Coevolutionary interactions between a defensive microbe and a pathogen within a *Caenorhabditis elegans* model host

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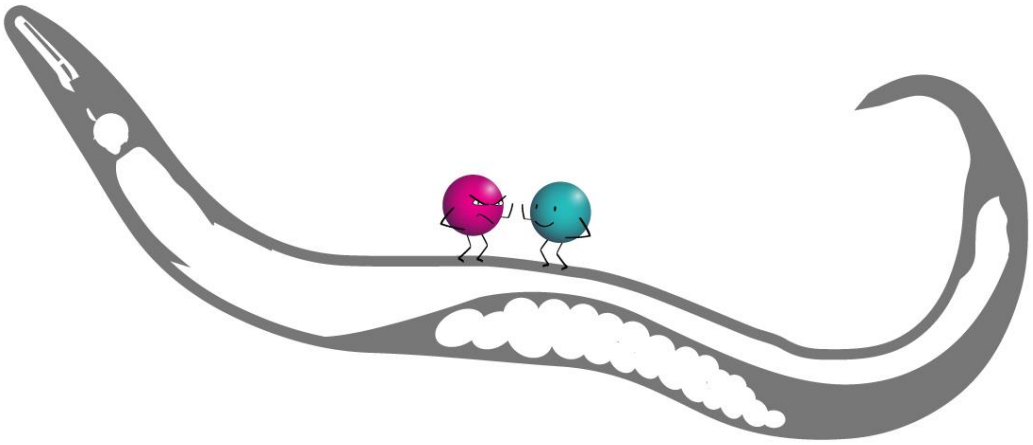


Image by Dan Guy

The enemy of my enemy is my friend

- *Ancient proverb*

Declaration

I declare that I wrote this thesis and that the work is my own except where explicitly stated in the text. This work has not been submitted for any degree or professional qualification except as specified.

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Publications

All published articles have been included in the Appendix. The general introduction and Chapters one and two of this thesis are published as:

Ford SA, King KC (2016) Harnessing the Power of Defensive Microbes: Evolutionary Implications in Nature and Disease Control. *PLoS Pathogens* 12(4): e1005465. doi:10.1371/journal.ppat.1005465

Ford SA, Kao D, Williams D, King KC (2016) Microbe-mediated host defence drives the evolution of reduced pathogen virulence. *Nature Communications* 7, 13430. doi:10.1038/ncomms13430

Ford SA, Williams D, Paterson S, King KC (2016) Coevolutionary dynamics between a defensive microbe and a pathogen driven by fluctuating selection. *Molecular Ecology* doi: 10.1111/mec.13906

Chapter three is in preparation for submission. During my DPhil I also contributed to one other published project (see appendix):

King KC, Brockhurst MA, Vasieva O, Paterson S, Betts A, **Ford SA**, Frost CL, Horsburgh MJ, Haldenby S & Hurst GD (2016) Rapid evolution of microbe-mediated protection against pathogens in a worm host. *The ISME Journal* 10*,* 1915–1924. doi: 10.1038/ismej.2015.259

Abstract

Microbes can protect their plant and animal hosts against infection by pathogens, parasites and parasitoids. These 'defensive microbes' can provide a powerful line of defence beyond the host response and are becoming attractive candidates for disease control. In this thesis, I investigated how defensive microbes can interact with pathogens over evolutionary time by measuring the effects of co-passaging a defensive microbe (*Enterococcus faecalis*) and a pathogen (*Staphylococcus aureus*) within the *Caenorhabditis elegans* model host. In Chapter 1, I found that co-passaging drove the evolution of reduced pathogen virulence as a by-product of adaptation to microbe-mediated defence. Moreover, I show that the mechanism of pathogen resistance to the defensive microbe can determine the direction of virulence evolution. In chapter two, I discovered that the co-passaged defensive microbe and pathogen populations had undergone coevolutionary interactions within host populations via fluctuating selection dynamics. I then showed that these dynamics resulted in patterns of pathogen local adaptation and increased genetic divergence. Finally, in chapter three, I revealed that these coevolutionary interactions significantly affected the costs and benefits of the defensive microbes to their hosts, but that the relationship between these costs and benefits prevented the transition of defensive microbes across the mutualism-parasitism continuum. Together, this thesis uncovers the potential for defensive microbes to shape the evolution of pathogens and demonstrates that defensive microbes can be an evolutionarily dynamic but stable form of host resistance towards infectious disease. As such, the data presented in this thesis have important implications for how we study host-parasite interactions in nature and question our current understanding of virulence evolution, pathogen local adaptation and the origin of defensive microbes.

