Report

Current Biology

Cooperation and Cheating among Germinating Spores

Highlights

- Microbial spore germination can be a cooperative, density-dependent social process
- *Myxococcus xanthus* spore germination involves multiple public-good molecules
- Glycine betaine mediates density dependence of germination under saline conditions
- Glycine-betaine non-producers cheat to germinate more efficiently in mixed groups

Authors

Samay Pande, Pau Pérez Escriva, Yuen-Tsu Nicco Yu, Uwe Sauer, Gregory J. Velicer

Correspondence

samayrp@gmail.com

In Brief

Pande et al. demonstrate that germination of *M. xanthus* spores is a socially multifaceted process mediated by multiple diffusible public-good molecules.

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Report

Cooperation and Cheating among Germinating Spores

Samay Pande,[1,3,4,*] Pau Pérez Escriva,[2] Yuen-Tsu Nicco Yu,[1] Uwe Sauer,[2] and Gregory J. Velicer[1]
[1]Institute for Integrative Biology, ETH Zurich, Universitaetstrasse 16, 8092 Zurich, Switzerland
[2]Institute of Molecular Systems Biology, ETH Zurich, Otto-Stern-Weg 2, 8093 Zurich, Switzerland
[3]Department of Microbiology and Cell Biology, Indian Institute of Science, C.V. Raman Avenue, 560012 Bangalore, India
[4]Lead Contact
*Correspondence: samayrp@gmail.com
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SUMMARY

Many microbes produce stress-resistant spores to survive unfavorable conditions [1–4] and enhance dispersal [1, 5]. Cooperative behavior is integral to the process of spore formation in some species [3, 6], but the degree to which germination of spore populations involves social interactions remains little explored. *Myxococcus xanthus* is a predatory soil bacterium that upon starvation forms spore-filled multicellular fruiting bodies that often harbor substantial diversity of endemic origin [7, 8]. Here we demonstrate that germination of *M. xanthus* spores formed during fruiting-body development is a social process involving at least two functionally distinct social molecules. Using pairs of natural isolates each derived from a single fruiting body that emerged on soil, we first show that spore germination exhibits positive density dependence due to a secreted "public-good" germination factor. Further, we find that a germination defect of one strain under saline stress in pure culture is complemented by addition of another strain that germinates well in saline environments and mediates cheating by the defective strain. Glycine betaine, an osmo-protectant utilized in all domains of life, is found to mediate saline-specific density dependence and cheating. Density dependence in non-saline conditions is mediated by a distinct factor, revealing socially complex spore germination involving multiple social molecules.

RESULTS AND DISCUSSION

Organismal fitness often depends greatly on population density, both negatively [9–11] and positively [12–14]. Positive density dependence of fitness may reflect the operation of cooperative biological traits that increase the absolute fitness of other organisms [13, 15]. Such cooperative interactions have been investigated primarily in the context of population growth [13] and survival [15–17]. However, many organisms [1, 5], including plants [18, 19], animals [20, 21], and microbes [2, 3], persist for periods in a specialized state of metabolic dormancy from which they must emerge, or germinate, before initiating growth and reproduction. The potential for social interactions to affect fitness during germination of dormant organisms has received less attention, although positive and/or negative effects of density on germination of plant seeds [18, 19], pollen [22], and microbial spores [23–28] have been documented.

Many microbes, both eukaryotic and prokaryotic, produce dormant spores that survive environmental stress better than growing cells [1–4] and, in some species, increase dispersal [1, 5]. For the energetically costly process of sporulation to culminate in growth under favorable conditions, spores must successfully undergo the metamorphic process of germination. Given that microbes live in diverse and crowded populations and communities, even small differences in the rate or extent of germination between genotypes could translate into substantial fitness differences and thus be subject to selection.

Microbes exhibit many social traits [3, 29, 30], but whether seemingly dormant spores interact socially, particularly during germination [31, 32], remains unclear. Some microbes, such as the bacterium *Myxococcus xanthus* and the eukaryotic amoeba *Dictyostelium discoideum*, sporulate within multicellular fruiting bodies [2, 3, 6], which might heighten the opportunity for social components of germination to evolve. Previous experiments showed that spherical *M. xanthus* "microcysts" formed under high-nutrient conditions in response to glycerol produce a diffusible factor at high density that promotes germination in water [25]. Although the processes and products of glycerol- versus starvation-induced sporulation differ greatly [33], this result suggests that germination of natural spores formed within fruiting bodies in response to starvation may be a social process [25]. Because individual fruiting bodies that emerge from natural habitats often contain genetically distinct spores [7, 8, 34, 35], social interactions during germination might occur between either identical clonemates or between different genotypes. We explore both of these possibilities using six natural strains of *M. xanthus* sampled from three fruiting bodies, including two focal strains derived from the same fruiting body known to differ greatly in their sensitivity to salinity, an important ecological parameter shaping microbial communities that varies greatly across microbial habitats [36, 37].

We first test for general effects of spore density on germination efficiency in pure culture and whether any such effects are mediated by diffusible molecules. In light of previously identified differences in sensitivity to salt between our focal strains, we then

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examine whether saline stress alters patterns of density-dependent germination. Further, we uncover the molecular identity of a social germination factor specific to conditions of saline stress and test whether "defection" from producing that factor confers a "cheating" phenotype to non-producers.

Germination Density Dependence Is Mediated by a Public-Good Molecule Secreted Prior to Extensive Loss of Stress Resistance

We tested whether spore density affects germination efficiency in pure cultures of six *M. xanthus* natural isolates when spore populations were incubated at low versus high initial spore density in nutrient-rich medium. These strains derived from three fruiting bodies that had emerged on soil collected from three forested locations separated pairwise by ~10 km, two strains each from fruiting bodies GH3.5.9, KF4.3.9, and MC3.5.9 [7]. Strains compared across these fruiting bodies are known to vary at >10,000 polymorphisms in their shared genome content whereas strains sampled from the same fruiting body differ at <10 polymorphisms [8]. Sonicated spores harvested from starvation-induced fruiting bodies of the six strains were resuspended at low and high densities (~10^5 and ~10^7 spores/mL, respectively), incubated in liquid CTT growth medium [38], and assayed for germination (i.e., loss of resistance to heat and sonication) after 12 h. Germination was strongly density dependent, as spore populations of all six isolates germinated almost completely by 12 h at high density whereas germination at low density was extremely low (Figure 1A). These results suggest that germination is generally density dependent in natural populations of *M. xanthus*.

