Title

Reconstructing the Early Evolution of Insect-Bacterial Mutualism through Natural Symbiotic Systems, Experimental Evolution, and Symbiosis Engineering

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Declaration of interest

None

Abstract

The evolutionary transition from free-living bacteria to mutualistic symbionts remains a central question in symbiosis research. Traditionally, this process has been inferred from comparative analyses of extant symbiotic systems, which provide evolutionary snapshots but offer limited insight into the processes connecting them. Recent studies of the stinkbug *Plautia stali* and its bacterial associates have begun to address this gap by integrating comparative analyses of natural systems, experimental evolution, and symbiosis engineering. Natural systems generate hypotheses about possible evolutionary trajectories, experimental evolution reconstructs the emergence of mutualistic traits and symbiont replacement, and synthetic approaches identify the biological changes that are sufficient to confer host benefits. Together, these complementary approaches suggest that mutualism can, in some cases, arise through relatively minor modifications of existing regulatory and metabolic networks and establish a new empirical framework for investigating the origins, dynamics, and evolution of insect-bacterial mutualism.

Keywords

Insect-bacterial mutualism; Experimental evolution; Symbiosis engineering; Symbiont replacement; Mutualism evolution

Highlights

Natural symbiotic systems provide evolutionary snapshots spanning free-living bacteria, culturable symbionts, and highly specialized obligate symbionts.

Experimental evolution in the *Plautia stali-Escherichia coli* system has enabled the reconstruction of the early stages of mutualism under laboratory conditions.

Rewiring of existing regulatory and metabolic networks, rather than acquisition of novel functions, can drive the transition to mutualism.

Experimental reconstruction of symbiont replacement reveals that successful host colonization and host benefit are not necessarily coupled traits.

Symbiosis engineering and experimental evolution provide convergent approaches for identifying the mechanisms underlying the origin of insect-bacterial mutualism.

Introduction

Insect-bacterial mutualisms are among the most important interspecies interactions in nature, underpinning host survival, ecological diversification, and evolutionary success through functions such as nutritional provisioning, defense against natural enemies, and environmental adaptation [1-4]. In particular, insects that rely on nutritionally imbalanced diets, such as plant phloem sap or other nutrient-poor food sources, frequently depend on symbiotic bacteria to supply essential amino acids, vitamins, and other metabolites that are scarce or absent in their diets. In many cases, these microbial partners are indispensable for host development and reproduction. Highly integrated symbioses, in which bacterial symbionts exhibit extreme genome reduction and strict host dependence, have been extensively studied and have provided fundamental insights into the long-term consequences of host-microbe coevolution [5]. However, these systems represent advanced snapshots along prolonged evolutionary processes and offer limited opportunities to directly investigate how free-living bacteria initially transitioned into beneficial symbiotic partners.

Consequently, research on the origins of insect-microbe symbiosis has traditionally relied heavily on comparative approaches. Comparative genomics and phylogenetic analyses have revealed which genes and metabolic pathways have been retained, modified, or lost during symbiont evolution, providing important insights into the evolutionary trajectories of established symbiotic associations. Nevertheless, such approaches are fundamentally limited in their ability to reconstruct the sequence of evolutionary events that gave rise to mutualism. For example, it remains difficult to determine the order in which key traits - such as reduction of host harm, enhancement of colonization ability, and acquisition of nutritional provisioning - were established, as well as whether these traits evolved independently or in an interdependent manner. Understanding the origin of insect-microbe symbiosis therefore requires approaches that can capture the processes of evolutionary change, rather than solely its end products.

In this context, experimental evolution offers a powerful framework for directly investigating evolutionary processes. While long-term evolution experiments in bacteria and studies of host-parasite systems have revealed fundamental principles of adaptation and coevolution [6-9], their application to animal-microbe mutualism has been relatively limited [10].

Recently, however, the stinkbug *Plautia stali* and its associated bacteria have emerged as a model system providing a particularly tractable platform that enables the integration of experimental evolution, comparative analyses of natural symbioses, and synthetic approaches. These studies open new avenues for empirically investigating the origins of mutualistic symbiosis. In this review, we discuss how these complementary approaches can be combined

to elucidate the transition from free-living bacteria to mutualistic symbionts and advance our understanding of the evolutionary origins of insect-bacterial mutualism (Figure 1).

