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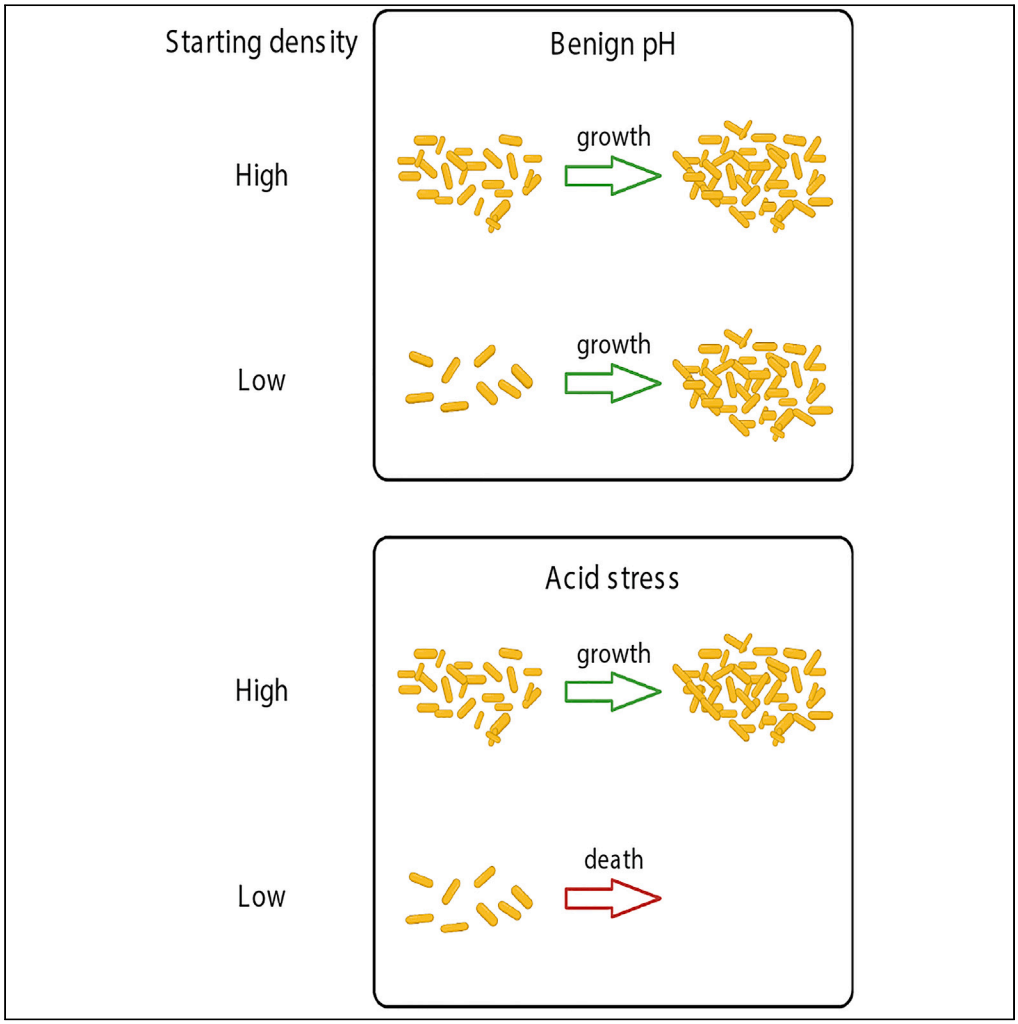

Francesca Fiegna, Samay Pande, Hansrainer Peitz, Gregory J. Velicer

gregory.velicer@env.ethz.ch

Highlights

Survival and growth of *Myxococcus xanthus* under acid stress is density dependent

Density-dependent *M. xanthus* growth under acid stress is mediated by public goods

Density-dependent growth is common across phylogenetically diverse bacteria

Mechanisms of density-dependent growth under acid stress can differ across species

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Widespread density dependence of bacterial growth under acid stress

Francesca Fiegna,[1] Samay Pande,[1,2] Hansrainer Peitz,[3] and Gregory J. Velicer[1,3,4,*]

SUMMARY

Many microbial phenotypes are density-dependent, including group-level phenotypes emerging from cooperation. However, surveys for the presence of a particular form of density dependence across diverse species are rare, as are direct tests for the Allee effect, i.e., positive density dependence of fitness. Here, we test for density-dependent growth under acid stress in five diverse bacterial species and find the Allee effect in all. Yet social protection from acid stress appears to have evolved by multiple mechanisms. In *Myxococcus xanthus*, a strong Allee effect is mediated by pH-regulated secretion of a diffusible molecule by high-density populations. In other species, growth from low density under acid stress was not enhanced by high-density supernatant. In *M. xanthus*, high cell density may promote predation on other microbes that metabolically acidify their environment, and acid-mediated density dependence may impact the evolution of fruiting-body development. More broadly, high density may protect most bacterial species against acid stress.

