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Exploring the Predictability of Evolution: Identifying Repeatable Adaptive Traits Across Microbial Communities

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An abstract of a thesis submitted to the Faculty of Emory College of Arts and Sciences of Emory University in partial fulfillment of the requirements of the degree of Bachelor of Science with Honors

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Abstract

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By Katherine Shen

Understanding the predictability of microbial evolution in host-associated environments is crucial for uncovering the mechanisms that drive ecological stability and diversity. In this study, we examined how bacterial communities evolved during association with the nematode Caenorhabditis elegans, focusing on the emergence of repeatable adaptive traits. Using single-species, pairwise, and multi-species (Community G) experimental evolution frameworks, we tracked changes in species composition, colony morphotypes, motility, and growth dynamics across five serial passages.

While Microbacterium oxydans (MO) maintained stable phenotypes across conditions, Chryseobacterium indologenes (CI) evolved alternate morphotypes exclusively in the complex community environment. These alternate CI morphotypes exhibited reduced motility and distinct colony morphologies compared to ancestral strains. Principal Component Analysis revealed early divergence followed by stabilization of community composition, suggesting strong initial selection pressures mediated by the host environment. Logistic growth assays indicated that alternate morphotypes had altered saturation densities, while reversion assays suggested a degree of phenotypic plasticity.

Overall, our findings highlight that microbial evolution in host-associated environments is shaped by species interactions, spatial structure, and host filtering. While some evolutionary outcomes, such as species persistence, were repeatable, others, such as the emergence of alternate morphotypes, were contingent on community complexity. This work advances our understanding of how ecological interactions drive microbial diversity and the predictability of evolution in host-associated systems.

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Exploring the Predictability of Evolution: Identifying Repeatable Adaptive Traits Across Microbial Communities

Introduction

Understanding how microbial communities evolve and adapt over time is essential for uncovering the mechanisms that drive ecological dynamics and host-microbe interactions. Microbial populations often exhibit convergent evolution—where distinct species develop similar traits under similar conditions—while also evolving divergent adaptations in response to specific environmental niches (Taylor et al., 2022). These patterns of convergence or divergence depend on the ecological and environmental contexts in which evolution occurs (Castledine et al., 2020).

This project investigates microbial adaptation during interactions with the nematode worm *Caenorhabditis elegans* (C. elegans), focusing specifically on how bacterial populations evolve on the worm surface and inside the worm gut. Prior work has shown that C. elegans can act as both a predator and a dispersal vehicle, creating distinct selective pressures in these two environments. By comparing evolved bacterial populations from the worm surface and worm gut, this study aims to understand how spatial location within the host and exposure to different selective pressures influence microbial evolutionary trajectories. Key questions include: Are the evolutionary outcomes repeatable across independent populations? Do similar alternate morphotypes and traits evolve in both the surface and gut environments, or does divergence occur?

Convergent evolution typically occurs when unrelated species experience similar environmental challenges, leading to analogous adaptations despite their distinct ancestries. For instance, in host-associated environments, bacteria often evolve traits such as enhanced colonization ability or resistance to host immune defenses, even if they belong to distinct phylogenetic groups (Meroz et al., 2024). In experimental evolution studies with C. elegans, bacterial species have been observed to repeatedly evolve morphotypic changes, such as increased biofilm production or altered motility, to improve persistence in the host gut (Scheuerl et al., 2020).

In contrast, divergent evolution is expected when populations experience distinct environmental conditions or encounter varying selective pressures, leading to different adaptive trajectories. For example, bacterial populations can evolve in heterogeneous microenvironments or in the presence of different microbial consortia, prompting divergence in evolutionary strategies. In spatially structured environments, *Pseudomonas* species diversify into morphologically and functionally distinct types, such as biofilm-forming "wrinkly spreaders" and motile planktonic variants (Rainey & Travisano, 1998), driven by local differences in oxygen, surface area, or density effects.

Evolution in multispecies communities can result in variable and context-dependent outcomes, shaped not only by abiotic selection pressures but also by dynamic interspecies interactions. These interactions introduce frequency-dependent selection, ecological feedback loops, and co-evolutionary pressures absent in monocultures (Morris et al., 2012; Lawrence et al., 2012). In monocultures, by contrast, strong and consistent selection pressures often lead to convergent evolution, where microbial populations independently evolve similar traits (Fiegna et al., 2015).

Spatial structure is another major driver of evolutionary dynamics. In structured environments like biofilms, local selection and limited dispersal favor diversification and niche specialization (Rainey & Travisano, 1998). In contrast, well-mixed environments, like the Long-Term Evolution Experiment (LTEE) with *E. coli*, tend to favor convergent evolution toward globally optimal phenotypes, though diversification can still emerge via frequency-dependent selection (Lenski et al., 1991; Rozen & Lenski, 2000).

Host-microbe interactions further shape microbial evolution by introducing novel selective forces. Bacterial motility, for instance, can be advantageous for gut colonization and survival (Yuen & Ausubel, 2018). The host gut imposes spatial structure and bottlenecks, influencing evolutionary outcomes through both selection and genetic drift (Gibson et al., 2019).

Previous work in this lab has shown that alternate bacterial morphotypes—distinct colony variants—can arise during experimental evolution with *C. elegans* (Duckett et al., 2024). These morphotypes likely confer advantages such as enhanced resource competition, dispersal, or survival in host-associated environments. Notably, alternate morphs previously observed exhibited decreased motility compared to ancestral isolates and morphologically ancestral-like variants from the same samples (Duckett et al.).

By incorporating both bacterial interactions and spatial constraints, the *C. elegans* system allows a more ecologically relevant study of microbial adaptation. Importantly, *C. elegans* provides two distinct environments: the external surface and the internal gut, each presenting different selective pressures. The external surface is more exposed, while the gut is a spatially structured, nutrient-rich, and immune-active environment.

This project focuses on the repeatability and divergence of morphotype evolution across these two environments. By comparing bacterial evolution on the worm surface versus within the gut, we aim to determine whether similar selective pressures drive convergent adaptations or if distinct environments promote divergent evolutionary trajectories. Identifying the conditions under which alternate morphs arise will provide insights into the predictability of microbial evolution and the role of ecological interactions in shaping phenotypic diversity.

Ultimately, understanding whether certain traits consistently emerge across different worm-associated environments will provide critical insights into the mechanisms driving microbial

diversity, the evolutionary strategies that sustain community stability and fitness, and the broader principles governing host-microbe co-evolution (Taylor et al., 2022).

Methods

Media and buffers

Nematode Growth Medium plates (NGM) for *C. elegans* cultivation were prepared by dissolving 3 g of NaCl, 2.5 g of peptone, and 17 g of agar in 975 mL of distilled water. The mixture was autoclaved at 121°C for 20 minutes and allowed to cool to approximately 55°C. After cooling, 1 mL of 1M $CaCl_2$, 1 mL of 5 mg/mL cholesterol (prepared in ethanol), 1 mL of 1M $MgSO_4$, and 25 mL of 1M potassium phosphate buffer (pH 6.0) were added aseptically. The medium was mixed thoroughly and poured into sterile Petri dishes (approximately 10–12 mL per 60 mm plate). Plates were allowed to solidify at room temperature before being stored at 4°C in sealed plastic bags to prevent desiccation. NGM Medium is made without adding agar.

Nutrient Agar (NA) was used for dilution plating of bacteria. This medium was prepared by dissolving 5 g of peptone, 3 g of yeast extract, 5 g of NaCl, and 15 g of agar in 1 L of distilled water. The solution was stirred until the components were fully dissolved and then autoclaved at 121°C for 20 minutes. After autoclaving, the medium was allowed to cool to approximately 50–55°C before being poured into sterile Petri dishes (approximately 20–25 mL per 100 mm plate). The plates were left to solidify at room temperature and then stored at 4°C in sealed plastic bags to prevent contamination and dehydration.

Stiernagle Medium (S medium) was used for maintaining *Caenorhabditis elegans* and culturing associated bacterial communities in liquid experiments. This medium was prepared by dissolving 5.85 g of NaCl, 1 g of K_2HPO_4, 6 g of KH_2PO_4, and 5 g of peptone in 900 mL of distilled water. The solution was stirred until fully dissolved and then autoclaved at 121°C for 20 minutes. After autoclaving, the medium was allowed to cool to room temperature. Once cooled, 25 mL of sterile 1 M potassium citrate buffer (pH 6.0), 1 mL of sterile 1 M $MgSO_4$, 1 mL of sterile 1 M $CaCl_2$, and 1 mL of sterile 5 mg/mL cholesterol in ethanol were added. The final volume was adjusted to 1 L with sterile distilled water. The prepared S Medium was stored at room temperature and used within two weeks to maintain sterility and efficacy.