The two strains from fruiting body MC3.5.9 (here referred to as strains A and E for simplicity [7, 39]) were examined more extensively with regard to both density dependence and the potential for strain interactions during germination. These strains were of particular interest based on prior experiments showing a large difference in salt tolerance between them (see STAR Methods). In time-course assays, sonicated spores were incubated at three densities (~10^5, ~10^6, and ~10^7 spores/mL) in nutrient-rich medium and assayed for germination (i.e., loss of resistance to heat and sonication) after 0, 12, and 18 h. Consistent with the assays of all six isolates (Figure 1A), the highest-density treatments of both strains germinated extensively by 12 h whereas both the intermediate- and low-density treatments did not (Figure 1B). From 12 to 18 h, the high-density treatments continued to near-complete germination and the intermediate-density treatments began germinating, but the low-density treatments still showed no evidence of germination after 18 h (Figure 1B).

In a similar experiment with the focal strains A and E performed only at the highest density and at a finer temporal scale, total viable cell counts, which include both ungerminated, stress-resistant spores and germinated, stress-sensitive cells, never decreased throughout the experiment (Figure S1A), indicating the absence of extensive cell death. Total viable counts only increased sometime after 12 h (Figure S1A), indicating that population increase due to division of germinated cells is not detectable for at least that period. Supernatant from high-density spore suspensions (10^7 spores/mL) of strain A sampled after 12 h of incubation in high-nutrient medium was found to induce germination of most spores at low density (10^5 spores/mL) (Figure S1B), indicating that density dependence of germination is at least partially mediated by a diffusible "public-good" [40] molecule secreted by the high-density population.

The diffusible germination factor was not carried over as extracellular material from harvested fruiting bodies because sonicated spores were washed in buffer solution prior to resuspension in germination-inducing nutrient medium. Rather, the assays reported above indicated that the germination factor was secreted by either spores still resistant to stress or by freshly germinated, stress-sensitive cells that had not yet divided in large numbers, or both. We further examined the dynamics of germination and supernatant-rescue potential with strain A, which had not germinated significantly at high density after 8 h in our initial assays but had germinated extensively by 12 h. To do so, we determined the onset and subsequent levels of germination in high-density, high-nutrient cultures at a finer temporal scale and correspondingly assayed for induction of low-density germination by supernatant from high-density spore cultures. Whereas the onset of significant germination in high-density spore cultures of strain A, as reflected by loss of stress resistance, was detected only after 9 h (Figure 1C), supernatant from those same cultures was found to be highly effective at inducing germination of low-density spores already from only 5 h of incubation (Figure 1D). Because supernatant was effective in inducing germination even before a large proportion of donor spores germinated, these results suggest that spore populations initially respond to the presence of growth substrate by at least secreting, and possibly also synthesizing, social germination molecules. The secretion of a public-good metabolite is then followed by large-scale germination resulting in loss of stress resistance.

A Cheatable Cooperative Trait Confers Resistance to Saline Stress during Germination

The focal strains A and E were known from prior experiments to differ in their sensitivity to saline stress during vegetative growth and development. For example, 1% NaCl greatly inhibits spore production during starvation-induced development by strain A but not by strain E (Figure S2A). We thus tested whether patterns of density-dependent germination by these strains are also differentially affected by salinity. Consistent with the greater sensitivity of strain A to salt during development, strain A spores that had formed in the absence of salt were found to be incapable of germinating under saline conditions, irrespective of their initial density (Figure 2A). In contrast, at high density, spores of strain E germinated as proficiently in 1% NaCl conditions as in the absence of added salt (Figures 1B and 2A).

Mixing of these two strains at high density under saline conditions was found to completely suppress germination by the chimeric populations as a whole when strain A was present at either high or intermediate frequency (0.9 and 0.5, respectively; Figure S2B). Such negative effects of genetic polymorphism on the total productivity of microbial groups [41, 42]—"chimeric load" [41]—can be due to either interference competition [43] or negative population-level effects of one genotype producing less of a cooperation molecule than the other (i.e., "defection" [44, 45]). To test which mechanism was responsible for the observed negative effects of strain A on strain E germination in