INTRODUCTION

Organismal density often greatly affects survival and reproduction, both positively and negatively.[1–4] Such density dependence of fitness can mediate selection both on dispersive and aggregative traits that modify density *per se* and on other traits with density-sensitive fitness effects. A positive relationship between fitness and density—the Allee effect—is observed under some ecological conditions in many species.[5–16]

Beneficial fitness effects of high density can be byproducts of simple organismal proximity. For example, organismal proximity can reduce heat or water loss, locomotion costs or predation risk.[1,10,17–21] Positive density dependence (PDD) of fitness can also derive from social traits that are selectively favored, at least in part, due to fitness benefits conferred on others by individuals expressing the focal trait.[22,23] Fitness benefits derived from conspecific interactions can be demonstrated experimentally by two distinct approaches. One approach is to demonstrate that interaction between individuals of distinct genotypes increase the fitness of a focal individual (or genotype) relative to its performance under identical conditions in the absence of inter-genotype interaction, even if total population density is held constant.[24,25] An alternative approach is to manipulate total population density and thereby demonstrate that average fitness increases with density.[26,27]

Microbes express many traits known or hypothesized to benefit their conspecifics, including quorum-sensing signals that regulate gene-expression responses to environmental cues.[28,29] However, relatively few studies have directly demonstrated PDD of population growth by manipulating cell density with strains not artificially engineered to exhibit such PDD.[7,28,30–35] Fewer still have investigated relationships between density and fitness in microbial populations under stress imposed by fundamental abiotic environmental parameters, such as pH, temperature, and hydration,[33,36–38] despite the major roles such forces have played in the evolution of microbial life. Here, we experimentally examine the fitness response of several bacterial species to variable density across environments varying in pH, an important selective force across microbial habitats that varies greatly across both large and small spatiotemporal scales and strongly affects microbial community composition.[39,40] Acidity in particular is a common microbial stressor, and some individual bacterial species have been found to exhibit PDD of survival[41] or growth[30] under acid stress. However, the phylogenetic generality of density-dependent growth under acid stress has not, to our knowledge, been tested systematically.

[1]Institute of Integrative Biology, ETH Zurich, Zurich, Switzerland

[2]Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore, India

[3]Department of Biology, Indiana University, Bloomington, IN, USA

[4]Lead contact

*Correspondence: gregory.velicer@env.ethz.ch

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We first examine interactive effects of density and pH on growth by *M. xanthus*, a bacterium that exhibits unusually sophisticated cooperative traits among prokaryotes[42] and pervades terrestrial soils.[43] *M. xanthus* employs two distinct motility mechanisms with social components,[44,45] preys on other microbial species,[46] and forms multicellular fruiting bodies upon starvation.[47,48] The effectiveness of several *M. xanthus* behaviors is positively density dependent, including motility requiring Type IV pili, fruiting-body development, spore germination, and growth on non-hydrolyzed macropeptides.[26,27,49,50] *M. xanthus* predatory efficiency has also been hypothesized to depend positively on density.[50]

After demonstrating that *M. xanthus* growth and development become increasingly dependent on density with increasing pH stress, we test whether such PDD of growth under acid stress is a common bacterial trait by examining four additional diverse bacterial species. We additionally investigate whether observed acid-stress Allee effects are mediated by diffusible "public good" secretions.

RESULTS

Myxococcus survival and growth under pH stress is strongly density dependent

Preliminary experiments with the reference strain GJV1 growing on agar surfaces that measured optical density (OD) over time suggested that *M. xanthus* growth rate is density dependent at some degrees of both acidic and basic pH stress. Especially in the acidic direction, growth rate appeared more sensitive to deviation from pH 7.5 (the pH at which the low-density populations grew best in those experiments) at low cell density than at high density. To allow direct estimates of viable-cell population size, we further investigated the effect of acid stress on density-dependent growth by *M. xanthus* using strain DK3470, which disperses more easily in liquid after growth on agar than GJV1 due to a mutation that reduces production of extracellular matrix components.[51,52] Based on our preliminary results with GJV1, we performed growth assays with DK3470 at pH 5.0, 6.0, and 7.5.

When cells were inoculated onto CTT agar at low density (LD, ~1.25×10^6 cells/ml) at the three pH levels, viable population size decreased in both acidic treatments (pH 5.0, 6.0) until no viable cells were detected at later time points, whereas populations grew robustly from the same LD at pH 7.5 (Figures 1and S2). Starting at 100-fold higher density (intermediate density, ID, ~1.25×10^8 cells/ml), viable populations decreased to non-detection at pH 5.0 but grew steadily at both pH 6 and pH 7.5. Populations initiated at the highest density (HD, ~1.25×10^{10} cells/ml) increased only slightly before reaching maximum population size. However, these HD populations reveal positive density dependence of survival in that they did not decrease substantially even at pH 5.0 as did the lower-density populations, but rather remained relatively stable throughout the experiment in all pH treatments. Thus, under even mild acid stress, cell density determines whether *M. xanthus* populations survive and grow. These outcomes reflect a strong Allee effect[6] in which populations increase or decrease depending on whether they are above or below a threshold density, respectively.