M9 Worm Buffer was used for washing and handling *C. elegans* during experimental procedures, such as transferring worms between plates and preparing worm digests. This buffer was prepared by dissolving 3 g of KH_2PO_4, 6 g of Na_2HPO_4, 5 g of NaCl, and 1 mL of 1 M $MgSO_4$ in 1 L of distilled water. The solution was stirred until completely dissolved, filter-sterilized through a 0.22 μm filter, and stored at room temperature. M9 buffer was used fresh or stored for no longer than one month to ensure optimal worm viability.

Strains

ID	Abbreviation	Taxonomic Name	Source
B-2879	AA	*Arthrobacter aurescens*	NRRL
B-24236	MO	*Microbacterium oxydans*	NRRL
B-14848	CI	*Chryseobacterium indologenes*	NRRL
B-1574	RE15M	*Rhodococcus erythropolis (mucoid)*	NRRL
B-1876	BS	*Bacillus sp.*	NRRL
ATCC 49188	OA	*Ochrobactrum anthropi*	ATCC
B-23392	SS	*Sphingobacterium spiritovorum*	NRRL

Table 1. Bacterial strains were used in this study.

Evolution experiment

Bacterial community conditions in the evolution experiment were: community G (all seven bacterial isolates shown in Table 1, with 6 replicates), pairs consisting of MO + each other bacterial species (6 total pairs, with 3 replicates each), single species MO (with 12 replicates) and OP50 control (3 replicates).

Bacterial cultures used in the evolution experiment (see Table 1) were first grown in 1 mL LB broth for 48 hours at 25 °C in static conditions. Cultures were then transferred to 1.5 mL microcentrifuge tubes and centrifuged at 9,000 × g for 2 minutes to pellet cells. The supernatant was discarded, and bacterial pellets were resuspended in 1 mL of sterile S medium. Cultures were then diluted to 10^8 CFU/mL and combined in equal volumes to form either pairwise mixtures, single species or community experiments.

For worm-associated evolution, synchronized L1 larvae of *Caenorhabditis elegans* (wild-type N2 strain) were obtained by bleaching gravid adults and incubating eggs overnight in M9 buffer at 20 °C. *C. elegans* N2 synchronized according to a standard bleach-NaOH protocol (Stiernagle,2006) and washed to remove dauer hormone. The larvae were then resuspended in M9 worm buffer with 0.1% Triton X-100 (M9TX01) at a concentration of 1-2 worms/µL. Approximately 100 L1 larvae were transferred to 6 cm NGM agar plates seeded with 50 µL of the indicated bacterial community. UV-killed *E. coli* OP50 was included as a supplemental food source during the initial inoculation to support early worm development.

Plates were incubated at 25 °C and monitored every 7 days for five weeks (five total passages). Each week, plates were scored for (i) worm population density (0–4 scale), (ii) presence of larvae, and (iii) percentage of bacterial lawn coverage. At the end of each seven-day passage, plates were washed to remove bacteria and worms for sampling and establishment of the next

passage of the evolution experiment, Each plate was washed with 2 mL M9TX01 to remove bacteria and worms, and the wash was transferred to a labeled 1.5 mL microcentrifuge tube. A single-use sterile culture scraper was used as needed to separate the adherent bacterial lawn from the agar surface. Tubes were balanced with additional buffer as needed, then spun briefly (1-2 seconds at 2000xg) to pellet most worms, and the supernatant (bacterial lawn) was removed for plating and preparation of frozen stocks. An additional wash with 1 mL M9TX01 was performed to remove the bulk of the residual bacteria, after which 900 µL was removed, leaving 100 µL of worms and diluted bacteria in each sample tube.,

To inoculate the next passage, 10 µL of the resulting resuspension was transferred to the center of a new NGM + UV-killed OP50 plate (6 cm).

To obtain a bacterial count from within the worms, non-adhered bacteria were first purged from the gut. For this, 100 µL S medium with 2x heat-killed OP50 was added to each tube and incubated at 25°C for 1 hour. The samples were then rinsed with 900 µL M9TX01, spun down, and the supernatant discarded, and the washing step was repeated. Worms were chilled on ice (~10 minutes) or at 4°C. Then, 1 mL ice-cold M9 buffer + bleach (1:2000 v/v) was added to each tube, and tubes were held on ice for 20 minutes to kill external bacteria. After surface bleaching, worms were rinsed twice with 1 mL M9TX01, then resuspended in 100 µL of 0.25% SDS + 300 mM DTT mixture for 8 minutes at room temperature to partially permeabilize the cuticle. Samples were washed twice with M9TX01, then resuspended in 1 mL M9TX01 in wells of a 24-well plate. The BioSorter was used to sort batches of 50 adults (or all available adults if fewer than 50), into wells of a 96-well 2 mL Axygen plate. Plates were then chilled at 4°C for 30-60 minutes before digestion.

The remaining worm sample was used for community analysis. Approximately 50 adult worms were isolated (or all available adults if fewer than 50), washed with M9TX01, surface-bleached to remove external bacteria, and homogenized using a sterile motorized pestle in 20 µL M9TX01. Homogenates were split: half was plated on nutrient agar (NA) for community composition analysis via colony morphology and CFU counts; the other half was mixed 1:1 with 40% glycerol and stored at –80 °C for future analysis. Up to six colony isolates per species per replicate were selected, grown in 100 µL LB in 96-well plates for 48 h at 25 °C, mixed 1:1 with 40% glycerol, and cryopreserved at –80 °C.

Worm digest and bacterial lawn samples were dilution plated onto nutrient agar (NA) for quantification and detection of alternate morphs, either on square plates (10 µL spots) or on 10 cm NA plates (100 µL/plate). Preliminary experiments showed that some bacterial combinations could not be plated on square NA plates, as the different colony types could not be reliably distinguished in the small grid. Plates were incubated at 25°C for at least 2-3 days before counting and determination of morphotypes. Colonies were counted once the plates were fully grown.

Glycerol stocks (20% glycerol v/v) were made from unused volumes of lawn and worm samples and were frozen at -80°C for further study.

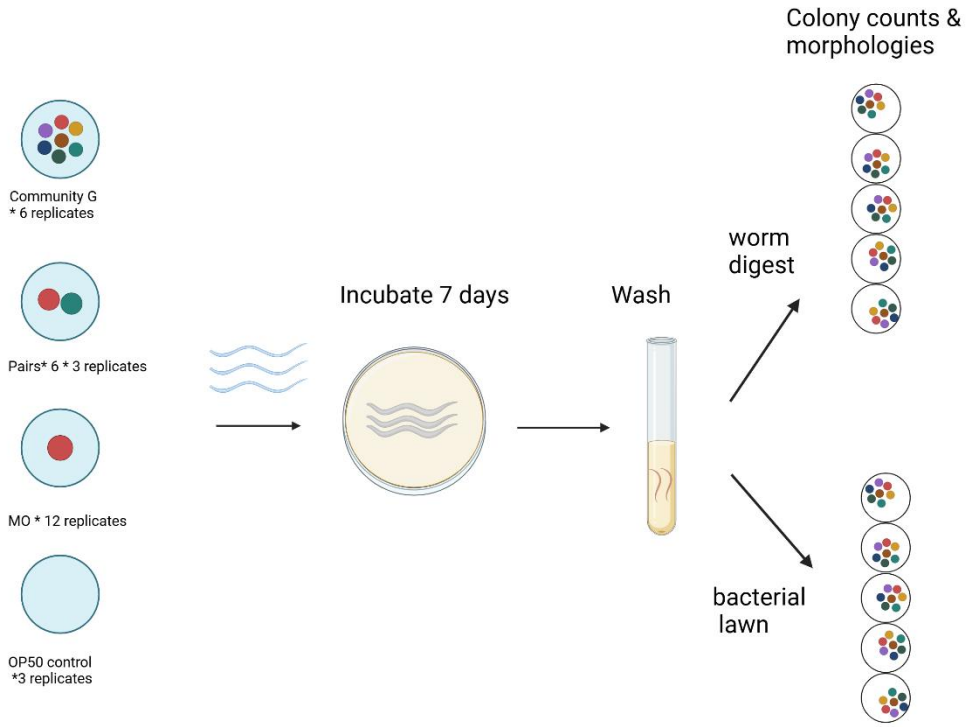

Figure 1. Experimental workflow for community-based evolution in *C. elegans*-associated bacterial communities.