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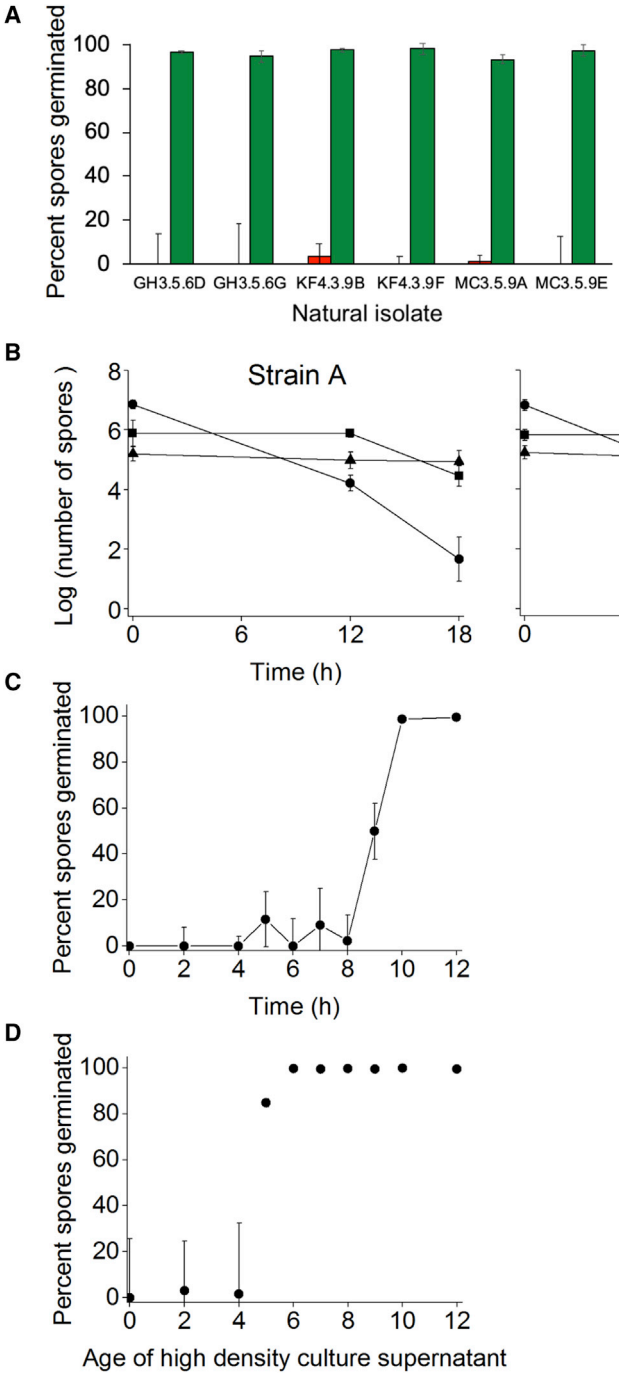

Figure 1. *M. xanthus* Spore Germination Is Density Dependent and Induced by a Diffusible "Public-Good" Molecule Secreted Prior to Extensive Spore Germination

(A) Germination of spores from six distinct natural isolates at high (green) and low (red) density is shown. Bars represent the percentage of spores germinated after 12 h in nutrient-rich medium. Differences between germination frequencies of spores at high versus low density are significant for all six strains (paired-sample t test for differences between arcsine-square-root-transformed germination frequencies, $p < 0.01$). Error bars represent 95% confidence intervals (n = 3).

(B) Viable spore counts of strains A and E over time after inoculation into high-nutrient growth medium at three initial densities of $\sim10^7$ (circles), $\sim10^6$ (squares), and $\sim10^5$ (triangles) spores/mL are shown. Error bars indicate 95% confidence intervals (some of which are smaller than their respective data symbols; n = 3) (also for panels [C] and [D]).

(C) Percentage of strain A spores inoculated into nutrient-rich medium at high density ($\sim10^7$ spores/mL) differentiated into stress-sensitive cells over time.

(D) Percentage of strain A spores inoculated at low density ($\sim10^5$ spores/mL) that had germinated after 12 h of incubation in supernatant taken from high-density spore cultures after variable periods of incubation in nutrient-rich medium.

See also Figure S1.

a saline environment, we used an antibiotic-resistant variant of strain A to test whether its performance during germination can be socially rescued by strain E.

At low frequency (~0.1) in a mixture with strain E, strain A spores were found to germinate extensively in the presence of NaCl (Figure 2B), despite their inability to do so in pure culture (Figure 2A). Thus, strain E produces a social factor of a type or at a level not produced by strain A that confers to strain A the ability to germinate under saline stress. However, suppression of strain A germination by salt was not merely complemented by strain E but strain A actually outperformed strain E at germination in mixed populations. Specifically, strain A increased significantly in frequency by ~10% among germinated cells relative to its initial frequency among ungerminated spores (paired-sample t test for change in frequency, $p = 0.034$; Figure 2B). This result differs greatly from the expectation derived from pure-culture assays that strain A frequency should have decreased greatly among germinated cells in the absence of social interaction (Figure 2B). This pattern conforms to a classic cheating relationship in which one genotype—the cheater—performs worse at a cooperative trait than another genotype—the cooperator—in pure groups but socially exploits and outcompetes the cooperator in mixed groups [10, 44].

Cheating under Saline Stress Is Mediated by Secretion of Glycine Betaine

We next tested whether the social factor secreted by strain E that rescues strain A germination in the presence of salt (Figure 2) is diffusible. To do so, strain A spores were incubated in supernatant from cultures of strain E spores (isolated after 10 h of incubation) under saline conditions. Indeed, strain E supernatant

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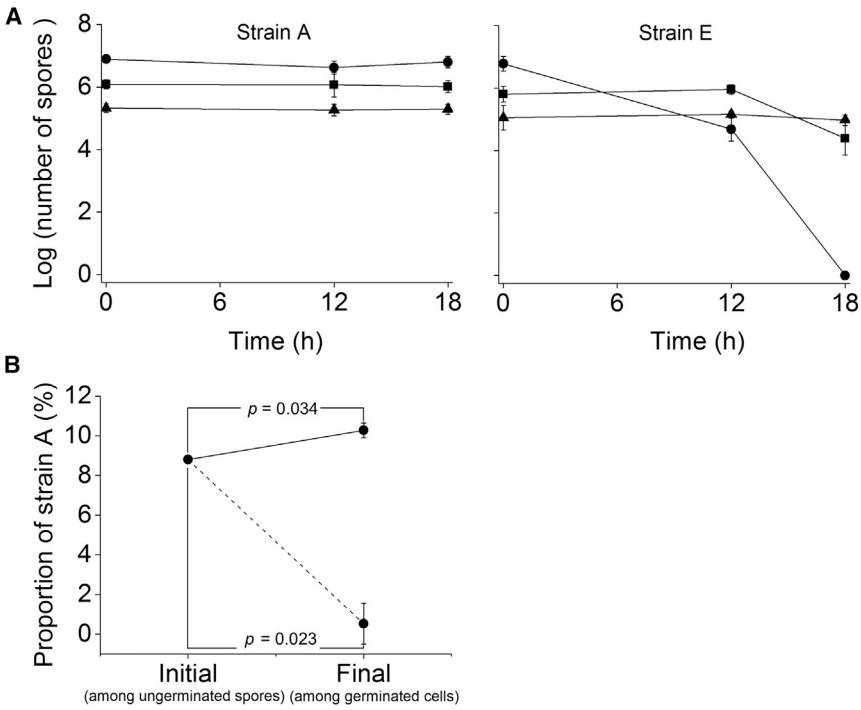