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General introduction

Harnessing the Power of Defensive Microbes: Evolutionary Implications in Nature and Disease Control

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*Published as: Ford SA, King KC (2016) Harnessing the Power of Defensive Microbes: Evolutionary Implications in Nature and Disease Control. *PLoS Pathogens* 12(4): e1005465. doi:10.1371/journal.ppat.1005465 (see appendix)

Microbes are vital to the functioning of multicellular organisms. This realisation has fuelled great interest in the effects of microbes on the health of plant [1–3] and animal hosts [4–6] and has revealed that microbe-mediated protection against infectious disease is a widespread phenomenon (Table 1) [7–11]. Defensive microbes can protect hosts from infection by parasites (including pathogens and parasitoids) by direct or host-mediated means (Box 1). Such protective traits have made these microbes attractive candidates for disease control. In fact, defensive microbes are already being applied in phage therapy and bacteriotherapy for humans, as well as to control vector-borne and agricultural diseases (Table 2).

Box 1. Mechanisms of Defensive Microbes

Direct

Hyperparasitism or predation: Microbes can parasitise or predate upon the parasite [39].

Interference competition: Microbes can produce toxic compounds, such as antibiotics or bacteriocins, that may either kill the parasite or reduce its growth rate [40–42].

Resource competition: Microbes can compete with parasites for host resources [10,42], usually via the rate of resource acquisition [40,41].

Host-mediated

Host immune-mediation: Microbes can elicit a host immune response to which the parasite is not resistant [40,42].

Host tolerance-mediation: Microbes can increase the fitness of their host during infection without reducing the fitness of the parasite by enhancing host tolerance (e.g., via tissue damage prevention and/or repair) [43,44].

Table 1. Defensive microbes in nature. Defensive microbes are listed with their host(s), the parasite(s) they protect against, their mechanism(s) of defence, their impact on parasite fitness, and their mode of transmission.

	Host(s)	Defensive microbe(s)	Parasite(s)	Mechanism(s)	Impact on parasite fitness	Transmission mode	Ref(s).
Crustacea	American Lobster	gram-negative bacterium	Fungi	Interference competition	Negative	Unknown	[80]
	Shrimp	*Alteromonas* bacteria	Fungi	Interference competition	Not measured	Unknown	[81]
Insects	Aphids	*Hamiltonella defensa; Regiella*	Parasitoids	Interference competition	Negative	Vertical	[82,83]
	Aphids	*Regiella*; *Rickettsia*; *Rickettsiella*; *Spiroplasma*	Fungi	Unknown	Negative	Vertical	[84–86]
	Beewolf wasp; Subterranean termite	*Streptomyces* bacteria	Fungi	Interference competition	Negative	Vertical and Horizontal	[87,88]
	Bumble bees	*Gilliamella*	Protozoa	Unknown	Negative	Horizontal	[16]
	Dampwood termite	Undefined microbiome	Fungi	Interference competition	Negative	Unknown	[89]
	Fruit flies	*Spiroplasma*	Parasitoids Nematodes	Interference competition	Negative	Vertical	[54,90,91]
	Fruit flies; Mosquitoes	*Wolbachia sp.*	Arboviruses Protozoans Fungi	Resource competition Immune mediation	Negative None	Vertical	[92–95]
	Invasive Whitefly	*Rickettsia*	Fungi	Unknown	Negative	Vertical	[96]
	Leaf cutting ants	*Actinomycete* bacteria	Fungi	Interference competition	Negative	Vertical and Horizontal	[97,98]
	Leaf-rolling Weevil	*Penicillium herquei*	Unknown microbes	Interference competition	Negative	Unknown	[99]
Plants	Cacao Tree	Foliar Endophytic fungi; Arbuscular Mycorrhizal fungi	Fungi Oomycetes	Resource and Interference competition Immune mediation	Negative	Horizontal	[100–103]
	Chestnut trees	Virus	Fungi	Hyperparasitism	Negative	Horizontal	[104]
	Plant species	Mycoparasitic fungi	Fungi	Hyperparasitism	Negative	Horizontal	[3,39]
Vertebrates	Hoopoe birds	*Enterococcus faecalis*	Bacteria	Interference competition	Negative	Unknown	[105,106]
	Humans	Undefined microbiome in breast milk	Bacteria	Unknown	Negative	Unknown	[107]
	Metazoan hosts (incl. humans)	Bacteriophage	Bacteria	Hyperparasitism	Negative	Unknown	[108]
	Metazoan hosts (incl. humans)	*Bdellovibrio bacteriovorus*	Bacteria	Predation	Negative	Horizontal	[109,110]
	North American Bullfrog	Undefined skin microbiome	Fungi	Interference competition	Negative	Unknown	[5]