Experimental evolution was conducted across five serial passages. Four treatment types were used: (1) a complex seven-species community (Community G, 6 replicates), (2) six pairwise combinations consisting of MO + each other bacterial species (3 replicates per pair, totaling 18 pairwise replicates), (3) monocultures (MO, 12 total replicates), and (4) an OP50 control (3 replicates).

Motility assays

To test which specific traits were selected during evolution, a motility assay for alternate and original morphs was conducted.

Two days prior to the assay, bacterial cultures were inoculated in LB media from glycerol stocks. These cultures were covered with Breath-Easy film and incubated at 25°C with shaking for 24 hours. On the following day, overnight LB cultures were diluted 1:100 into liquid NGM media and incubated under the same conditions to ensure a consistent inoculum. On the morning of the

assay, the final inoculum was prepared by diluting the overnight NGM cultures 1:100 in fresh NGM media and incubating for 4 hours.

NGM motility agar was prepared immediately before use by dissolving 1.5 g of NaCl, 1.25 g of peptone, and 1.75 g of agar in 500 mL of distilled water. After adding a stir bar, the mixture was autoclaved and allowed to cool to approximately 50-60°C. Once cooled, sterile components were added, including 12.5 mL of 1 M KPO_4 buffer, 500 µL of 1 M $MgSO_4$, 500 µL of 1 M $CaCl_2$, and 500 µL of vortexed cholesterol.

Agar plates were poured under sterile conditions using a laminar flow hood, with a heat stir available to maintain the agar's temperature between 50-60°C during pouring. A serological pipette was used to ensure consistent agar volumes in each plate, and freshly poured plates were allowed to solidify and dry with lids off for 20 minutes inside the laminar flow hood. A 30°C incubator containing a water tray to maintain humidity was prepared prior to the assay.

After the agar solidified, 1 µL of bacterial inoculum was carefully pipetted onto the center of each plate. Plates were then incubated face-down in stacks of no more than four. At pre-determined intervals, motility was measured by recording the diameter of the bacterial spread using a ruler.

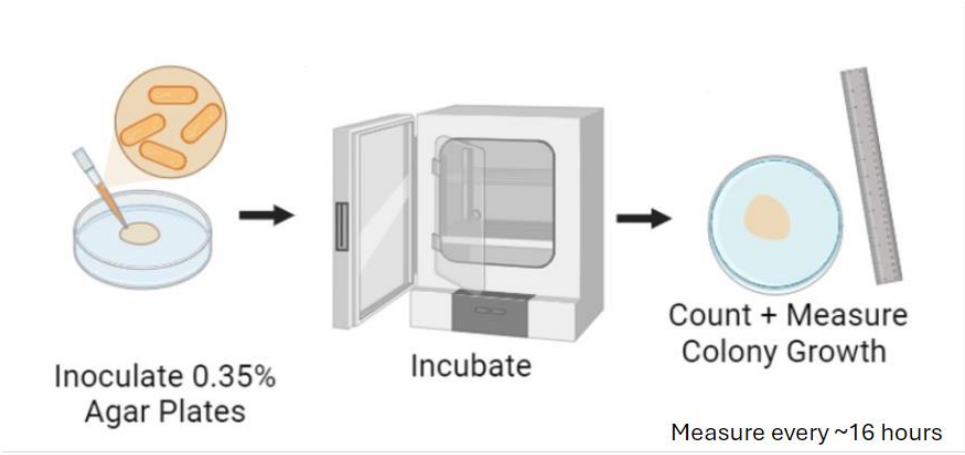

Figure 2. Experimental workflow for C. *indologenes motility assay*

Revertants collection and Isolation

To investigate the frequency and characteristics of reversion from the alternate morphotype, potential revertants were collected from motility assays conducted on low-agar plates. Collection was performed on day six or seven, depending on the appearance of "fluffy" sectors within alternate morphotype spots. In some cases, sectors emerged as early as day five, and collection was carried out if convenient. The frequency of reversion varied between samples, with some

motility spots exhibiting no visible reversion, while others developed distinct "fluffy" sectors, indicating possible morphological shifts.

Revertant collection involved using a sterile wooden dowel to gently sample the very outer edge of each "fluffy" sector. Up to six fluffy sectors were collected per motility spot, though typically only one to three were obtained. Each revertant was labeled according to its origin to maintain traceability, and labels were also recorded on plates for reference. To document morphological characteristics at the time of isolation, photographs of each sector were taken prior to collection. After collection, each revertant was inoculated into fresh LB medium using the same sterile dowel. Inoculation was performed in deep 96-well plates for efficiency, with 150–200 µL of LB used per well in 1.2 mL deep-well plates.

To provide a baseline for comparison, controls were included in the experiment. Three replicates of both the original and alternate morphotypes were inoculated and grown under the same conditions, with technical triplicates included for reproducibility. The plate was incubated at 25–30°C overnight to allow sufficient bacterial growth. The following day, culture densities were assessed before plating, and if needed, dilution plating was performed using serial dilutions (D3–D8) to optimize colony isolation. Revertants were then plated on nutrient agar (NA) for further morphology assessment. Square NA plates were used where necessary to facilitate visual comparisons. Plates were incubated at 25°C for four days, as this timeframe provided optimal conditions for distinguishing morphologies.

Following incubation, colonies were examined to determine whether revertant resembled the original morphotype or retained alternate characteristics.

Figure 3. “Fluffy session” defined as revertant were collected (M. Oxydans is used as an example here)

Data analysis – Diversity of evolving microbial communities

Community composition was assessed by plating both worm-associated and lawn-associated bacterial fractions on nutrient agar and identifying colonies by morphology. Colony types were matched to known reference isolates using morphology and historical validation. For each replicate at each passage, the relative abundance of each colony morphotype was calculated based on CFU counts. These data were used to analyze both species persistence and morphotypic variation over time. Community G was compared to pairwise and monoculture conditions to assess the effect of species interactions on diversity maintenance.

Data analysis - Motility

To assess whether motility evolved in response to community interactions or passaging, a surface motility assay was performed using soft 0.25% NGM agar. Bacterial spread was measured in centimeters at regular intervals (e.g., 24 h, 46 h). Measurements focused on isolating from three morphotypes: ancestral, alternate, and original. Each morphotype was tested across different passages and community backgrounds. Differences in motility across passages, community types, and morphotypes were evaluated using linear regression models. The dependent variable was spread diameter, and independent variables included morphotype, passage number, and community replicate. Boxplots and line graphs were generated using ggplot2 to visualize motility trends, revealing that alternate morphs showed significantly reduced motility relative to both ancestral and original types, and that community context did not consistently alter motility.

Data analysis – Logistic Growth

To assess whether alternate morphotypes differed from original morphs or the ancestral strain in growth dynamics, optical density (OD_{600}) measurements were taken over time in liquid culture. Growth curves were used to calculate two parameters for each isolate: the exponential growth rate (r) and the carrying capacity (K), represented by the maximum OD_{600} value achieved (saturation density).

For each community replicate, Wilcoxon signed-rank tests were used to compare growth rates and saturation densities between light (original) and dark (alternate) morphotypes. Nonparametric tests were chosen because preliminary analysis indicated that the data were not normally distributed. Two-tailed tests were applied, and p-values less than 0.05 were considered statistically significant. In cases where p-values approached but did not reach significance (e.g., $p = 0.057$), trends were noted. Statistical analyses were conducted using R (version 4.4.1) with the wilcox.test function.

Results

Bacterial Composition in Single Species, Pairwise, and Community Conditions

To understand how bacterial interactions shape community composition during evolution, we asked whether patterns of species persistence differ between pairwise combinations and a more complex multi-species context. To address this, we tracked community composition over five passages by plating lawn and worm-associated samples and identifying colony morphotypes to estimate the relative abundance of each species. If contamination was observed in a replicate, that replicate was not passed further. If one species within a pair went extinct, passaging was likewise halted.