Figure 2. Strain A Exploits a Social Germination Factor Produced by Strain E under Saline Stress to Gain a Germination Advantage

(A) Strain E is germination proficient under saline stress in pure culture whereas strain A is not. Viable spore counts of strains A and E over time after inoculation into high-nutrient growth medium supplemented with 1% NaCl at three initial densities of $\sim 10^7$ (circles), $\sim 10^6$ (squares), and $\sim 10^5$ (triangles) spores/mL are shown. Error bars indicate 95% confidence intervals (some of which are smaller than their respective data symbols; n = 3).

(B) Strain A cheats on strain E during germination under saline stress. Spores of strains A and E were mixed with A in the minority (~9%) and exposed to nutrients (CTT) and salt stress (1% NaCl) for 12 h. Based on pure-culture assays (Figure 2A), in the absence of social interaction between the strains, strain A frequency would be expected to be much lower among stress-sensitive, germinated cells than its initial frequency among spores (dashed line). However, strain A frequency is actually increased among germinated cells. Error bars represent 95% confidence intervals (some of which are smaller than their respective data symbols; n = 3). p values are from paired-sample t tests for differences between the arcsine-square-root-transformed initial and final frequencies of strain A cells.

See also Figure S2.

induced most strain A spores to germinate, whereas extremely few strain A spores germinated in unconditioned medium (t test for difference between arcsine-square-root-transformed frequencies of spore germination in unconditioned versus conditioned media; p = 0.004; Figure 3A).

To identify the social molecule involved in this interaction, we carried out metabolomic analysis of supernatant generated by both strains under both saline and non-saline conditions. When incubated in saline conditions, spores of strain E secreted three compounds that were not detected in either the supernatant of strain A spores under saline conditions or the supernatant of strain E spores under non-saline conditions—aminobutanoic acid, glycine betaine, and 2-aminomuconate (Table S1). Previous studies have shown that glycine betaine is an effective osmo-protectant produced by a wide variety of prokaryotes and eukaryotes [46, 47], including by *M. xanthus*, in which vegetative cells upregulate glycine-betaine production in response to saline stress [48]. We thus quantified and compared the amount of glycine betaine produced by spores of strains A and E after incubation in saline conditions by a more focused method of targeted mass spectrometry. Consistent with our initial results, supernatant from strain E spores had high levels of glycine betaine (189 μM on average), whereas strain A supernatant did not (Figure S3A). Together, these experiments suggested that glycine betaine is the diffusible factor that complements the ability of strain A to germinate under saline conditions.

We tested this hypothesis directly by adding glycine betaine to cultures of strain A spores under saline conditions and found that, like strain E supernatant, glycine betaine induced germination by the vast majority of strain A spores (~98%), which did not germinate in its absence (t test for difference between arcsine-square-root-transformed frequencies of spore germination in unconditioned versus conditioned media; p = 0.016; Figure 3B). We also tested for a change in glycine-betaine concentration when spores of strain A are incubated in the culture supernatant of strain E. In these experiments, glycine-betaine concentration decreased greatly within 12 h (Figure S3B), suggesting that strain A takes up glycine betaine produced by strain E.

Glycine-Betaine Synthesis Is Costly

The ability of strain A to exploit glycine betaine produced by strain E to gain a relative fitness advantage in mixed groups under saline conditions (Figure 2) suggests that glycine betaine is costly to produce and that energy saved by non-production can increase the germination rate of non-producers in the presence of producers. To test whether glycine-betaine production per se has a fitness cost, we replaced the promoter of the methyltransferase gene *MXAN_7068* responsible for glycine-betaine synthesis by a vanillate-inducible promoter [48, 49]. This allowed us to regulate the amount of glycine betaine synthesized by strain E^{van} (Figure S3C). Under saline conditions in pure culture, strain E^{van} germinated extensively only when glycine-betaine synthesis was induced by addition of vanillate, whereas in the absence of vanillate only a small fraction of strain E^{van} spores germinated (Figure S3C). These results demonstrate that glycine-betaine production is important for strain E germination.

Strain E^{van} should not incur significant costs of glycine-betaine synthesis in the absence of vanillate. Such cost avoidance might result in a fitness advantage over a cost-incurring producer in mixed groups in environments in which glycine betaine is beneficial. To test this hypothesis, we competed strain E^{van} with strain E during germination under saline conditions and found that strain E^{van} germinates at a higher efficiency than strain E in mixed groups (t test for differences between arcsine-square-root-

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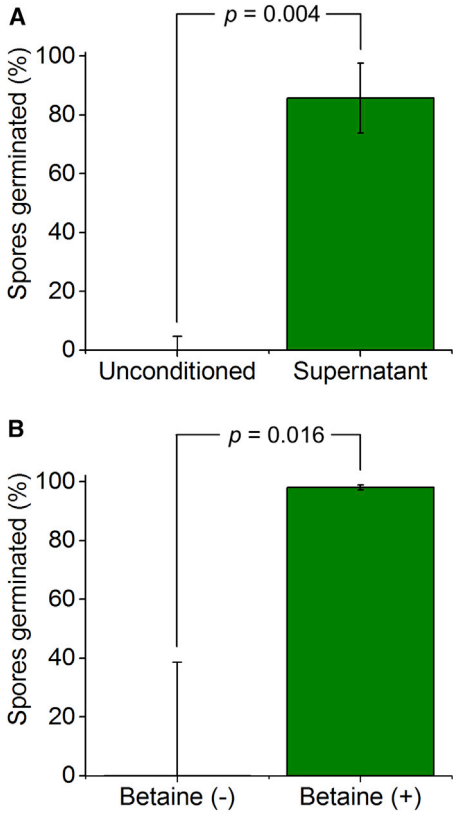

Figure 3. Strain E Supernatant and Glycine Betaine Both Rescue the Germination Defect of Strain A in the Presence of NaCl

The proportions of strain A spores that germinated in a high-salt environment after 12 h in medium conditioned by (A) strain E supernatant and (B) glycine betaine versus unconditioned medium are shown. Error bars represent 95% confidence intervals (n = 3). p values reflect paired-sample t tests for differences between arcsine-square-root-transformed frequencies of spore germination in unconditioned versus conditioned media. See also Figures S3 and S4.