Table 2. Applications of defensive microbes in infectious disease control. Defensive microbes are listed with their host(s), the parasite(s) they protect against, their mechanism(s) of defence, their impact on parasite fitness, and their mode of transmission.

Host(s)	Defensive microbe(s)	Parasite(s)	Mechanism(s)	Impact on parasite fitness	Transmission mode	Ref(s).
Fish	Probiotics (oral)	Bacteria	Multiple mechanisms	Negative	Horizontal	[111]
Mammals (incl. humans)	Faecal transplant undefined microbiome	Bacteria	Multiple mechanisms	Negative	Horizontal	[6,76]
	Phage	Bacteria	Hyperparasitism	Negative	Horizontal	[78,79,112]
	Probiotics (oral)	Bacteria Fungi	Multiple mechanisms	Negative	Horizontal	[77,113]
Mosquitoes	*Wolbachia sp.*	Arboviruses Protozoans	Resource competition Immune mediation	Negative	Vertical and Horizontal	[61,74]
Plants	Foliar Endophytic fungi; Arbuscular Mycorrhizal fungi	Fungi Oomycetes	Resource competition Interference competition Immune mediation	Negative	Horizontal	[2,3,103]

Despite the impact defensive microbes can have on host and parasite fitness, our current perspective of host–parasite evolution is largely based upon pairwise species interactions [12]. By combining knowledge of defensive microbe–parasite interactions at the mechanistic level with evolutionary theory, we can predict how defensive microbes might alter the evolution of host and parasite traits, such as resistance and virulence. This will not only shape how we understand patterns of host–parasite coevolution in nature but will inform our decisions in utilising defensive microbes as disease control agents. We propose three potential evolutionary implications of defensive microbes on host–parasite interactions.

Defensive Microbe–Parasite Coevolution

Microbes can be an evolvable component of host defence (Box 2). Given that they can have short generation times, high mutation rates, and large within-host population sizes, defensive microbes might adapt to parasites much faster than their longer-lived eukaryotic hosts are able [11]. Consequently, coevolution between defensive microbes and parasites could provide

"real-time" disease control, whereby evolutionary changes in parasites are met by rapid reciprocal evolution in defensive microbes. For defensive microbes utilising direct mechanisms of interaction with parasites (Box 1), coevolution is likely to depend upon the population size, generation time, and gene flow of the defensive microbe population. For defensive microbes utilising host-mediated mechanisms of interaction with parasites (Box 1), coevolution is more likely to be limited by the rate of host evolution, since those mechanisms employ host-encoded traits. Exploring the potential for defensive microbe–parasite coevolution will be fundamental for predicting the long-term efficacy of microbe-mediated defence in the face of parasite evolution.

Box 2. Evolution of Defensive Microbes

Microbe-mediated defence is hypothesised to evolve from two sources of selection [11]. Firstly, microbe-mediated defence can be directly favoured when microbial fitness depends strongly on host fitness [45], such as when microbes are vertically transmitted (e.g., *Wolbachia* in Table 1) or when the host exercises "partner choice" or "host sanctions" and selectively cultures defensive microbes [8, 9, 11]. It is thought that beewolf wasps selectively acquire protective *Streptomyces* bacteria to ensure continued defence [46]. Alternatively, microbe-mediated defence might be selected as a byproduct of intraspecific and interspecific microbial interactions [11,47] (e.g., via competition over resources) or hyperparasitism (e.g., hyperparasitic viruses parasitise fungal pathogens of plants in Table 1). Hosts may also impose partner choice by exploiting microbial competition, termed "competition-based screening" [48].