In single-species plates, *Microbacterium oxydans* (MO) persisted across passages without detectable changes in colony morphology or growth characteristics. No alternate morphotypes or obvious evolutionary adaptations were observed over the course of the experiment. MO maintained a stable phenotype throughout, suggesting that in the absence of competitors or complex community interactions.

In the pairwise conditions, one species often dominated while the other was driven to extinction (*Bacillus sp., Rhodococcus erythropolis (mucoid), Sphingobacterium spiritovorum* data were not shown because of extinction*)* suggesting that direct competition limited coexistence. Notably, *Arthrobacter aurescens* (AA) consistently declined across all replicates, indicating a competitive disadvantage. In contrast, *Microbacterium oxydans* (MO) and *Ochrobactrum anthropi* (OA) were maintained across multiple pairwise treatments, suggesting they possess traits that support persistence in simple competitive environments. Passaging was stopped when one species became extinct - for example, AA pairs were stopped at passage 4 because AA became extinct at this point. Several pairwise combinations were excluded from analysis due to early extinction or contamination.

Importantly, no alternate colony morphotypes were observed for MO in either single-species or pairwise conditions, consistent with prior findings that MO maintains a stable phenotype when evolved alone or with one competitor. In these conditions, *Chryseobacterium indologenes* (CI) also did not show emergence of alternate morphotypes.

In the community condition (Community G), more complex and stable coexistence was observed. Although AA still declined, MO, OA, and CI were maintained across all replicates, and overall community diversity was higher than in the pairwise settings. As in the simpler settings, MO did not evolve alternate morphotypes in the community. However, CI evolved distinct alternate morphotypes during community evolution, suggesting that interactions within a multi-species environment specifically promoted phenotypic diversification of CI but not MO.

These findings highlight how community context influences evolutionary outcomes: while pairwise competition often leads to exclusion, multi-species environments may support more stable and diverse communities. Several pairwise combinations were excluded from analysis due to early extinction or contamination; in these cases, one species disappeared after the first passage, or plates were compromised, making continued tracking impossible. These limitations

further justify the focus on Community G for multi-species analysis, as it provided the most robust and interpretable dataset for examining long-term evolutionary dynamics.

The community condition also revealed variation in bacterial composition. Additionally, worm digests exhibited lower species richness compared to lawns, implying that host-mediated selection might further refine microbial community composition by favoring species with stronger colonization or persistence traits.

The patterns seen in MO persistence across all experiments are consistent with prior studies, suggesting that MO has robust survival mechanisms that allow it to thrive under both single and community conditions without significant phenotypic change. MO fractions were similar across community replicates compared to previous studies.

Some limitations included contamination events: Community G replicates 4–6 in passage 5 were stopped due to contamination.

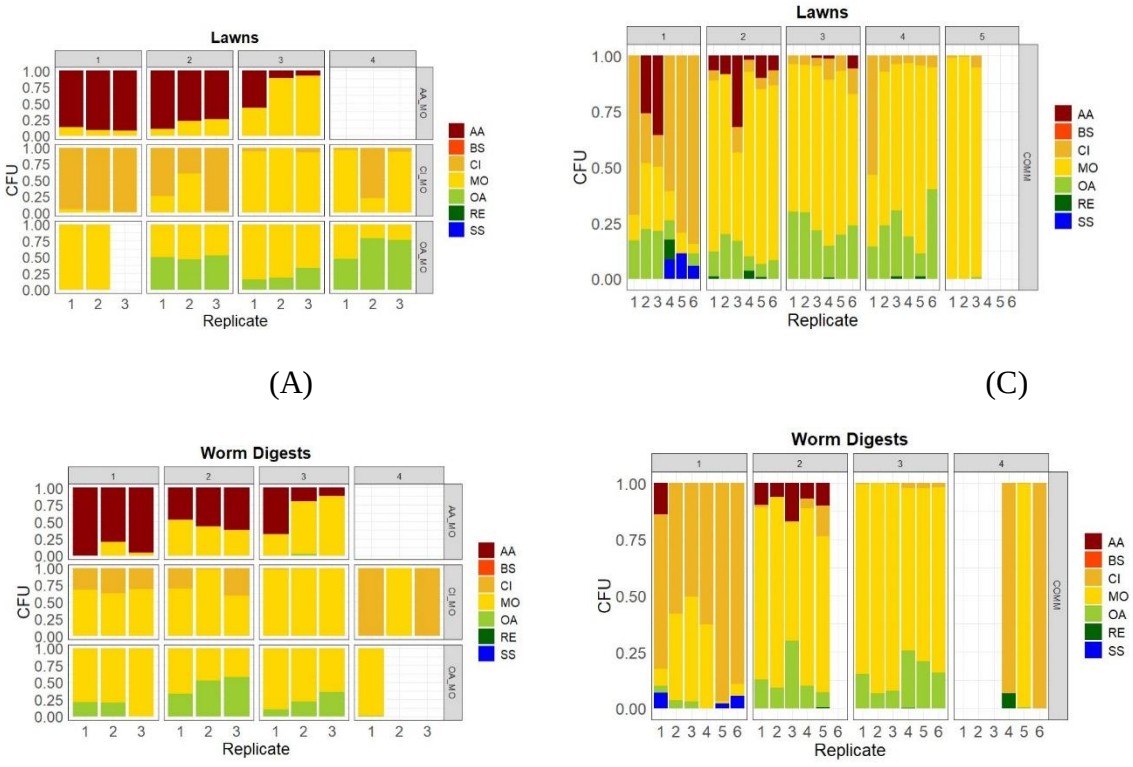

Figure 3. Composition of bacterial pairs and bacterial community G during experimental evolution.
Data shown are relative abundance of member taxa within each condition and replicate, over

passages 1–4 of the evolution experiment. Pairs were established in triplicate; Community G was established with six replicate plates. X-axes represent replicate groups; Y-axes show the relative CFU counts of different bacterial species.

(A-B) Pairwise combinations of bacteria:
(A) Lawn compositions showed relatively consistent ratios of the two strains across replicates.
(B) Lawn compositions varied across generations and replicates, particularly for CI and OA.

(C-D) Community G:
(C) Worm-associated communities showed more stable ratios between MO, OA, and CI across generations.
(D) Lawn communities showed more variation across both generations and replicates.

Principal Component Analysis (PCA) was performed to visualize shifts in microbial community composition across different passages in both lawns and worm-associated communities. The results indicate substantial divergence between early and late passages, with a large gap between the first two generations and more constrained variation in later passages. This suggests that early generations experienced rapid restructuring of community composition.

Additionally, a notable reduction in diversity was observed within the first two passages, consistent with rapid early competitive exclusion. Following this initial loss of diversity, alternate morphotypes of CI, but not MO, emerged in Community G replicates.

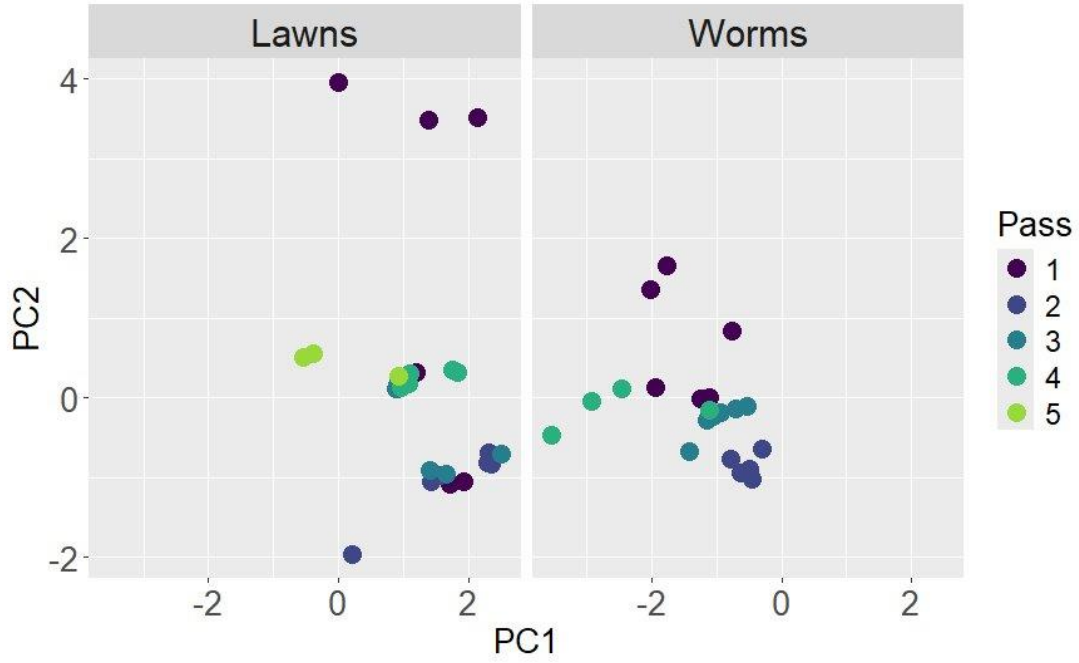

Figure 4. PCA diagram shows the relationship between community replicates for lawns and worms' colony.