A pH-regulated diffusible secretion mediates PDD of *M. xanthus* growth under acid stress

We tested whether the exported social molecule(s) mediating density-dependent growth of *M. xanthus* under acid stress is diffusible or cell-bound. To do so, we supplemented populations initiated at LD and pH 6.0—conditions that normally lead to population decline (Figure 1)—with filter-sterilized supernatant from cultures that had been initiated at LD or intermediate density (ID) (same densities as in the Figure 1 experiment) and incubated at pH 6.0, 7.5, and 8.5 for 18 h. As expected, supernatant from all populations initiated at LD, regardless of the pH at which those populations were incubated, failed to prevent population decline of cultures initiated at LD and pH 6.0, as did control supplementation with CTT growth medium (Figure 2). However, supernatant from both ID populations incubated at the two lower pH levels induced significant growth of LD populations at pH 6. These outcomes demonstrate that density-dependent survival and growth by *M. xanthus* is mediated by a diffusible secretion. Unlike supernatant derived from ID cultures grown at pH 6.0 and 7.5, supernatant from ID cultures growing at pH 8.5 failed to induce growth of LD pH 6.0 cultures. This latter outcome reveals that the production and/or stability of the diffusible *M. xanthus* PDD factor is regulated by environmental pH.

The secreted PDD factor might protect from acid toxicity either by increasing environmental pH or by inducing changes in cellular state that render cells less sensitive to unchanged acidity. To test between these possibilities, we compared the pH of supernatant from ID, pH 6.0 cultures after 18 h of incubation to pH measurements of the original medium but found no significant change ($p > 0.05$, one sample

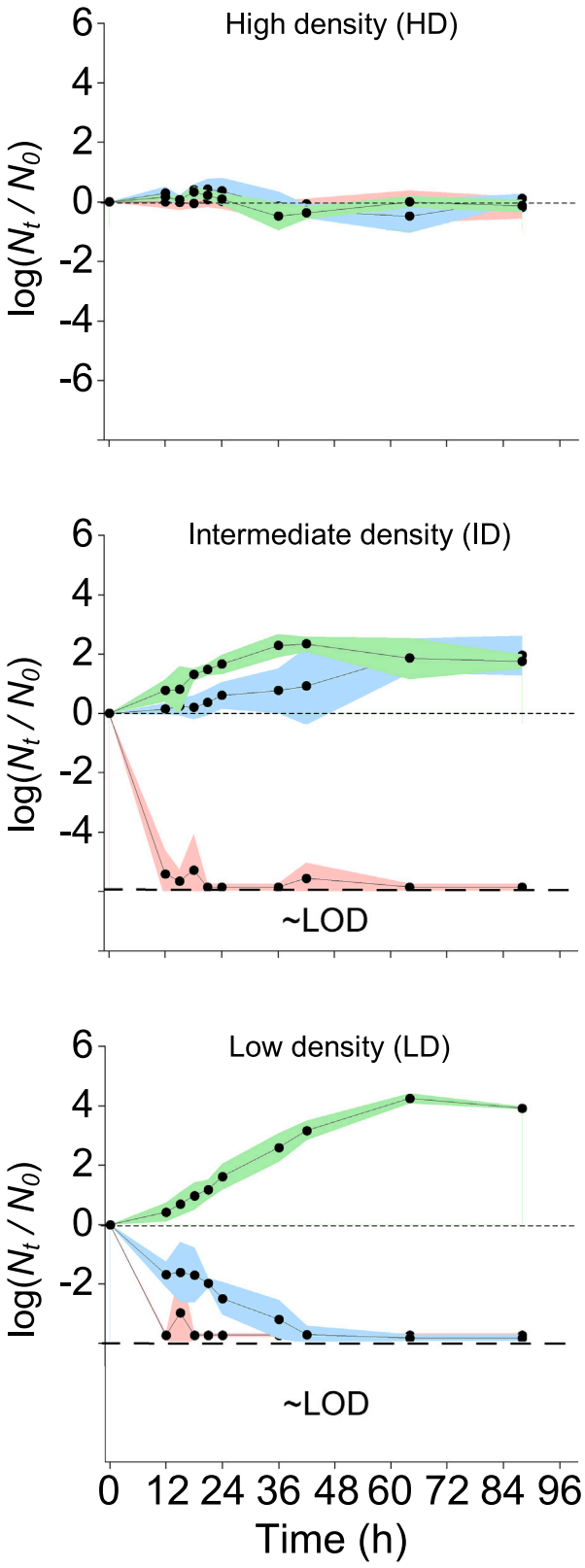
High density (HD)
Intermediate density (ID)
Low density (LD)
log(Nt / N0)
6
4
2
0
-2
-4
-6
~LOD
0 12 24 36 48 60 72 84 96
Time (h)

Figure 1. High density allows growth of *M. xanthus* under acid stress that kills low-density populations
Log-transformed ratios of population size (N_t) of *M. xanthus* strain DK3470 over time relative to starting size (N_0) are shown for populations inoculated in media of pH 5.0 (red shading), 6.0 (blue) or 7.5 (green) at high (HD), intermediate (ID), and low (LD) density (see STAR Methods). Shading represents 95% confidence regions (n = 3).

t-test). This outcome indicates that the secreted PDD factor exerts its protective effects by a mechanism other than altering environmental pH.

