum-dependent public good, by placing the *amyE* gene, coding for secreted α-amylase enzyme, under the regulation of a copy of the *srfA* promoter (*Methods* and Fig. S5). This allowed us to use liquid media with starch as the main carbon source for the cooperative growth assay.

We find that at zero relatedness (purely well-mixed cycles), both the *ΔcomA* strain and the minority pherotype initially invaded into the population, but eventually only the *ΔcomA* strain prevails (Fig. 4*B*, *Bottom Right*). In contrast, at an intermediate level of relatedness ($R = 0.5$), the *ΔcomA* strain is selected against after the entire selection scheme is completed, whereas each of the pherotypes invaded from rarity into the population (Fig. 4*B*, *Top*). Finally, in pure clonal growth (relatedness of one), the *ΔcomA* strain is quickly eliminated from the population, whereas the relative frequency of the minority pherotype remains approximately constant (Fig. 4*B*, *Bottom Left*). The nonmonotonic dependence of pherotype coexistence on relatedness is due to the combination of two effects—at low relatedness, the cheater mutant overcomes both pherotypes, whereas if relatedness is sufficiently high, it will be eliminated. On the other hand, the invasion rate of the minority pherotype approaches zero as relatedness approaches unity (36).

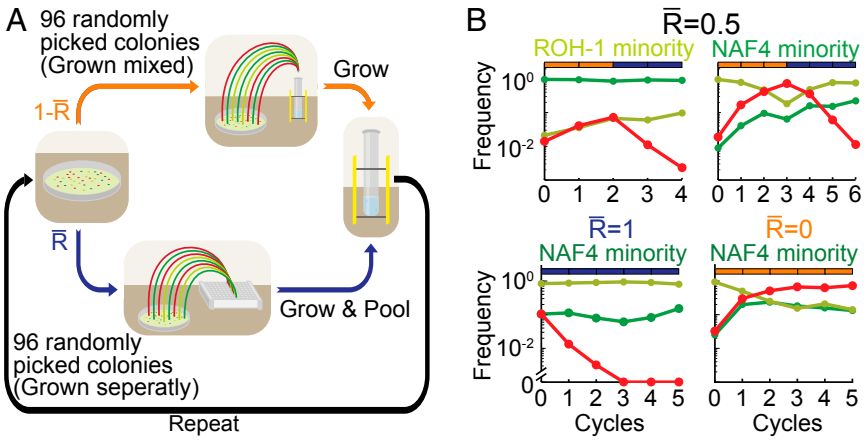

Fig. 4. Pherotypes coexist whereas cheaters are eliminated in the presence of population bottlenecks. (*A*) A scheme of the selection process. In each cycle, 96 single colonies are either mixed together into a single test tube (no bottlenecks, orange) or used to inoculate 96 separate wells (strong bottlenecks, blue). The frequency of clonal cycles in the process is defined as $\bar{R}$. Cells are grown in minimal medium containing soluble starch as a main carbon source and express the gene encoding for the starch-degrading exoenzyme α-amylase under the control of the *srfA* promoter (*Methods* and main text). (*B*) Results of different selection schemes with varying $\bar{R}$. Shown are the absolute frequencies of the *ΔcomA* (red, AES1341), $comQXP_{RO\text{-}H\text{-}1}$ (light green, AES3014), and $comQXP_{NAF4}$ (dark green, AES3013) genotypes during coculture of the three genotypes, as a function of the cycle. The $\bar{R}$ parameter used in each experiment is marked above each graph as well as the identity of the minority pherotype. Blue and orange rectangles at the top of each graph are used to mark the cycles with and without population bottlenecks, respectively.

Discussion

In this work, we showed that *comQXP* pherotype allelic diversity can be maintained by negative frequency-dependent selection through mutual facultative cheating between strains encoding for different pherotypes. We then demonstrated how kin selection through population bottlenecks could simultaneously explain both the observed standing genetic variation of pherotypes and the rarity of quorum-sensing null mutants. Importantly, the bottleneck structure is a generic mechanism, which illustrates the ability of the structured population to select for pherotype variability. We do not know how well the bottleneck model applies to the natural life history of *B. subtilis*.