Alternate Morphotypes

Although these experiments were set up to investigate replicability of alternate morphotype emergence in *M. oxydans*, we do not observe any alternate morphs in this species. Instead, we observed emergence of alternate morphs in *Chrysobacterium indologenes* (CI).

Motility assays were set up to investigate the repeatability of alternate morphotype emergence in *Microbacterium oxydans* (MO). However, no alternate morphotypes were observed in this species under the tested conditions. In contrast, during the same motility assays, new alternate morphotypes emerged in *Chryseobacterium indologenes* (CI). These morphs appeared 6–7 days after the motility assays were initiated and were first observed at passages 3, 4, and 5 in Community G replicates 1, 2, 5, and 6.

Prior to this experiment, alternate morphotypes of CI had not been documented in our lab's evolution experiments. The newly observed morphotypes were distinguished by the presence of fluffy, irregular regions along the colony edges, differing from both the ancestral and previously known morphologies. These edge-fluffy morphotypes appeared to have potentially higher spreading speeds compared to the original alternate morphs previously isolated earlier in the evolution experiment. Their distinct morphology suggests that these variants may possess enhanced motility traits or altered surface interactions, although further characterization would be needed to confirm these functional differences.

Logistic growth

To assess whether alternate morphotypes differed from original morphs or the ancestral strain in growth dynamics, we measured optical density (OD600) over time and compared growth rates and saturation densities across replicate communities. Growth curves revealed that all isolates—ancestral (ANC), original/light morphs, and alternate/dark morphs—exhibited typical logistic growth on liquid media, but with noticeable variation in both growth rate and final OD among groups (Figure 1). The ancestral strain consistently reached the highest OD600 values across time, followed by light morphs, with dark morphs generally showing the slowest or lowest growth performance.

To quantify these differences, we calculated growth rate (r) and carrying capacity (K, represented by saturation OD600) and compared them between light and dark morphs within each community replicate using Wilcoxon tests (Figure 2). In most communities, there was no statistically significant difference in growth rate between morphotypes. However, in Community G5, there was a trend toward higher growth rates in light morphs ($p = 0.057$), suggesting a potential but non-significant performance difference. In contrast, clear and significant differences in saturation density were observed in some replicates. Notably, in Community G1 ($p = 0.00016$) and G6 ($p = 0.0047$), light morphs reached significantly lower OD600 values than their dark

counterparts. This suggests that although growth rates were similar, dark morphs in these communities reached higher densities in stationary phase. However, as OD-to-CFU mapping was not carried out, it is also possible that the difference in optical density reflects a difference in cell size or secretion of extracellular factors rather than a change in cell counts.

These findings indicate that some alternate morphotypes exhibit meaningful physiological differences from original morphs and their ancestors, particularly in their saturation densities, but that these differences were neither consistent nor universal. While differences in growth rate were subtle, reduced saturation OD in light morphs in some replicates may suggest trade-offs between dispersal traits (e.g., motility) and resource accumulation or growth efficiency. These growth assays help to clarify whether phenotypic changes observed on solid media (e.g., colony morphology or motility) correspond to measurable shifts in overall growth dynamics in liquid culture.

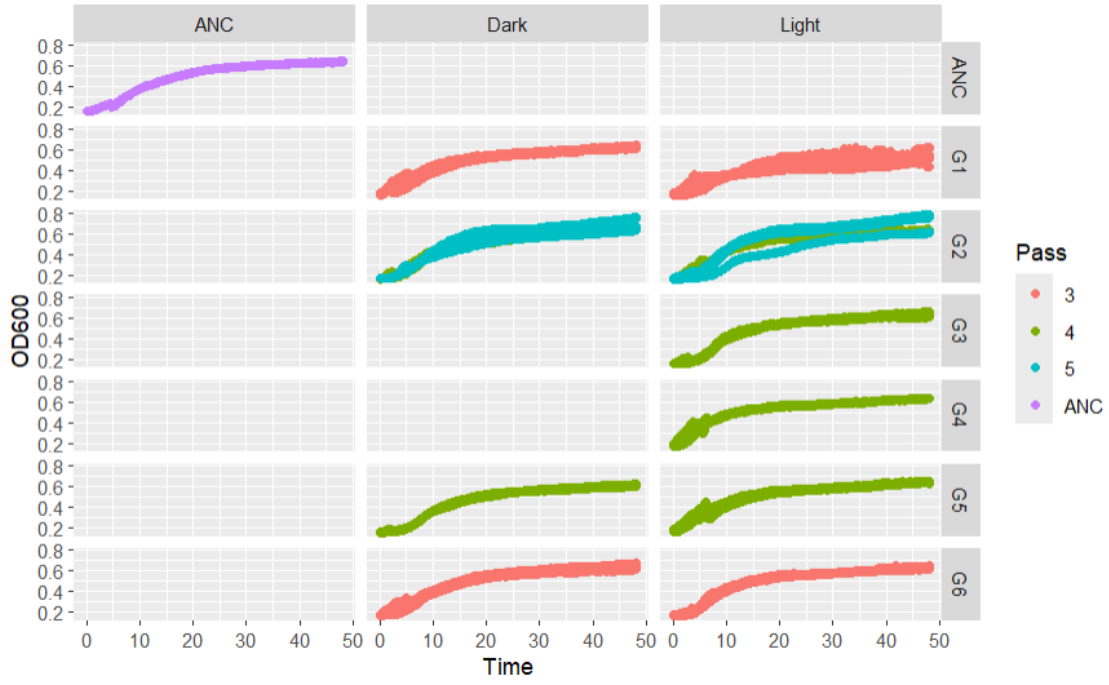

Figure5. Growth curves of ancestral, dark morph, and light morph isolate across replicate communities.
OD600 was measured over time in liquid culture to compare growth dynamics between Chryseobacterium indologenes morphotypes (ancestral, dark, and light) evolved in different replicate populations (G1–G6).

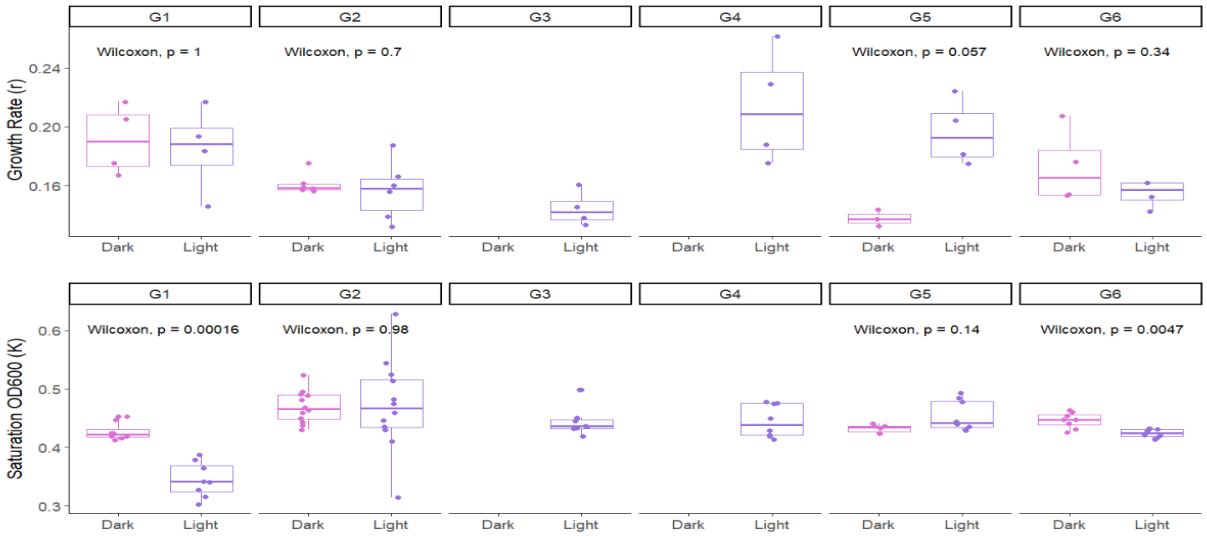

Figure 6. Growth rate (r) and saturation density (K) of evolved Chryseobacterium indologenes (CI) morphotypes across replicate communities.
Boxplots show comparisons of growth rate and final OD_{600} between dark and light morphs isolated from replicate Community G populations. Dark morphs refer to the alternate colony morphotypes that evolved during experimental evolution, while light morphs correspond to original-like colony morphotypes. Wilcoxon signed-rank p-values indicate the statistical significance of each comparison. The ancestral CI strain was not included in these comparisons, as the focus was on differences between the two newly evolved morphotypes rather than comparisons with the ancestor.