PDD of aggregative fruiting-body development increases with acid stress

In light of the acid-stress Allee effects on vegetative growth documented above, we asked whether PDD of *M. xanthus* fruiting-body development already known to occur at benign pH[26,29] increases in strength with increasing acid stress. Populations of the developmentally proficient strain GJV1 were incubated on TPM starvation agar at five pH levels (pH 5.5, 6.0, 6.5, 7.0, and 7.5) and three initial cell densities (HD, ID, and LD, see STAR Methods) and developmental phenotypes were observed. While dark fruiting bodies formed at all five pH conditions in the HD treatment, the degree of developmental aggregation was found to be more sensitive to acid stress at lower densities (Figure 3). ID populations showed only very slight aggregation at the lowest pH and LD populations did not even minimally aggregate at the two lowest pH levels. Thus, the negative effect of decreasing cell density on developmental aggregation is greater under acid stress than at benign pH levels.

Acid-stress Allee effects are common across the eubacteria domain but appear to be mediated by diverse mechanisms

Our results with *M. xanthus,* together with results from prior studies,[30,53,54] led us to ask whether acid-stress Allee effects are common across the eubacteria domain. Pilot experiments identified acidic pH ranges at which growth of four non-myxobacterial species—*Arthrobacter globiformis*, *Micrococcus luteus, Pseudomonas fluorescens,* and *Rhizobium vitis*—still occurred at least at high density but was reduced relative to less acidic pH. Subsequently, at one pH level for each species at which high-density growth was reduced, changes in population size by all four species over a 32 h period were measured in cultures initiated from two starting densities differing by a factor of 10^4 (see STAR Methods). PDD of growth under acid stress was clearly evident in all four species (Figures 4 and S3). In three species (*A. globiformis*, *P. fluorescens,* and *R. vitis*), the LD populations decreased while the HD populations increased (strong Allee effects), while in *M. luteus* the HD populations increased more than did LD populations (a weak Allee effect) (Figure S3).

To examine whether acid-stress Allee effects are mediated by similar mechanisms across bacterial species, we tested whether, as occurs in *M. xanthus* (Figure 2), supernatant from HD cultures of the four non-myxobacterial species mitigates the disadvantage of LD cultures subjected to acid stress relative to HD cultures. HD cultures treated with any supernatant consistently performed better under acid stress in these experiments than the respective LD cultures treated with the same supernatant (Figure S4), either remaining stable or declining less than the corresponding LD cultures, which consistently decreased significantly due to acid stress. However, unlike HD supernatant from *M. xanthus,* HD supernatants from the other species gave either no evidence (*A. globiformus, M. luteus,* and *R. vitis*) or only weak evidence (*P. fluorescens*) of improving the performance of acid-stressed LD cultures (Figure S4). These results suggest that the Allee effects observed in most of these species are mediated either by (i) cell-bound rather than diffusible molecules or (ii) diffusible molecules that rapidly diffuse away into agar after cultures were spotted onto experimental plates and thus have little protective effect under our experimental conditions.

DISCUSSION

Molecules mediating social interactions play major roles in microbial life histories [e.g[2,22,23,25,44,45,47,55,56]]. Having found that social molecules greatly mitigate acid stress by the complexly social bacterium *M. xanthus,* we investigated whether social protection from toxic acidity is a widespread trait among prokaryotes. Indeed, all five phylogenetically diverse species examined here exhibited clear PDD of growth under acid stress. Moreover, additional examples of social protection from pH stress have previously been reported in yet other individual species.[30,38,53,57,58] Collectively with those earlier reports, our phylogenetically broad experiments suggest that social forms of protection from toxic acidity are the norm rather than the exception among eubacteria.

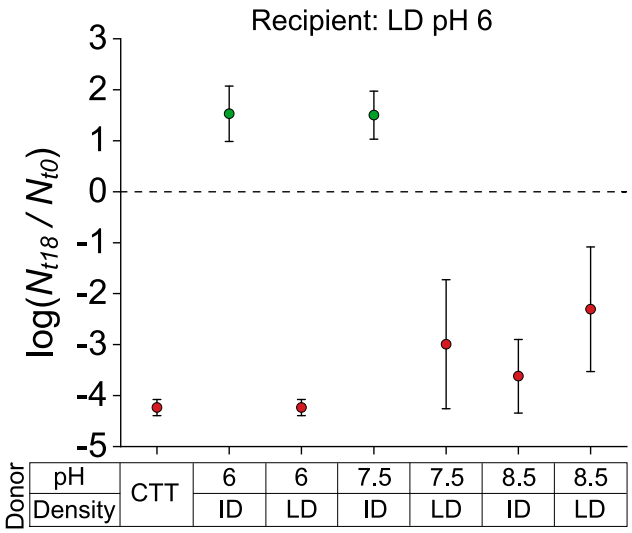

Figure 2. *M. xanthus* growth under acid stress is mediated by a diffusible public good

Supernatants from intermediate-density (ID) cultures of DK3470 grown at pH 6.0 or 7.5 protect low-density (LD) populations from acid stress (pH 6.0) and thereby allow population growth (green circles). Control media (CTT), supernatants from LD cultures grown at three tested pH values and supernatants from ID cultures grown under basic conditions (pH 8.5) do not protect LD cultures from population crashes caused by acid stress (red circles). Error bars represent 95% confidence intervals (n = 3).