Evolutionary Hypotheses from Natural Symbiotic Systems

One promising avenue for investigating the early stages of symbiosis evolution is the study of natural symbiotic systems that retain bacterial partners with characteristics of free-living microorganisms. In the stinkbug *P. stali*, distinct geographic populations harbor different gut symbionts, some of which remain culturable and retain traits associated with a free-living lifestyle [11]. Moreover, experimental screening of soil-derived bacteria identified free-living relatives of the symbionts that retain the capacity to support normal development of *P. stali*, demonstrating that host-beneficial traits can exist outside established symbiotic associations. These findings suggest that traits required for host association and host benefit may already exist in environmental bacterial populations, providing a plausible starting point for the evolution of insect-microbe mutualism.

Collectively, these observations provide a series of evolutionary snapshots of bacteria associated with a single host species, ranging from free-living environmental strains capable of supporting host development to culturable symbionts and highly specialized unculturable symbionts. While these bacteria cannot be assumed to represent a direct evolutionary progression, they provide a framework for investigating how host-beneficial traits, host dependence, and symbiotic specialization may emerge during the evolution of mutualism. Such systems therefore offer valuable hypotheses regarding the phenotypic and genomic transitions that accompany the shift from a free-living lifestyle to obligate symbiosis. Comparative analyses of natural symbiotic systems therefore provide not only descriptions of established associations but also evolutionary scenarios that can subsequently be tested through experimental approaches.

Experimental reconstruction of mutualism through evolution in an artificial symbiotic system

While natural symbiotic systems provide snapshots of potential evolutionary states, experimental evolution offers a means to reconstruct and dissect the evolutionary processes that may connect them. Using the stinkbug *P. stali*, we previously found that replacement of the native obligate symbiont with *Escherichia coli* occasionally yielded insects that successfully developed into fertile adults, but only at a very low frequency [11]. This finding suggested that the host is not exclusively dependent on its native symbiont; rather, under certain conditions, it can provide a suitable niche within its symbiotic organ for alternative bacteria and tolerate them as symbiotic partners. It provided a starting point for our experimental evolution project.

In the project, we established multiple host lineages harboring a hypermutable strain of *E. coli* and maintained them through serial passage. Remarkably, several independently evolving lineages acquired mutualistic properties that significantly improved host survival and adult emergence rates. The evolved *E. coli* strains also exhibited phenotypic characteristics commonly observed in insect symbionts, including reduced growth rate, loss of motility, and diminished extracellular matrix production [12].

Genomic and transcriptomic analyses revealed that these independently evolved lineages repeatedly acquired mutations in global regulatory pathways associated with carbon catabolite repression (CCR). Introduction of the evolved mutations into ancestral *E. coli* was sufficient to reproduce the mutualistic phenotype, demonstrating that these genetic changes contributed to bacterial adaptation to the host environment. Subsequent analyses further showed that altered tryptophan metabolism, leading to tryptophan accumulation through reduced expression of the tryptophanase gene *tnaA*, played a critical role in improving host fitness [13].

These findings indicate that the initial transition toward mutualism may depend less on the acquisition of novel functions than on the rewiring of pre-existing regulatory and metabolic networks. In other words, mutualistic interactions can emerge through changes in the allocation and regulation of existing physiological functions, without evolving entirely new genetic functions. This study therefore provides experimental evidence that the earliest stages of symbiosis evolution can be initiated by a relatively small number of genetic changes.

Experimental reconstruction of symbiont replacement

Bacteria that evolve increased compatibility with their hosts may acquire not only the ability to confer benefits but also the capacity to displace pre-existing symbiotic partners. We recently demonstrated that one lineage of experimentally evolved *E. coli* was capable of competitively excluding the native symbionts of *P. stali* [14]. Remarkably, this evolved strain displaced not only culturable symbionts that are thought to represent relatively early stages of symbiotic specialization, but also the unculturable symbiont fixed in natural populations on mainland Honshu, Japan, despite its long-term association with the host. These findings suggest that even seemingly stable mutualistic associations may remain susceptible to invasion and replacement by newly emerging symbionts.

Importantly, however, the evolved *E. coli* strain did not confer host fitness benefits comparable to those provided by the native symbionts. This observation indicates that the ability to invade and persist within the host and the ability to improve host fitness are not necessarily coupled traits. Rather, they may evolve independently and be subject to distinct selective pressures. As a result, the symbionts that spread most successfully within host

populations are not necessarily those that confer the greatest benefits to their hosts. Understanding the evolution of mutualistic symbiosis therefore requires consideration of both host fitness effects and competition among symbionts.