Motility

As motility of community members was altered (CG, MO) in the previous experiment, we next sought to determine whether morphotypes could be characterized by differences in this trait. *C. indologenes* are non-flagellate, gram-negative bacteria; like other Flavobacteriales, these bacteria show T9SS-dependent gliding motility on the surface of soft agar plates. Surface motility assays were therefore conducted on soft (0.25-0.35%) NGM agar to determine whether the alternate morphology was associated with changes in motility.

Colonies of *Chryseobacterium indologenes* (CI) were isolated from lawns and categorized into three morphotypes: Type 1, Type 2, and Type 3. Type 1 colonies were only observed at Passage 3 in Community G1. Four Type 1 colonies (A1–A4) were isolated from G1 lawns during this passage.

Type 2 colonies were more widespread and collected from multiple communities and passages. At Passage 3, Type 2 colonies were isolated from Community G1 (A5–A8) and Community G6 (A10, A11, B3, and B4). At Passage 4, Type 2 colonies were found in Community G2 (B7 and B8) and Community G5 (C9–C11). In Passage 5, additional Type 2 colonies were collected from

Community G2 (D1–D4). Overall, Type 2 morphotypes were present across Passages 3, 4, and 5, and were isolated from Communities G1, G2, G5, and G6.

Type 3 colonies were the most common and widely distributed across later passages. At Passage 3, Type 3 colonies were isolated from Community G6 (A9, A12, B1, and B2). At Passage 4, Type 3 colonies were collected from Community G2 (B5 and B6), Community G3 (B9–B12), Community G4 (C1–C4), and Community G5 (C5–C8). In Passage 5, Type 3 colonies were again isolated from Community G2 (D5–D8).Additionally, an ancestral colony (C12) was included as a control reference.

Isolates of different types showed different patterns during expansion (Figure 3). Based on visual differences on motility plates, we separated these isolates into three motility types, which mapped directly onto the “dark” and “light” types indicated from colony morphology on 1.5% plates. The ancestral strain exhibited both ring-like structures and branching patterns. The same overall structure was retained in the Type 3 (original or “light” colony variant) morph. In contrast, the Type 2 (“dark” colony variant) alternative morph showed defined concentric rings but lacked pronounced branching, indicating a shift in surface spreading behavior. The Type 1 alternate morph (from community G1 only) exhibited limited pronounced branching but are more different than those other two types.

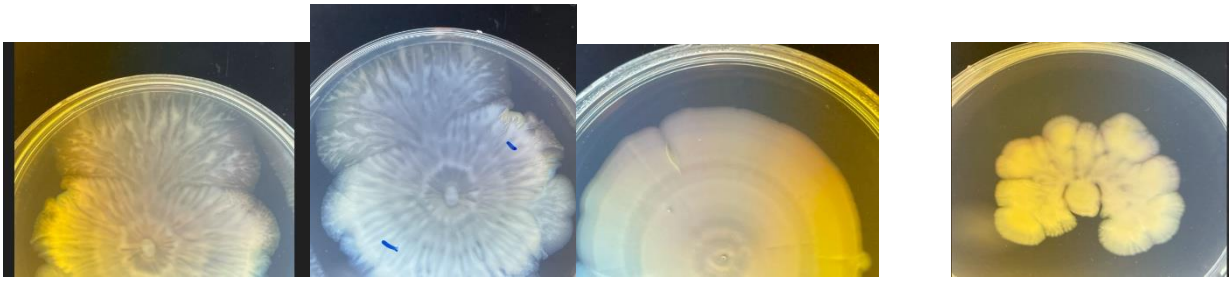

Figure 7. Motility Frontiers of *Chryseobacterium indologenes* Morphotypes.
The images show distinct colony morphologies of *C. indologenes* isolates from motility assays. From left to right: (1) ancestral strain, (2) light morph (Type 3 Community G6, Pass 3), (3) dark morph (Type 2, Community G2, Pass 4) , and (4) dark morph (Type 1, Community G1, Pass 3). Dark morphs correspond to the evolved alternate morphotypes, while the light morph resembles the original ancestral colony appearance. This arrangement highlights morphological variation between ancestral, light, and dark evolved forms.

Representative images of motility phenotypes were captured to compare the spreading behavior of ancestral, alternate, and original morphotypes of *Chryseobacterium indologenes* on soft agar. While specific isolates IDs were not recorded for these images, the selected colonies were chosen because they visually represented the typical phenotype of their respective group. Each

plate was incubated for 7 days at 25°C before imaging to allow for full expansion and expression of motility patterns. Images were taken using a standard smartphone camera under ambient laboratory lighting. These photos highlight clear differences in colony morphology and spreading dynamics: ancestral and original (light) morphs often formed branching or ring-like patterns, while alternate (dark) morphs tended to produce more compact, unbranched colonies with reduced surface coverage. These images illustrate consistent visual trends observed across replicates and support quantitative findings of reduced motility in evolved alternate morphotypes.

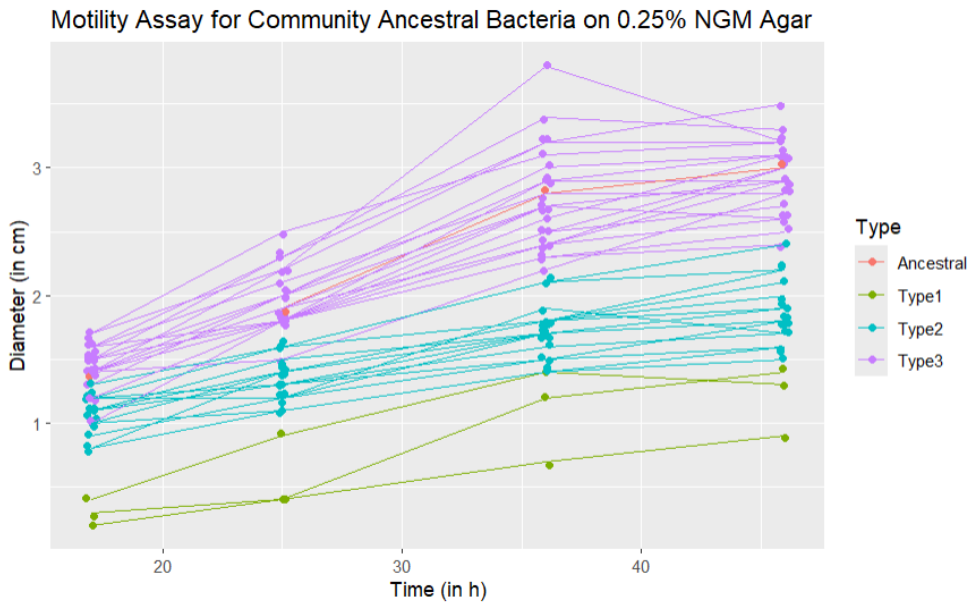

Figure 8. Motility in diameter (cm) of *C. indologenes* isolates across time (h) on 0.25% NGM agar plates.