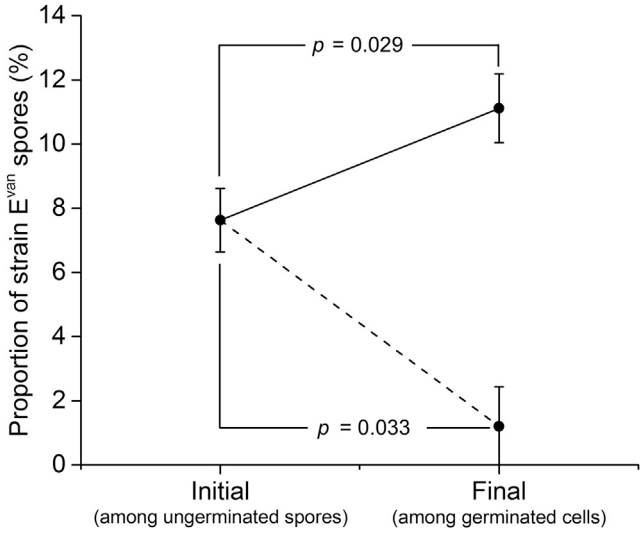

Figure 4. Defection from Glycine-Betaine Production in Mixed Groups with Producers Confers a Germination Advantage under Saline Stress

Mixed pairwise germination competitions between strains E and E^{van} were performed in the presence of 1% NaCl. In the absence of supplemental glycine betaine, E^{van} frequency is increased among stress-sensitive germinated cells prior to population growth relative to the initial E^{van} frequency among ungerminated spores (solid line). Performance of the two strains in pure culture generates the expectation that E^{van} frequency should decrease greatly in these competitions in the absence of social exploitation (dashed line). Error bars represent 95% confidence intervals (n = 3). p values are for paired-sample t tests for differences between initial and final arcsine-square-root-transformed frequency values. See also Figure S3D.

transformed strain E^{van} frequency among pre-germinated spores versus among germinated cells after 12 h, $p < 0.03$; Figure 4), despite its germination defect in pure culture (Figure S3C). This fitness advantage is likely due to an ability of E^{van} to allocate metabolic cost savings from glycine-betaine non-production toward more rapid germination when glycine betaine is provided by strain E.

However, when glycine betaine was experimentally added to germination competitions between strain E and strain E^{van}, E^{van} frequency neither increased nor decreased among germinated cells relative to its initial frequency among spores (Figure S3D). This result suggests that glycine-betaine production is autoregulated in response to its extracellular concentration, such that neither strain E nor strain E^{van} incurs production costs upon glycine-betaine supplementation and both can thus germinate equally well.

Glycine Betaine Does Not Mediate General Density Dependence

The observation that spore germination by strain A is density dependent in the absence, but not the presence, of salt suggests that glycine betaine is not the social factor mediating density dependence for either strain A or E in the absence of salt. In theory, however, glycine betaine might be responsible for the density-dependent germination of strain E under both saline and non-saline conditions and that of strain A under non-saline conditions but fail to be produced by strain A under saline stress, thus resulting in the patterns observed in Figures 1 and 2. To test this latter (and in our view unlikely) hypothesis, glycine betaine was added to low-density spore cultures of both strains in the absence of added salt. As expected, glycine betaine did not rescue the failure of either strain to germinate at low density (results not shown). This result implies that *M. xanthus* spore germination involves at least two distinct social factors, one responsible for general density dependence under benign germination conditions and another—glycine betaine—necessary for germination under saline stress. It will be of interest to identify the chemical nature of the former and also investigate whether there are yet more social germination factors in this species.

Many microbes secrete diffusible public goods—molecules that benefit not only the individuals that produced them but also others within diffusion range [40]. For example, public-good molecules are involved in nutrient scavenging [50], quorum sensing [51], antibiotic degradation [52], extracellular catabolism (invertase) [53], bio-surfactants, and biofilm exo-polysaccharides. We have found that diffusible factors also promote nutrient-induced germination of bacterial spores harvested from *Myxococcus* fruiting bodies. These findings indicate that

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yet another life-cycle phase of the highly social myxobacteria, which exhibit positive density dependence of motility [54], growth on macromolecular substrates [55], fruiting-body development, and sporulation [17], involves cooperation. Social germination among our focal strains of *M. xanthus* involves at least two distinct public-good molecules that mediate positive density dependence of germination efficiency, one under benign laboratory conditions and one—the widespread osmo-protectant glycine betaine—under salt stress.

Glycine-betaine production was found to impose an individual-level metabolic cost that reduces germination efficiency relative to non-producing or low-producing cheaters that exploit production by others under salt stress (Figures 2 and 4). Our two focal strains, one a glycine-betaine producer and one a non-producer, were isolated from the same fruiting body that emerged on a natural soil sample. This suggests that cheating on public-good production by some genotypes during germination may contribute to the genetic diversity commonly found within natural social groups of *M. xanthus* [3]. (Cooperators and cheaters can both be maintained in a population when cheating occurs below a threshold cheater frequency [56, 57].) More broadly, because glycine betaine is produced by many species [46], non-producers may sometimes also benefit from inter-specific complementation.