A similar phenomenon has been demonstrated in an experimental system involving the grain beetle *Oryzaephilus surinamensis* and *Sodalis praecaptivus*, a culturable and genetically tractable bacterium closely related to many insect symbionts [15]. In this system, an artificially introduced symbiont was shown to replace an ancient native symbiont within only a few host generations. These studies demonstrate that symbiont replacement is not merely an inference derived from comparative phylogenetic analyses but an experimentally tractable process that can be reconstructed and mechanistically dissected under controlled conditions. More broadly, these findings suggest that symbiosis evolution should not be viewed solely as a process that optimizes host benefit. Instead, it represents a multilayered evolutionary process shaped by interactions among host selection, symbiont competition, and ecological opportunity. From this perspective, symbiont replacement in nature is not simply taxonomic turnover, but rather the outcome of competition and selection within host-associated microbial ecosystems.

Engineering symbiosis without evolution

In recent years, efforts to understand the origins of mutualism have expanded beyond experimental evolution to include synthetic approaches that directly engineer beneficial host-microbe interactions. For example, *S. praecaptivus* was converted from a harmful associate into a beneficial partner by engineering the overproduction of aromatic amino acids [16]. Remarkably, a bacterium that initially impaired host performance was transformed into an engineered symbiont that enhanced host development through a relatively simple metabolic modification. These findings demonstrate that host-beneficial traits need not arise exclusively through prolonged evolutionary processes but can emerge from relatively modest alterations in metabolic flux.

A similar pattern has been observed in our stinkbug-*E. coli* system. Modification of *metJ*, a transcriptional repressor involved in methionine metabolism, was sufficient to generate host-beneficial phenotypes [17]. Importantly, neither of these examples involved the acquisition of novel metabolic pathways. Rather, substantial changes in host-microbe interactions resulted solely from alterations in the regulation of pre-existing metabolic networks.

Experimental evolution and symbiosis engineering represent two distinct but convergent approaches to understanding the origins of mutualism. Whereas experimental evolution is used to investigate how mutualism evolves, synthetic approaches are used to test

what is required for mutualism to emerge. Despite their methodological differences, both approaches converge on a common conclusion: relatively small changes in metabolic regulation can profoundly alter the nature of host-microbe interactions. These findings suggest that the transition to mutualism may be more evolutionarily accessible than has traditionally been assumed.

Future perspectives

An important challenge for future studies will be to determine how broadly these mechanisms can be generalized across mutualistic host-microbe systems. A previous study has shown that the fitness effects of both native symbionts and experimentally evolved *E. coli* differed depending on host species, indicating that a strategy promoting mutualism in one host may not necessarily be effective in another [18]. Comparative analyses across multiple symbiotic systems will therefore be essential for distinguishing general principles of symbiosis evolution from lineage-specific adaptations.

At the same time, advances in analytical technologies are likely to further enhance the value of comparative studies of natural symbiotic systems. Integrative multi-omics approaches combining genomics, transcriptomics, proteomics, metabolomics, and phenomics may allow increasingly precise inferences regarding the evolutionary status of individual symbionts. Comparative analyses spanning free-living bacteria with host-beneficial potential, culturable symbionts that retain some degree of environmental competence, and highly specialized unculturable symbionts may offer an increasingly refined picture of the early stages of symbiosis evolution.

Recent studies have also revealed that multiple metabolic changes contributing to mutualism do not necessarily act additively. For example, mutations affecting methionine metabolism and tryptophan metabolism each improved host performance, yet their combination did not generate a proportionally greater benefit. This observation suggests that the effects of individual metabolic changes emerge within the context of complex metabolic and physiological networks formed between hosts and symbionts. Consequently, future efforts in symbiosis engineering may require a more systems-level perspective capable of predicting the behavior of integrated host-microbe networks, rather than focusing solely on individual genes or metabolic pathways.

In combination, natural symbiotic systems provide evolutionary snapshots and hypotheses, experimental evolution reconstructs the processes that may connect them, and symbiosis engineering tests the mechanistic requirements for mutualism. The convergence of these complementary approaches has begun to provide an empirical framework for addressing the longstanding question of how free-living bacteria evolve into insect-associated mutualistic

symbionts. As iterative cycles of natural history observations and manipulative experimentation become increasingly sophisticated, they will not only deepen our understanding of the origins of mutualism but also provide a more comprehensive view of the mechanisms underlying its stabilization, breakdown, and replacement [19].