The motility assay conducted on 0.25% NGM agar revealed distinct differences in bacterial spreading ability across the ancestral strain and evolved morphotypes (Figure 4). All isolates were motile on soft agar, and alternate morphs were consistently less motile than original morphs. Type 3 evolved isolates (“light” morphs, like ancestor) showed similar motility to the ancestor. In contrast, the evolved alternate morphotypes (Type 1, Type 2) displayed significantly reduced motility, with replicate G1 alternate morphs (Type 1) showing even less motility than other alternate morphs.

A linear regression was performed to assess the relationship between motility diameter and the factors community replicate, passage, and morphotype (type of revertant). The results showed that only morphotype had a statistically significant effect on motility diameter (p-value = 1.651e-

07), indicating that the type of revertant was the primary driver of variation in motility, whereas community replicate and passage did not have a significant impact.

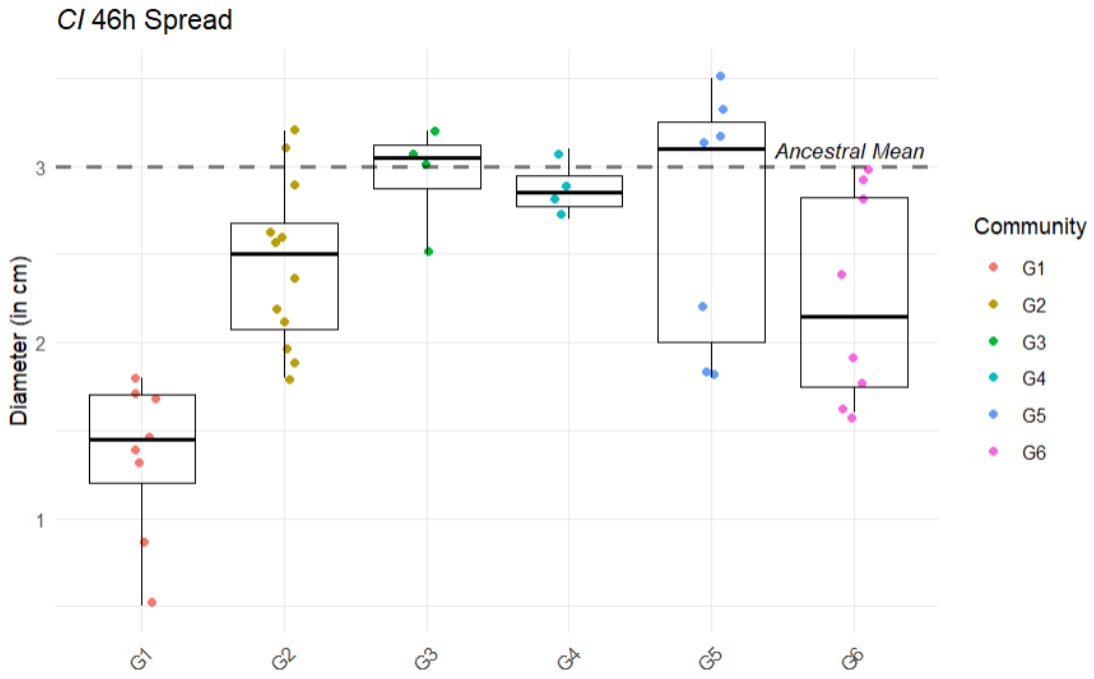

Figure 10. Motility Spread of *Chryseobacterium indologenes* (CI) at 46 Hours Across Replicate Communities.

The box plot shows the motility spread (diameter in cm) of *C. indologenes* measured after 46 hours on soft agar for individual evolved colonies isolated from replicate Community G populations (G1–G6). Each point represents the motility diameter of a single colony (clone); approximately 8–10 colonies were tested per community. The dashed line indicates the mean motility diameter of the ancestral strain for reference. Colonies were sampled from passages 3, 4, and 5, but passage information is not distinguished visually in this plot.

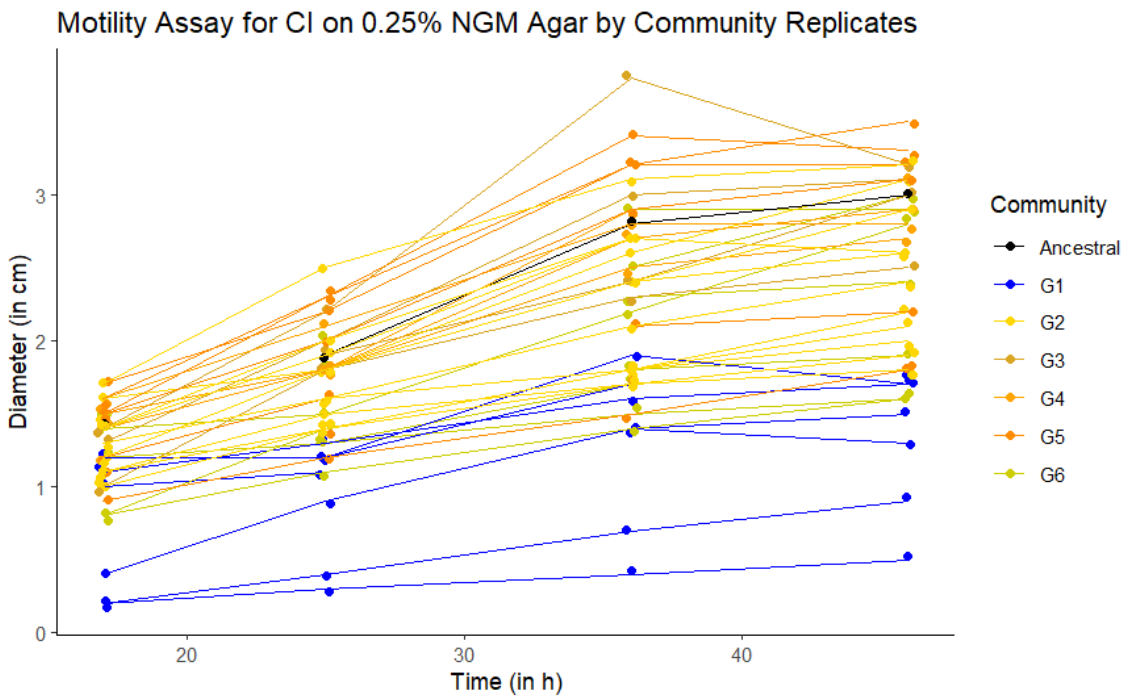

Figure 11. Motility Assay for *Chryseobacterium indologenes* (CI) Across Different Communities on 0.25% NGM Agar.
The plot shows motility spread (diameter in cm) of *C. indologenes* over time (18, 26, and 46 hours) across different evolved Community G replicates. Each line represents a single colony (clone); approximately 9–10 colonies were tested per community. The ancestral isolate is shown in black for comparison. Colonies tested included a mix of dark and light morphs corresponding to type 1, type 2, and type 3 morphotypes that evolved during experimental evolution.

All groups showed broadly overlapping trends in motility, with no clear divergence in spreading ability across communities or morphotypes. The consistency in motility suggests that evolutionary pressures in different community environments did not lead to major changes in motility traits. The slight variations observed are likely due to stochastic fluctuations rather than distinct adaptive differences.

Reversion

To investigate the stability of alternate morphotypes and the potential for phenotypic reversion, motility assays were conducted on low-agar (0.25%) NGM plates and monitored over several days for the emergence of revertant sectors. By days 6 or 7 of incubation, some colonies derived from alternate morphotypes developed distinct “fluffy” sectors at their outer edges. These sectors resembled the ancestral or original morphotype in appearance, suggesting potential reversion events. Reversion was observed in several replicates of Community G, including G1 passage 3, G6 passages 3 and 4, G1 passage 4, G5 passage 4, and G2 passage 5. In colonies where revertants appeared, typically 1 to 3 fluffy sectors could be collected per plate. These sectors

were isolated using sterile wooden dowels and inoculated into LB media in deep-well 96-well plates for overnight growth. However, due to inconsistent growth across replicate plates and issues with motility assay setup in some samples, the resulting data were not sufficient for robust quantitative comparison across groups. As a result, reversion was qualitatively observed but not systematically recorded for statistical analysis. The inconsistent appearance of revertants and limited sample sizes prevented meaningful comparisons between community replicates or passages. Nonetheless, the repeated emergence of revertant-like sectors in multiple samples suggests that alternate morphotypes retain some degree of phenotypic plasticity, and further genomic or transcriptomic analysis will be needed to understand the mechanisms underlying these apparent reversions.