Yet despite the prevalence of social protection from acid stress across bacteria, this phenotype has clearly evolved by diverse molecular mechanisms. *Streptococcus mutans* cells protect themselves against oral-cavity acidity (that they themselves increase) by secreting a quorum-sensing peptide that in turn triggers stress-protective physiological change.[38] *Corynebacterium ammoniagenes* eliminates toxic acidity in urea-containing medium by secreting urease.[30] *M. xanthus* does not appear to significantly alter its environmental pH (at least under our experimental conditions), but does secrete a protective diffusible factor. The four non-myxobacterial species examined here, however, appear to employ non-diffusible protective molecules (or diffusible protectants of a different character than that of *M. xanthus*), as supernatant from high-density cultures of these species did not protect acid-stressed low-density populations from population decline, as occurred in *M. xanthus*. Thus, just drawing from our own experiments and the examples of *S. mutans* and *C. ammoniagenes*, different forms of bacterial social protection against acid stress appear to involve both diffusible and non-diffusible factors and to involve both predominantly cell-level protection and environmental modification. More detailed investigations of the species examined here and other species are likely to reveal yet greater mechanistic diversity.

High conspecific cell density is the norm for microbes growing as colonies in structured habitats, for example in biofilms,[38,59,60] and is thus expected to be a major selective force in most natural contexts, including under both stressful and benign conditions. To fully understand the social lives of microbes, fitness effects of density both (i) within stressful ranges of diverse abiotic parameters, such as hydration level, oxygen level, pH, salinity, starvation, and temperature and (ii) while subject to biotic antagonisms, such as interference competition,[28] immunity,[31] and predation[7] need to be quantified across a broad range of microbes and the molecular mechanisms of discovered Allee effects and their evolutionary causes correspondingly investigated. Intriguingly, the yeast *Saccharomyces cerevisiae* has recently been found to socially protect itself from temperature stress by secretion of glutathione.[33] Costly production of such abiotic-stress protectants should often be cheatable by non-producing or low-producing genotypes.[27] Thus, genetic diversity among conspecifics for variation at protectant-production levels due to such exploitation is expected to be common.

Considering the myxobacteria, traits previously shown to depend positively on cell density have been associated, whether directly or indirectly, with motility,[61] aggregative fruiting-body development[26,62] or predation[50]—the social behaviors for which the myxobacteria are best known. Additionally, due to strong positive frequency dependence of interference competition between natural isolates of *M. xanthus*, high

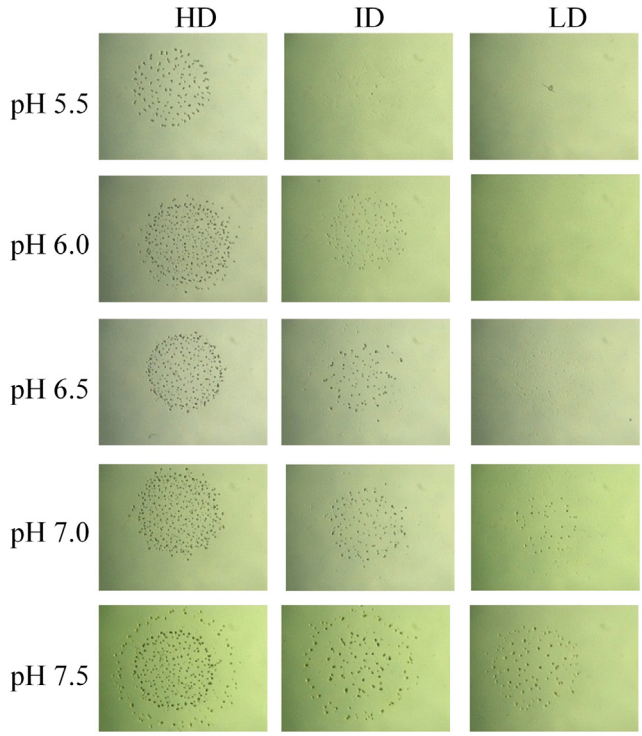

Figure 3. Strength of PDD during aggregative multicellular development by *M. xanthus* increases with acid stress

Developmental phenotypes after five days of starvation for populations initiated at three cell densities (HD, ~5 x 10^9 cells/ml; ID, ~10^9 cells/ml; LD, ~3 x 10^8 cells/ml) and five pH values are shown. Dark spots are fruiting bodies in which many light-refractive spores have formed whereas light spots are aggregates with fewer, if any, stress-resistant spores.

local density should also promote competitiveness in direct cell-cell combat when diverged cell groups meet in the soil.[63] Here, we show that simple vegetative growth by *M. xanthus* under acid stress is dependent on a social trait. Social resistance to pH stress appears to be independent of other complex *M. xanthus* social behaviors, as it was documented most extensively here using a strain defective at TFP-based motility and aggregative development.

These findings with *M. xanthus* also have implications for understanding microbial "eco-evo-devo",[64] or how ecological conditions shape the evolution of microbial developmental systems. We found that acid stress amplifies dependence of *M. xanthus* fruiting-body development on cell density beyond that observed at benign pH.[26,29] Specifically, the minimum cell density required to allow developmental aggregation increases with acid stress (Figure 3). This suggests that stressfully acidic natural conditions select more strongly for traits promoting high local density upon nutrient depletion than do less stressful pH conditions. Thus, acid stress and potentially other forms of abiotic stress may impact how the relationship between density and development (Figure 3)—including output of spores that subsequently germinate and grow—evolves. Such environmental stresses may thereby select on density-determining traits such as motility behavior and adhesin production, not only in the myxobacteria but also in other aggregative developmental systems as well.