The question of how much metabolic activity occurs within "quiescent" spores has received attention in some species [58, 59]. Our results strongly suggest that starvation-induced *M. xanthus* spores themselves release a social germination factor prior to the onset of morphological transformation. Whether this factor is actively synthesized by spores or rather is merely released by spores after having been previously generated during vegetative growth or spore formation remains to be determined.

Ramsey and Dworkin hypothesized that the primary benefit of fruiting-body formation by myxobacteria might not be enhanced spore dispersal but rather high spore density upon germination in low-nutrient environments [25]. If fruiting bodies actually evolved due to such a benefit, this would require that both sporulation and density-dependent germination existed prior to the origin of fruiting-body formation, which is not known. Although our results with spores formed during fruiting-body development do not confirm this hypothesis as correct, they do strengthen its plausibility and expand its relevance to a broader range of conditions, including germination in nutrient-rich and saline environments. Given that density can affect germination from dormant states in some plants [18, 19], slime molds [23, 24], and other bacteria [26, 28], further investigation of potential social interactions during spore germination across a broad range of microbes, including humans pathogens, is of interest.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2020.08.091.

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AUTHOR CONTRIBUTIONS

S.P. and G.J.V. conceived the study and designed the experiments. S.P. performed all experiments except the metabolomic analysis and generation of pMRNY3629. P.P.E. and U.S. designed, performed, and reviewed the mass spectrometry analysis. Y.-T.N.Y. designed and generated plasmid pMRNY3629 and designed the experiments to generate plasmid pMRNY7068 and strain E^{van}. S.P., P.P.E., and G.J.V. analyzed and interpreted the results. S.P. and G.J.V. wrote the manuscript. All authors amended the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and Virus Strains		
See Experimental Model and Subject Details	[7, 8, 39]	N/A
Chemicals, Peptides, and Recombinant Proteins		
Kanamycin	Sigma-Aldrich	Cat# K1377-25G
BD Bacto casitone	Fischer scientific	Cat# 225910
BD Bacto agar	Fischer scientific	Cat# 214030
Tris base	Sigma-Aldrich	Cat# T1503-1KG
Magnesium sulfate	Sigma-Aldrich	Cat# M1880-1KG
Potasium phosphate	Fluka	Cat# 60356
Oligonucleotides		
Forward primer: 5′AACATGTGAGTGAACGGCGGTTTTCGCACG3′	This study	N/A
Reverse primer: 3′CACCACGACCGCGACCTGTTGC5′	This study	N/A
Recombinant DNA		
pMRNY3629	This study	N/A
pMRNY7068	This study	N/A
Software and Algorithms		
SPSS 23.0	SPSS, Chicago, IL, USA	https://www.ibm.com/sa-en/marketplace/spss-statistics
Origin	OriginLabs corps, Northampton, MA, USA	http://www.originlab.com/

RESOURCE AVAILABILITY

Lead Contact

Further information and request for resources should be directed to and will be fulfilled by the Lead Contact Samay Pande (samayrp@gmail.com).

Materials Availability

Unique reagents used in this study will be freely available.

Data and Code Availability

The published article includes all the data generated during this study.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Strains

Strains used in this study were derived from three fruiting bodies (GH3.5.6, KF4.3.9 and MC 3.5.9) isolated from three undisturbed wooded locations in Indiana, USA as described previously [7]. These fruiting bodies were selected because they were previously known to harbor endemic diversity which originated from distinct local ancestors [7]. Strains GH3.5.6.c46 [7] (aka GH3.5.6.D [39]), GH3.5.6.c5 [7] (aka GH3.5.6G [39]), KF4.3.9c9 [7] (aka KF4.3.9B [39]), KF4.3.9c47 [7] (aka KF4.3.9F [39]), MC3.5.9c2 [7] (aka MC3.5.9A [39], here 'strain A'), and MC3.5.9c25 [7] (aka MC3.5.9E [39], here 'strain E')) and are among those strains previously characterized for interactions during starvation-induced development on nutrient-limited TPM medium [39]. A previously reported kanamycin-resistant variant of strain A that exhibited no significant marker effect on sporulation [39] or spore germination was also used here. Differences in saline sensitivity between strains A and E were discovered in experiments performed prior to this study investigating the effects varying several ecological parameters on interactions between strains during development.

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Semantics

Here we use a broad definition of 'cooperation' in the context of intra-specific social interactions as simply behavior that is beneficial to conspecifics [13, 15], regardless of exactly what combination of forces were responsible for the evolutionary origin of the behavior (which usually are not clearly known). We use 'cheating' to mean a fitness advantage gained by a focal individual/genotype by exploitation of cooperative trait expressed by others and associated with low or non-expression of the cooperative trait by the cheater.

METHOD DETAILS

Culture conditions

All agar plates were incubated at 32°C and 90% rH, all liquid cultures were incubated at 32°C, 300 rpm and all spore suspensions were incubated at 32°C. All *M. xanthus* strains were stored frozen in CTT liquid medium [38] with 20% glycerol. To obtain spores for germination assays, strains were inoculated onto CTT 1.5% agar medium [38] from frozen stocks, incubated for 3-5 days, inoculated from the colony edge into 8 mL liquid CTT, and incubated for ~24 h at 300 rpm. Cultures were then centrifuged at 20°C for 15 min at 5000 rpm and pellets were resuspended with fresh TPM liquid buffer [60] to a density of $\sim 5 \times 10^9$ cells/ml prior to being spotted on TPM starvation agar (1.5% agar). Starvation plates were then incubated for 5 days, after which the cell populations were harvested with a sterile scalpel, transferred into 1 mL ddH_2O and heated for 2 h at 50°C. After heat treatment, spores were sonicated twice for 10 s. All experiments were performed in three independent replicate sets.