Facultative cheating was previously defined and identified in the context of fruiting-body forming bacteria and amoebas (18, 37). The complexity of fruiting-body development, however, hinders the elucidation of the molecular mechanisms underlying this behavior (36). In this work, facultative cheating is directly associated with a specific molecular mechanism on the one hand and with observed population genetic diversity patterns on the other. It is important to note that not all bacteria show intraspecific variability in quorum-sensing pherotypes. Pherotype diversity can be constrained by the molecular diversity available to the quorum-sensing signal, by mechanisms that prevent the diversification process (17), or by additional mechanisms that select against exploitation (38).

Pherotypes can be considered as a type of kin-recognition "tags," whose existence, evolution, and impact on the fate of cooperation have been a focal interest in social evolution and signaling theory (e.g., refs. 39–41). Notably, it has been suggested that variability in several bacterial traits, such as the production of bacteriocins (42), contact-dependent inhibition toxins (43), or colony segregation (44, 45), is maintained through kin-recognition mechanisms. Typically, kin-recognition tags are considered to direct cooperative behavior only among organisms with the same tag or to direct aggressive behavior only toward organisms with a different tag. These interactions naturally lead to positive frequency-dependent selection for the majority tag, which tends to reduce tag variability (39). Under such conditions, tag variability is under a constant threat of elimination and can be maintained only by combining population structure and dynamically complex interactions with tag-bearing cheaters, which tend to eliminate the advantage of the most frequent tag in the population (40–42, 46). In contrast, quorum sensing controls only the decision to cooperate, but not the beneficiaries of cooperation. Under these conditions, interaction between tags (pherotypes) directly leads to negative frequency-dependent selection by mutual facultative cheating. Although a structured population is still required in that case to eliminate obligate cheaters, the additional complications are avoided and tag variability is directly favored.

An alternative explanation for the maintenance of pherotype diversity [and kin tags in general (41)] is that it can arise from apostatic selection (47) between the signaling bacteria and another organism that uses the quorum-sensing system to identify the bacteria and attack them. We find this to be unlikely due to the cytoplasmic location of multiple receptors with diverging pherotypes (more discussion in *Supporting Information*).

From an ecological perspective, facultative interactions can occur between different species in a multispecies population, and these may explain some of the diversity found in microbial communities (48). Recent work has demonstrated the ecological richness that can be attained by the chemical diversity of antibiotic production and degradation (49, 50). Our work demonstrates that a similar ecological richness may arise from the diversity of chemical signaling.

Methods

Detailed information on growth media and strain construction is given in *SI Methods* and Table S2.

Swarming Assays. Swarm plates were made of Spizizen minimal media (SMM) containing 0.005% glucose (wt/vol) and supplemented with 0.7% (wt/vol) Bacto-agar. Briefly, cells were grown for 1 d in minimal media before their inoculation and then placed in a humid incubator set to 30 °C for the designated time. Cells were then collected from the plates into a fixed volume of 5 mL. Cell yield was measured using optical density whereas population proportions were measured using a flow cytometer. Further details are in *SI Methods*. Relative fitness of genotype 1 over genotype 2 is determined as $(p_e/(1-p_e))/(p_i/(1-p_i))$, where p_e, p_i are the frequencies of genotype 1 at the end and beginning of the experiment, correspondingly.

Structured Population Assay. Growth was conducted in liquid SMM media with soluble starch as the major carbon source (0.2% wt/vol with addition of 0.01% glucose). Each cycle of growth was either clonal or mixed, where the overall frequency of each mode of growth over the entire experiment is determined by the average R parameter. At the beginning of each growth cycle, 96 colonies are randomly picked from a plate and subsequently are either mixed into a single test tube (no bottleneck cycle) or clonally grown in

separate wells of a 2-mL 96-well plate (bottleneck cycle). Cells were allowed to grow for 24 h and then pooled and plated on an LB plate, to initiate the next growth cycle.

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