In the myxobacteria, spores are often formed within fruiting bodies. Hypothesized benefits of sporulating within elevated, dense groups rather than more individualistically outside of fruiting bodies include increased probability of dispersal to growth-conducive environments and increased spore quality and survival.[2,42,65] Another idea proposes that the high density of spores within fruiting bodies enhances germination and subsequent growth after encountering nutrient levels conducive to growth.[42,50,66] Consistent with the latter hypothesis, spore germination was recently found to depend positively on density under both benign conditions and under saline stress.[27] Our results suggest that tight packing of spores benefits spore germination and subsequent growth if fruiting bodies encounter acidic conditions.

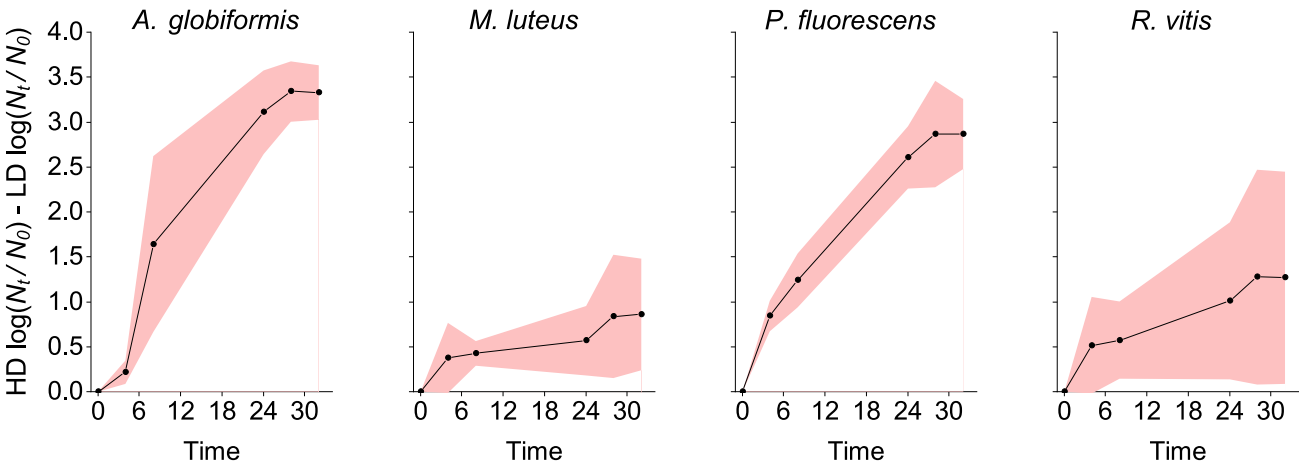

Figure 4. Growth under acid stress depends on density across highly diverged bacterial species

Growth differences between high-density (HD) vs. low-density (LD) populations of four bacterial species under acid stress are shown. Values greater than 0 indicate that the respective HD populations increased more than the respective LD populations by the relevant time point. The y axis unit reflects a 10-fold difference in the degree of population size change from the start of the experiment. (See also Figure S3.) Shaded areas represent 95% confidence regions (n = 3).

Microbes often modify environmental pH and in so doing can strongly impact microbial interactions.[30] For example, *Escherichia coli* generate acidic byproducts from metabolizing glucose, which has been shown to inhibit host colonization by *Vibrio cholerae*.[67] Our findings suggest that environmental acidification by potential prey may make myxobacterial predatory fitness density-dependent by a different mechanism than previously proposed. Earlier experiments showed that the growth rate of *M. xanthus* liquid cultures on large macromolecules that require extracellular hydrolysis for optimal metabolic use is density dependent, presumably because higher concentrations of extracellular enzymes in high density cultures facilitate more rapid access to hydrolized peptides.[50] This has often been cited as evidence that myxobacterial predation efficiency is likely to also be density dependent, although the evidence for this hypothesis has remained indirect. Our results suggest that prey metabolism rather than (or in addition to) predator metabolism may cause predation-fueled growth of myxobacteria to be density dependent when myxobacteria encounter prey colonies that have acidified their local environment.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.106952.

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AUTHOR CONTRIBUTIONS

All authors designed experiments, analyzed data, and drafted manuscript components. F.F., S.P., and H.P. performed experiments; and F.F., S.P., and G.J.V. revised the manuscript. H.P. performed early experiments that first revealed effects of density on *M. xanthus* growth and development under acid stress.