Acknowledgements

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13**. Wang Y, Moriyama M, Koga R, Oguchi K, Hosokawa T, Takai H, Shigenobu S, Nikoh N, Fukatsu T: **Tryptophanase disruption promotes insect-bacterium mutualism**. *Nat Microbiol* 2026, **11**:759-769. https://doi.org/10.1038/s41564-026-02264-z.

The study by Wang et al. demonstrates that disruption of *tnaA* is sufficient to promote mutualistic interactions between bacteria and insect hosts, by increasing tryptophan availability and improving host fitness. This finding provides direct experimental evidence that relatively simple regulatory or metabolic modifications, rather than acquisition of novel functions, can drive the early transition from free-living bacteria to mutualistic symbionts.

14**. Cai W, Moriyama M, Nishide Y, Koga R, Fukatsu T: **Symbiotic *Escherichia coli* strains can better colonize host stinkbugs and outcompete natural symbiotic bacteria, but confer less fitness benefits**. *bioRxiv* 2026. https://doi.org/10.64898/2026.04.01.715892.

Cai et al. demonstrated that an experimentally evolved *E. coli* lineage, originally selected for improved performance within the host, acquired the ability to displace native symbionts and establish itself within host populations. Importantly, this enhanced competitive ability was not accompanied by a corresponding increase in host fitness, revealing that traits promoting symbiont persistence can evolve independently of traits that improve host benefit. The study provides experimental evidence that symbiont replacement is a tractable evolutionary process and highlights the importance of considering both host fitness effects and competition among symbionts when investigating the early evolution of mutualism.

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Krüsemer et al. demonstrated that introduction of the facultative symbiont *S. praecaptivus* into

a grain beetle host led to the rapid displacement of an ancient obligate symbiont. This study provides direct experimental evidence that symbiont replacement can occur over remarkably short timescales. Together with our recent findings in stinkbugs, these results suggest that even long-established mutualistic associations remain evolutionarily dynamic and susceptible to invasion by novel symbiotic partners.

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This study shows that disruption of the methionine synthesis repressor *metJ* can enhance the mutualistic performance of *E. coli* in the stinkbug *P. stali*. Together with previous findings on *tnaA*, these results support a model in which modest regulatory changes are sufficient to promote mutualism. Notably, the lack of additive effects in the Δ*metJ* Δ*tnaA* double mutant indicates that the fitness consequences of metabolic modifications depend on interactions within the host-symbiont metabolic network, highlighting the non-linear and systems-level nature of mutualism evolution.

18*. Sugiyama R, Moriyama M, Koga R, Fukatsu T: **Host range of naturally and artificially evolved symbiotic bacteria for a specific host insect**. *mBio* 2024, **15**:e01342-24. https://doi.org/10.1128/mbio.01342-24.

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Figure legends

Figure 1. Conceptual framework for reconstructing the early evolution of insect-bacterial mutualism.

Natural symbiotic systems provide evolutionary snapshots spanning different degrees of

symbiotic specialization. Comparative analyses of these systems generate hypotheses about the traits and transitions associated with symbiosis evolution, whereas experimental evolution and symbiosis engineering enable these hypotheses to be tested and reconstructed under controlled conditions. Together, these complementary approaches establish a conceptual framework for identifying the key traits and evolutionary processes that drive the transition from free-living bacteria to mutualistic symbionts. The red, blue, and green solids shown on the left and right sides schematically represent hypothetical evolutionary trajectories of symbiosis-related traits. Changes in trait magnitude are illustrated by variation in the volume of each solid and, in cross-section, by the size of its projected area.