Spore germination assays

To adjust the density of heat treated and sonicated spores to $\sim 10^7$ spores/ml, optical density (at 600 nm) of the resuspended spore suspension was adjusted using standard curves (optical density versus spore density). To obtain spore suspension of $\sim 10^6$ spores/ml and $\sim 10^5$ spores/ml, spore suspension of $\sim 10^7$ spores/ml was diluted using ddH_2O.

Spores were resuspended in 100 μL of fresh liquid CTT or CTT + 1% NaCl medium to a density of 10^7 spores/ml (or dilutions thereof for low-density treatments) and incubated at 32°C in Tecan 200pro (Tecan Inc Austria) for specified periods. Spore suspensions before incubation (t_0) and after defined time points were transferred into 900 μL TPM liquid medium, heated for 2 h at 50°C and sonicated twice for 10 s. Sonicated cultures were dilution plated onto CTT soft agar plates with or without 40 μg/ml kanamycin. Heat treatment and sonication kill all cells that have germinated sufficiently to have lost resistance to these treatments, such that colonies appearing in CTT soft agar after dilution plating are derived from spores that had not yet germinated at the time of heat/sonication treatment. Differences between the colony counts from t_0 culture and later time points reflect the proportion of the initial spore population that had germinated at each time point.

Effects of density and salt on germination

To test for density-dependent germination among all six natural isolates simultaneously, spores were inoculated at $\sim 10^7$ and $\sim 10^5$ spores/ml in CTT medium and incubated for 12 hours. In some experiments focusing on strains A and E (Figures 1B and 2), spores were inoculated at three different densities ($\sim 10^7$, 10^6 and 10^5 spores/ml) in either CTT or CTT + 1% NaCl). For the assays of temporal germination dynamics, independent wells were disruptively sampled at specified time points.

Effects of conditioned medium on germination

To obtain supernatant for conditioning the germination medium (CTT + NaCl), spores were resuspended in 1 mL CTT + 1% NaCl medium at a density of $\sim 10^7$ spores/ml, incubated for 10 h, centrifuged for 15 min at 5000 rpm and filter sterilized through a 0.22 μm filter. Filtered supernatant was then mixed at a 1:1 ratio with unconditioned CTT + 1% NaCl medium. Washed spores were then added to this conditioned medium for to the appropriate density for germination assays.

To obtain the supernatant of high density spore suspension, spores of strain A ($\sim 10^7$ spores/ml) were resuspended in 1 mL CTT medium, incubated for 10 h, centrifuged for 15 min at 5000 rpm and filter sterilized through 0.22 μm filter. Filtered supernatant and fresh CTT medium was mixed in equal proportion and washed spores of strain A were added to the conditioned medium at the density of $\sim 10^5$ spores/ml.

Temporal analysis of high-density supernatant effects on low-density germination

To obtain the supernatant of high density spore suspension from different time points during incubation, spores of strain A ($\sim 10^7$ spores/ml) were resuspended in 1 mL CTT medium, incubated for designated time points (as shown in Figure 1), centrifuged for 15 min at 5000 rpm and filter sterilized through 0.22 μm filter. Different spore suspension was used for extracting supernatant from different time points. Next, Filtered supernatant was then mixed at a 1:1 ratio with unconditioned CTT + 1% NaCl medium. Washed spores were then added to this conditioned medium at the density of $\sim 10^5$ spores/ml for germination assays. The percentage of low-density spores germinated at each time point during incubation in supernatant from high density cultures was calculated by estimating the number of stress-resistant spores present after 0 and 12 h incubation in the focal supernatant sample. The t_{12} value was then subtracted from the t_0 value to give an estimate of the number of spores germinated and the percent-germinated scores was calculated as

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$$\%\ germinated = 100 * \frac{number\ of\ spores\ germinated}{initial\ number\ of\ spores}$$

Germination competition experiments

For co-germination assays, spores of competing strains were mixed in 1:9, 1:1 and 9:1 ratios in CTT and CTT + 1% NaCl. In each competition mixture the kanamycin-resistant variant of strain A was mixed with kanamycin-sensitive strain E, which allowed quantification of the relative germination efficiencies of the two strains. At both time points (both t_0 and t_{12}), the difference between colony counts on CTT versus CTT-kanamycin soft-agar media gave the colony-count estimate for strain E. The frequencies of each competitor at both t_0 and t_{12} were estimated in both untreated samples and samples subjected to heat and sonication. This allowed estimation of competitor frequencies among both ungerminated spores and among any germinated cells at both time points and calculation of frequency changes in both categories over the 12 h incubation. Paired-sample t tests were performed to test whether relative frequencies of the competing strains among ungerminated spores and among germinated cells changed significantly during incubation relative to their initial frequencies among ungerminated spores at t_o.

Effect of salt on spore production

For sporulation assays, strains were inoculated in CTT 1.5% medium from frozen stocks, incubated for 3-5 days, inoculated from colony edge in liquid CTT medium and incubated for 24 h at 300 rpm. Cultures were then centrifuged for 15 min at 5000 rpm and then resuspended in liquid TMP medium at a density of 5×10^9 cells/ml. 100 μL of this cell suspension was spotted on TMP 1.5% agar medium with or without 1% NaCl, incubated for 5 days at 32°C. After 5 days spores were harvested with sterile scalpel, transferred to 1 mL ddH_2O, heat treated for 2 h at 50°C, and dilution plated in CTT 0.5% agar medium. Dilution plates were incubated for 6 days at 32°C, after which colonies were counted to estimate spore productivity of strain A and E on TPM 1.5% agar medium with and without 1% NaCl.

Rescue of germination under saline stress by glycine betaine

To test the effect of glycine betaine on strain A germination in high salt environments, strain A spores were inoculated in 100 μL CTT-NaCl medium with and without 50 μM glycine betaine (Sigma). The proportion of the strain A populations germinated in these media was estimated after 12 h incubation as the difference in total viable cell counts before versus after heat and sonication.