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		
DK3470	Mates et al.[54]	NA
GJV1	Shimkets et al.[51]	NA
Arthrobacter globiformis	Lazazzera et al.[55]	NA
Micrococcus luteus	Lazazzera et al.[55]	NA
Pseudomonas fluorescens	Lazazzera et al.[55]	NA
Rhizobium vitis	Lazazzera et al.[55]	NA
Chemicals, peptides, and recombinant proteins		
BD Bacto casitone	Fischer scientific	Cat# 225910
BD Bacto agar	Fischer scientific	Cat# 214030
Tris base	Sigma-Aldrich	Cat# T1503-1 KG
Magnesium sulfate	Sigma-Aldrich	Cat# M1880-1 KG
Potassium phosphate	Fluka	Cat# 60356
Membrane filters	Whatman/GE Healthcare	Cat#10414006
Hydrocloric acid	Sigma-Aldrich	Cat# H1758-500 ML
Sodium hydroxide solution	Sigma-Aldrich	Cat#41,541-3
Deposited data		
Dryad	NA	https://doi.org/10.5061/dryad.c2fqz619b

RESOURCE AVAILABILITY

Lead contact

Further information and request for resources should be directed to and will be fulfilled by the lead contact Gregory J Velicer (gregory.velicer@env.ethz.ch)

Materials availability

Unique reagents used in this study will be freely available.

Data and code availability

- Data have been deposited at Dryad and are publicly available as of the date of publication. DOIs are listed in the key resources table
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Strains

M. xanthus strain GJV1 is a direct descendant of the lab-reference strain DK1622, from which it differs by only several point mutations.[68] GJV1 is proficient at two *M. xanthus* motility systems - type IV-pili driven S-motility and A-motility, which can drive single-cell movement.[44,69,70] Strain DK3470 has a mutation that blocks production of extracellular matrix compounds required for cell-cell cohesion, full motility and development.[71] Strains of the Gram-positive species *A. globiformis* (high-GC subdivision) and *M. luteus* (low-GC subdivision) and of the Gram-negative species *P. fluorescens* (gamma subdivision) and *R. vitis* (alpha subdivision) used here are the same as those reported by Morgan et al.[72]

METHOD DETAILS

Media and culture conditions

For all species, CTT liquid (1% casitone; 8 mM MgSO4; 10 mM Tris-HCl, pH 7.6; 1 mM potassium phosphate)[73] and CTT agar were used as growth media, agar plates were incubated at 32 °C and 90% rH and liquid cultures were incubated at 32 °C while shaking at 300 rpm. For *M. xanthus* development assays, TPM liquid (identical to CTT liquid except lacking Casitone, the sole carbon source in CTT) and TPM agar (1.5%) plates were used. Media pH was adjusted with NaOH or HCl to the appropriate level before autoclaving. For *M. xanthus* growth cultures, samples from frozen stocks were inoculated and incubated CTT hard (1.5%) agar plates for three days. An inoculum from each plate was then transferred into eight mL CTT liquid in a 50 mL flask and grown overnight. These exponential-phase cultures were then transferred into larger volumes of CTT (culture/flask volume always 0.16) and allowed to grow further until populations were sufficiently large to initiate all treatments of an experiment. Cell densities of exponential-phase cultures were estimated with a TECAN Genios plate reader. Cultures were then centrifuged (5000 rpm, 15 min) and resuspended to predicted densities of ~1.25 x 10^6, 1.25 x 10^8 or 1.25 x 10^{10} cells/ml with CTT liquid. For other species, samples from frozen stocks were inoculated directly into CTT liquid.

Growth assays

Growth-rate assays with strain DK3470 were based on direct colony counts after dilution plating of harvested and dispersed cultures. 10 mL aliquots of CTT 1.5% agar at pH 5.0, 6.0 and 7.5 were poured into 50 mL Erlenmeyer flasks and allowed to solidify one day prior to the beginning of the growth assay. Flasks were kept covered at room temperature. 400 μl of resuspended DK3470 culture containing approximately 1.25 x 10^6 (low density, LD), 1.25 x 10^8 (intermediate density, ID) or 1.25 x 10^{10} (high density, HD) cells/ml were spread onto the agar surface and allowed to dry in a laminar flow hood for approximately 30 min. Due to destructive sampling, multiple flasks were prepared for each combination of pH and initial cell density. Immediately after the 30′ drying period and after periods of incubation shown in Figure 1, 1 mL of CTT liquid and ~10 glass beads (3 mm diameter) were placed in each flask and flasks were shaken for 10′ at 300 rpm to disperse cells. Suspended cultures were then dilution-plated into CTT 0.5% agar plates and resulting colonies were counted after incubation at 32 °C (Figure S1).

For the four non-myxobacterial species, preliminary experiments identified acidic pH ranges at which growth in CTT liquid occurred but was substantially reduced in populations inoculated at a high initial density. Starting from initial densities (detailed below) differing by a factor of 10^4, growth at a pH value in the relevant range for each species was subsequently analyzed: *A. globiformis* at pH 4.35, *M. luteus* at pH 5.75, *P. fluorescens* at pH 4.6, and *R. vitis* at pH 4.25. Five mL of CTT 1.5% agar adjusted to the relevant pH for each species were poured into 6-well plate wells 24 h prior to initiating growth assays. After agar solidified, a polyamide membrane filter (NL 16 Whatman membrane filters, 25 mm diameter, 0.2 μm pore size) was placed on the agar surface of each well. Multiple wells were prepared for each replicate assay of each treatment because one filter was destructively sampled at each time point of each assay. Cultures were grown on filters in these assays because cells could not be easily harvested directly from the agar surface due to agar instability caused by acidity.