Discussion

The results of this study demonstrate that microbial evolution within the *C. elegans* system is shaped by both community interactions and host-mediated selection pressures. The emergence of alternate bacterial morphotypes, shifts in species composition, and changes in motility provide valuable insights into the predictability of microbial evolution and the factors influencing community stability. In agreement with previous findings on species interactions, our data show that while some evolutionary outcomes are repeatable, others are highly context-dependent, depending on both competitive dynamics and environmental constraints (Castledine et al., 2020; Lawrence et al., 2012).

The host-associated environment imposed a strong filtering effect, leading to greater compositional stability in worm-associated bacterial populations compared to those on lawns. This suggests that certain bacterial species are better adapted to persist in the host gut, potentially due to selective pressures such as nutrient competition, immune evasion, or spatial structuring (Samuel et al., 2016; Gibson et al., 2019). Principal Component Analysis (PCA) revealed that early-generation communities exhibited significant divergence, followed by stabilization in later passages, likely reflecting an initial phase of competitive exclusion before the establishment of stable microbial populations. This pattern aligns with prior research demonstrating that host-associated microbiomes tend to become more specialized and less variable over time as selective pressures refine community composition (Meroz et al., 2021).

Community interactions played a crucial role in shaping evolutionary outcomes. In pairwise conditions, competitive exclusion was frequently observed, with one species dominating while the other was driven to extinction. However, in community conditions, species coexistence was more prevalent, suggesting that higher-order interactions can buffer competitive pressures and promote microbial diversity. Coexistence are consistent in terms of the bacteria strains that can coexist but not the ratio they exists. The consistent decline of *Arthrobacter aurescens* (AA) across both pairwise and community conditions suggests that it was at a competitive disadvantage, whereas *Microbacterium oxydans* (MO) and *Ochrobactrum anthropi* (OA) were able to persist. These results support prior findings that species interactions can constrain or

facilitate evolutionary trajectories depending on ecological context (Fiegna et al., 2015; Scheuerl et al., 2020). The variation in *Chryseobacterium indologenes* (CI) abundance between pairwise and community conditions further highlights the influence of species interactions on microbial persistence and adaptation.

Motility emerged as a key adaptive trait, with alternate morphs consistently exhibiting reduced motility compared to their ancestral forms. Similar trends have been documented in other microbial evolution experiments, where motility is lost in favor of traits such as branching or concentric rings that provide competitive advantages in structured environments (van Ditmarsch et al., 2013; Rainey & Travisano, 1998). Additionally, the evolution of alternate morphotypes primarily in community conditions but not in pairwise or single-species experiments suggests that their emergence is driven by interactions between multiple bacterial species rather than independent selection pressures. This aligns with previous studies indicating that microbial communities undergoing co-evolution can generate novel phenotypic diversity that is absent in isolated populations (Duckett et al., 2024).

The presence of apparent revertants in the motility assays suggests that some alternate morphs may retain the potential for converting back to original morphs. However, it is not clear that these regions indicate true revertants – regions of apparently increased motility might also might be caused by slight variations in the plate surface, for example wetter regions on the plates. Even if the apparent revertants have restored the original phenotype, the mechanism would remain to be determined; in particular, whether these changes are driven by genetic changes or regulatory shifts remains an open question. Instances of reversible phenotypic changes have been observed in long-term microbial evolution experiments, where selection pressures fluctuate over time, leading to the retention of plasticity in certain traits (Lenski et al., 1991). Future genomic analyses of evolved morphotypes and their revertants will provide further insights into the stability and genetic basis of these adaptations.

These findings contribute to broader discussions on the predictability of microbial evolution. While some aspects, such as the decline of *A. aurescens* and the persistence of *M. oxydans*, were consistent across replicates, others, such as *C. indologenes* abundance and the emergence of alternate morphs, were more variable. This suggests that while strong selective pressures drive convergence in certain traits, other aspects of microbial evolution remain contingent on species interactions and environmental conditions (Taylor et al., 2022).

Overall, this study provides evidence that microbial evolution in host-associated environments is shaped by a combination of competition, host filtering, and adaptive trade-offs. The emergence of alternate morphotypes, shifts in motility, and differences in species persistence highlight the complex interplay between selection pressures and microbial community dynamics. By integrating experimental evolution with host-microbe interactions, we gain deeper insights into the factors that drive microbial diversity and evolutionary trajectories. Future work should focus

on genomic analyses of alternate morphs and revertants to uncover the underlying genetic mechanisms and assess whether similar evolutionary patterns occur across different microbial communities.

While this study provides key insights into microbial evolution within host-associated environments, several questions remain unanswered, warranting further investigation. One important avenue for future research is the genomic analysis of alternate morphotypes and revertants. Whole-genome sequencing of these variants could reveal the genetic basis of observed phenotypic shifts, determining whether changes in motility and morphology are driven by single nucleotide polymorphisms, large structural variations, or regulatory mutations. Additionally, transcriptomic analysis during different stages of adaptation could provide a clearer understanding of the molecular mechanisms governing bacterial interactions and adaptation.

Another key direction is exploring the repeatability of evolution in different host-associated microbial communities. While this study focused on C. elegans, similar experiments could be conducted in other model hosts, such as Drosophila melanogaster or zebrafish, to assess whether the same evolutionary patterns emerge in different host environments. Comparing how microbial communities evolve across hosts with varying immune responses, gut structures, and nutrient profiles would help determine the generalizability of the observed adaptations. Furthermore, experiments using synthetic microbial consortia with defined species compositions could allow for more controlled testing of how specific interactions shape evolutionary outcomes.

Experimental evolution over longer timescales could also provide deeper insights into the stability of alternate morphs and the role of long-term ecological dynamics. It remains unclear whether the observed alternate morphs would persist indefinitely or if additional evolutionary pressures could lead to further diversification or convergence. Expanding the number of evolutionary passages beyond five generations could help determine whether certain morphotypes remain stable or undergo additional adaptation over time. Similarly, examining the impact of fluctuating environmental conditions, such as changes in nutrient availability or host immune challenges, would provide a more comprehensive understanding of how microbial communities respond to variable selection pressures.

Despite the strengths of this study, several limitations should be acknowledged. First, while the experimental evolution design allowed for the identification of adaptive changes over multiple generations, the relatively short timeframe may not have captured the full extent of microbial diversification. Future studies with extended evolutionary timescales would be needed to assess the long-term stability of the observed adaptations. Additionally, although community dynamics were analyzed in the presence and absence of *C. elegans*, the complexity of natural microbial ecosystems is likely greater than what was replicated in this experimental system. In nature, microbial communities are influenced by a broader range of abiotic and biotic factors, such as

fluctuating host diet, environmental stressors, and additional microbial taxa that were not included in this study.

Another limitation is the focus on a subset of bacterial strains, which may not fully represent the diversity of microbial interactions that occur in more complex host-associated microbiomes. Future research could incorporate metagenomic approaches to analyze more diverse bacterial communities and identify broader evolutionary trends across different microbial lineages. Finally, while motility and morphological changes were key traits examined in this study, other potential adaptations, such as metabolic shifts or antibiotic resistance, were not assessed. Investigating how alternate morphotypes differ in metabolic activity, resistance to host immune defenses, or cooperative interactions with other bacteria would provide a more holistic view of microbial adaptation in host-associated environments.

This study provides key insights into how microbial evolution is shaped by both host-associated selection pressures and community interactions. The emergence of alternate morphotypes, shifts in motility, and species persistence patterns highlight the dynamic interplay between competition, adaptation, and host filtering. These findings contribute to our understanding of the predictability of evolution, demonstrating that while some evolutionary outcomes are repeatable, others are contingent on ecological context and species interactions. By integrating experimental evolution with host-microbe dynamics, this research sheds light on the mechanisms that drive microbial diversity and stability in host-associated environments.

Future work should focus on the genetic basis of these adaptations, the long-term stability of alternate morphs, and whether similar evolutionary patterns emerge in other microbial ecosystems. Expanding these studies across different host environments and microbial consortia will further refine our understanding of the evolutionary forces shaping microbial communities. Ultimately, this research underscores the complexity of microbial evolution and provides a foundation for future studies exploring the broader implications of species interactions in shaping evolutionary trajectories.

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