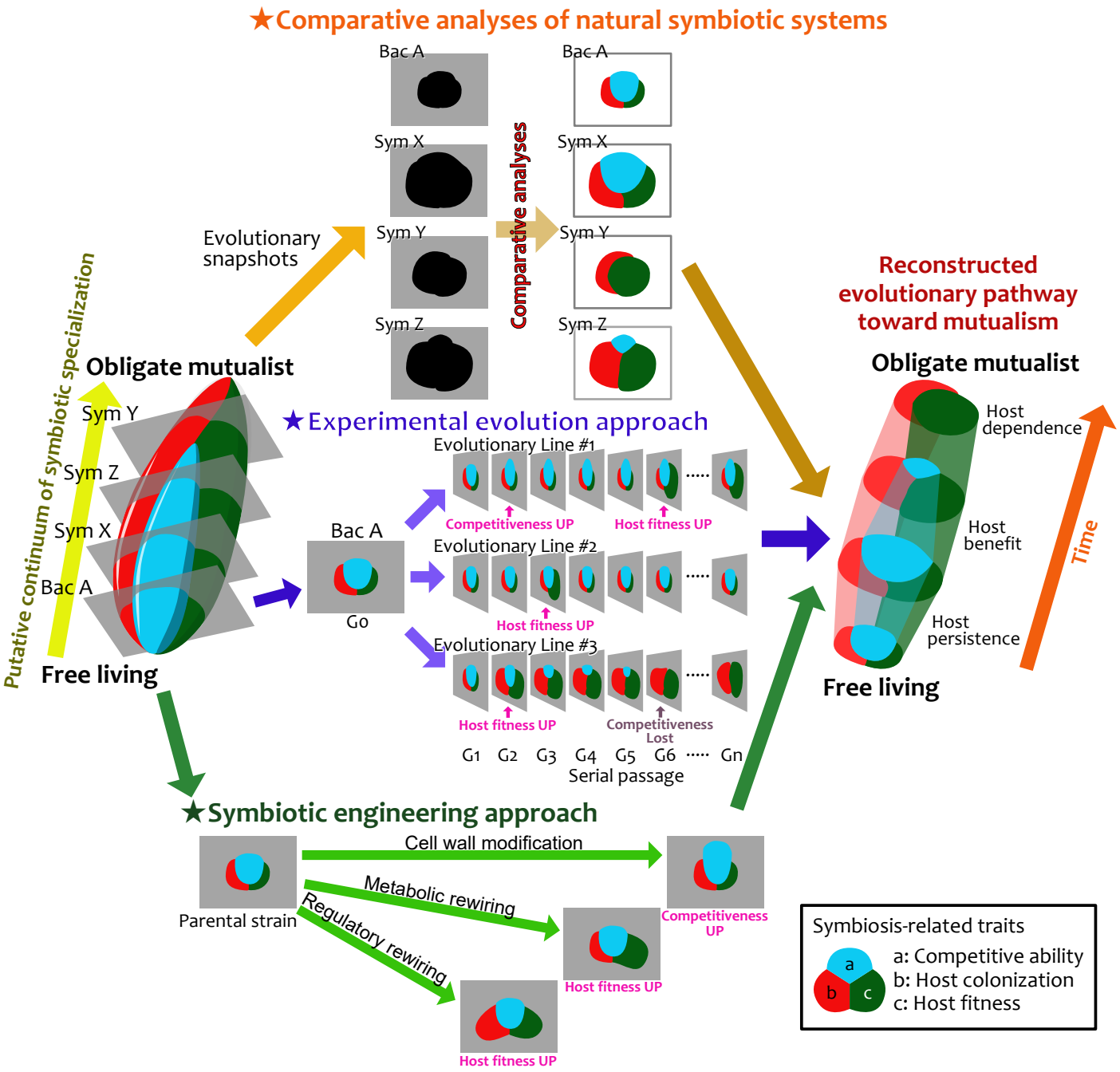
★Comparative analyses of natural symbiotic systems
Bac A
Sym X
Sym Y
Sym Z
Evolutionary snapshots
Comparative analyses
Reconstructed evolutionary pathway toward mutualism
Obligate mutualist
Putative continuum of symbiotic specialization
★Experimental evolution approach
Evolutionary Line #1
Competitiveness UP
Host fitness UP
Evolutionary Line #2
Evolutionary Line #3
Host fitness UP
Competitiveness Lost
G0
G1 G2 G3 G4 G5 G6 ····· Gn
Serial passage
Host dependence
Host benefit
Host persistence
Time
Free living
★Symbiotic engineering approach
Parental strain
Cell wall modification
Metabolic rewiring
Regulatory rewiring
Competitiveness UP
Host fitness UP
Host fitness UP
Symbiosis-related traits
a: Competitive ability
b: Host colonization
c: Host fitness

Figure 1