Cultures of each species were inoculated from freezer stocks and grown overnight in CTT liquid, centrifuged at 5000 rpm for 15 min and resuspended with CTT liquid to high density (HD, average cross-replicate densities of ~1.6 x10^9 cells/ml for *A. globiformis*, ~5.8 x10^8 cells/ml for *M. luteus*, ~1.3 x10^9 cells/ml for *P. fluorescens* and ~1.7 x10^9 cells/ml for *R. vitis* based on dilution plating immediately after resuspension) and diluted by a factor of 10^4 to generate low density (LD) cultures. 20 μL aliquots of each HD and LD culture were spotted on filters and allowed to dry in a laminar flow hood. Plates were incubated at 32 °C and 90% rH. Immediately after spots were dry (t_0) and after 4, 8, 24, 28 and 32 h, filters were removed and placed in a 50 mL flask containing 5 mL of CTT liquid and approximately 10 glass beads (3 mm diameter). Flasks were shaken at 300 rpm, 32 °C for 15 min to disperse cell populations, which were then dilution-plated into CTT 0.5% agar. Colonies were counted after 3–5 days of incubation at 32 °C, 90% rH.

Differences in the degree of growth between HD and LD populations at each time point N_t in the same replicate were calculated as: $\log_{10}(N_t(HD)/N_0(HD) - \log_{10}(N_t(LD)/N_0(LD)$.

Supernatant assays

M. xanthus

Following the same protocol as for the growth assay, we let *M. xanthus* cultures grow on CTT 1.5% agar in flasks at pH 6.0, 7.5 and 8.5 starting from low and high initial densities (~1.25 x 10^6 (LD) and ~1.25 x 10^8 (ID) cells/ml, respectively). After 18 h, we added 1 mL of CTT liquid to each flask and shook all flasks for 30 min at 300 rpm prior to collection of cell-free supernatant from each resuspended culture by filtration (0.2 μm filters).

Cultures of *M. xanthus* growing in CTT liquid were subsequently spun down and resuspended to LD and ID with the six treatments of collected supernatants described above (derived from ID and LD cultures grown on agar at pH 6.0, 7.5 and 8.5) and CTT liquid as a control. 400 μL of each resuspended culture were spread on CTT 1.5% agar adjusted to pH 6.0, 7.5 or 8.5. After 18 h of incubation, 1 mL of CTT liquid and ~10 glass beads (3 mm diameter) were added to each flask and flasks were shaken for 10′ at 300 rpm prior to dilution plating to obtain colony counts.

Non-myxobacteria

Non-myxobacterial species were inoculated in liquid CTT media, incubated overnight at 32 °C at 300 rpm, centrifuged at 5000 rpm for 15 min and resuspended in CTT liquid to the respective high-density (HD) value for each species and then diluted by a factor of 10^4 to generate the respective low-density (LD) treatment. 20 μL aliquots of these HD and LD cultures were then individually spotted on a polyamide membrane filter placed on CTT 1.5% agar with pH adjusted to both pH 7.5 (control) and the same acidic pH value used for the acid-stress growth assays described above and the resulting cultures were incubated for 18 h. To generate supernatant for the next step of the experiment, filters were then removed from the agar beds, placed in 1 mL CTT liquid, incubated at 100 rpm on a gel rocker for 30 min, and the filter-treated CTT liquid was then filter sterilized using a 0.2 μm filter. This method with membrane filters was used for harvesting supernatant for the non-myxobacterial species because the pH values used for these species made agar too soft to harvest supernatant with the same method as used for *M. xanthus*.

The resulting culture supernatants were used to then resuspend LD and HD cultures of the respective non-myxobacterial species, and growth assays were performed to measure the influence of diffusible substances on the growth of LD and HD cultures. To do so, cultures of *A. globiformis, M. luteus, R. vitis* and *P. fluorescens* that had been incubated in CTT liquid for 48 h were spun down and resuspended to HD and LD using the supernatant derived from either HD or LD cultures growing at either the respective acid-stress pH or at pH 7.5, as described above. 20 μL of these resuspended cultures were then spotted on a filter placed on CTT agar at the respective acidic pH for each species and additional samples from the same resuspended cultures were dilution-plated to obtain T0 population size estimates. Plated samples were then incubated for 18 h. To obtain population size estimates at T18, filters were placed in 12-well-plate wells containing 1 mL CTT liquid and cultures were dispersed by repeated pipetting prior to dilution plating.

Fruiting-body development

Development assays were performed in Greiner 6-well plates containing 5 mL pH-adjusted TPM 1.5% agar per well, which was poured one day prior to inoculation and kept at room temperature overnight. Vegetatively growing cultures of GJV1 were centrifuged, resuspended in TPM liquid to ~5 x 10^9 cells/ml and subsequently diluted into two lower density treatments of ~1 x 10^9 and ~3 x 10^8 cells/ml. Ten μL aliquots for each density treatment were placed on TPM agar and allowed to dry in a laminar flow hood. After five days of incubation, developmental phenotypes were photographed. Three temporally independent replicate assays were performed for each density-pH combination with two technical replicates performed in adjacent wells within each independent replicate.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data analysis was done using SPSS 23.0 (SPSS, Chicago, IL, USA). Details of the statistical tests used are mentioned in the figure legends. All experiments were performed in three temporally separated independent replicates.