Strain E^{van} construction

Plasmid pMRNY3629 is a derivative of pMR3629 [49], which features a vanillate-inducible gene expression system, a tetracycline-resistance marker and a 1.38 kb insert [61] of *M. xanthus* genomic sequence spanning the *Mxan_0018-Mxan_0019* gene region of strain DK1622. To generate pMRNY3629, the *Mxan_0018-Mxan_0019* segment was excised from pMR3629 by HindIII restriction-enzyme digestion followed by re-ligation of the vector. Insertion of a gene (or partial gene) of interest under the vanillate-inducible promoter in pMRNY3629 promotes integration of the resulting plasmid into the native locus of the cloned genetic fragment.

Plasmid pMRNY7068 was generated by inserting a 402 bp 5′-terminal fragment of *Mxan_7068* between the NdeI and BglII sites of pMRNY3629. To do so, the *Mxan_7068* fragment was amplified using colony PCR (forward primer: 5′AACATGTGAGTGAACGGCGGTTTTCGCACG3′, reverse primer: 3′CACCACGACCGCGACCTGTTGC5′) cloned into the PCR-Blunt vector (Thermo Fisher Scientific corporation, california, USA), excised using NdeI and BamHI and ligated into pMRNY3629 after restriction by NdeI And BamHI. Correctness of insert sequence was confirmed by sequencing.

pMRNY7068 was transformed into strain A using a previously described protocol [62]. Colonies from selective media were picked, grown to high density, stored frozen and analyzed for their ability to germinate in CTT + 1% NaCl with and without vanillate induction. To do so, spores were incubated in media containing 0, 50 and 100 uM vanillate and the number of viable spores was estimated both immediately and (t_0) and after 12 h of incubation (t_{12}). One of these transformants was named E^{van} and used for quantitative assays (Figure S3C).

Mass spectrometry measurements

To obtain conditioned cultured supernatant for mass spectrometry, spores of strain A and E were resuspended at a density of $\sim 10^7$ spores/ml in 1 mL fresh liquid CTT medium either with or without 1% NaCl and incubated for 10 h at 32°C. Supernatant was obtained by centrifugation of the medium 15 min at 5000 rpm and followed by filter sterilization through a 0.22 μm filter. We also quantified glycine betaine concentration over time in 10-h supernatant of strain E in the presence of strain A spores over a period of 10 h. Supernatent for analysis was obtained by centrifugation of spore cultures for 15 min at 5000 rpm and filter sterilization through a 0.22 μm filter.

Untargeted mass spectrometry analysis

For untargeted analysis, diluted samples were injected into an Agilent 6550 time-of-flight mass spectrometer (ESI-iFunnel Q-TOF, Agilent Technologies) operated in both negative and positive mode, at 4 Ghz, high resolution, and with a mass / charge (m/z) range of 50−1,000. The mobile phase was 60:40 isopropanol:water (v/v) and 1 mM NH4F at pH 9.0 for negative mode and 60:40 methanol water with 0.1% formic acid at pH 3 for positive mode. For online mass axis correction in both modes mobile phases were

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supplemented with hexakis(1H, 1H, 3H- tetrafluoropropoxy)phosphazine and 3-amino-1-propanesulfonic acid for online mass correction. After processing of raw data as described in Fuhrer et al. [63], m/z features (ions) were annotated by matching their accurate mass-to-sum formulas of compounds in the *Myxococcus xanthus* and *Escherichia coli* KEGG database with 0.003 Da mass accuracy and accounting for deprotonation [M-H+]-. Notably, this metabolomics method cannot distinguish between isobaric compounds, e.g., metabolites having identical m/z values (e.g., glycine betaine versus valine), and in-source fragmentation cannot be accounted for.

Targeted mass spectrometry analysis

In order to quantify changes in glycine betaine concentration, supernatant from each sample was diluted in water by a factor of 20. Diluted samples were injected into an Agilent 6550 time-of-flight mass spectrometer (ESI-iFunnel Q-TOF, Agilent Technologies). Ions were targeted for MS/MS fragmentation as [M+H]+ electrospray derivatives with a window size of ± 2 m/z in Q1. Fragmentation of the precursor ion was performed by collision-induced dissociation at 10 and 20 eV collision energy, and fragment-ion spectra were recorded in scanning mode by high-resolution time-of-flight MS. Peaks with intensity of at least 10% of the highest non-saturated peak intensity in the MS/MS spectra were extracted.

To distinguish between glycine betaine and valine present in the CTT medium, which both have the same m/z ratio (117.079 m/z), the fragmentation method described above was performed on standards of both compounds (Sigma-Aldrich, Schnelldorf, Switzerland)). Standards were prepared in 20x-diluted CTT medium to mimic the ionization matrix of the supernatant samples. In brief, the ionization potential of a focal molecule is affected by the totality of molecules present in the sample. This is often referred as the ionization matrix effect. To have comparable ionization potential and therefore ion intensities for a given ion concentration between the standard samples and the supernatant samples, standard samples were prepared in the same media as the supernatant samples. Fragment ions m/z 72.10, 58.10 and 59.10 allow distinction of both ions which is consistent with available spectra from MassBank of North America (MoNA, http://mona.fiehnlab.ucdavis.edu/) (accession keys KO002504 and PB000388) (Figure S4). The intensities of betaine-fragment ions of 59.1 m/z s were fitted with a second-degree polynomial function (Figure S4B). Limit of detection ion intensity was established based on the following formula:

$$LOD = \overline{CTT\ medium} + 3.3 * \sigma(CTT\ medium)$$

Where $\overline{CTT\ medium}$ is the average ion intensity of the 20x-diluted CTT medium, and $\sigma(CTT\ medium)$ its standard deviation.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data analysis was done using SPSS 23.0 (SPSS, Chicago, IL, USA). Details of the statistical test used are mentioned in the figure legends. All experiments were performed in three temporally separated independent replicates. Percentage data was converted to frequency followed by arcsine square root transformation before statistical analysis.