Ultimately, defensive traits may differ in their efficacy over evolutionary time [11]. It is likely that defensive traits that evolve via partner choice or maternal inheritance are more evolutionarily persistent than those that evolve as a by-product of microbial interactions. This

difference is because host defence is a directly selected trait in microbes in the former but a by-product of another interaction in the latter. Above all, evolutionary persistence will depend upon the presence of parasites [49,50]. From the defensive microbe's perspective, high parasite prevalence can select for their persistence in the host population [50,51]. However, parasites are not always highly prevalent and can have a natural periodicity (e.g., seasonality) or be spatially variable [15,52]. How this variation alters the intensity of selection for and maintenance of defensive microbes has been explored theoretically [53] and in field patterns [54] but has rarely been tested directly [4].

The host-associated costs of harbouring defensive microbes can affect the potential for such microbes to evolve and, as such, their position on the mutualism–parasitism continuum [55]. In many cases, defensive microbes have been found to be physiologically and ecologically costly to their hosts [4,56–58]. Nevertheless, if the benefit of protection to the host outweighs any costs, then the evolution of defensive microbes will be favoured [59]. Alternatively, a host–parasite interaction would arise if defensive microbes become more costly than beneficial [59]. For example, a microbe that protects a host as a by-product of competing for resources with a parasite could itself evolve increased host exploitation, and so virulence, by means of competition. If the cost (virulence) of this microbe now outweighs its benefit (defence), this defensive microbe is now a parasite.

There are a variety of methods, traditionally used to study host–parasite interactions, that can be extended to look for evidence of defensive microbe–parasite coevolution. These include: (1) testing for signals of selection at the molecular level [13], (2) identifying coevolutionary dynamics via translocation or time-shift experiments [4,12,14,15], and 3) detecting specificity in defensive microbe–parasite interactions [16–21]. Although tests 2 and 3 cannot be performed in human hosts, it is possible for defensive microbes and parasites of humans to be

studied in model organisms, e.g., mice [22] or *Caenorhabditis elegans* [23], in which such studies could be carried out. To date, research has found that defensive microbes often interact specifically with parasites, suggesting defensive microbe–parasite coevolution [16–21]. In the case of aphid protection against parasitoids by *Hamiltonella defensa,* the specificity of microbe-mediated defence [17,19] is associated with a high diversity of toxin genes within the locus responsible for parasitoid resistance [24], indicative of rapid gene turnover due to coevolution [25]. Consistent with these data, mathematical models have revealed conditions under which defensive microbes coevolve with parasites to the point that the host becomes irrelevant [26].

Evolution of Host Dependence on Defensive Microbes

Given that defensive microbes protect hosts from parasite-induced fitness costs, they could reduce selection for costly immune-based or behavioural-based host defence mechanisms [10]. Unless defensive microbes employ host-encoded traits (e.g., host immune or tolerance mediation), selection favouring host defences could wane, making hosts dependent on microbe-mediated protection over evolutionary time. It has been speculated that the loss of immune genes in pea aphids and honeybees that possess defensive microbes are a result of the evolution of host dependence [10,27,28]; however, further research is required to test this hypothesis. Ultimately, a dependence on microbes for defence could increase host susceptibility to infection, making defensive microbe perturbation or loss (e.g., via antibiotics or diet changes) dangerous for the host [29,30].

Phylogenetic comparisons can be used to test the hypothesis that defensive microbes remove selection for host-based defences. Here, the innate resistance of host populations that harbour defensive microbes can be compared with those that do not. We can predict that host populations associating with defensive microbes should have a lower innate resistance to

parasites. In support of this prediction, natural *Trachymyrmex* ant populations that harbour protective antibiotic-producing bacteria exhibit significantly reduced cleaning behaviour than populations without the bacteria [31]. The innate resistance of host populations can be assessed by removing the defensive microbe and measuring resistance to parasitic infection and/or the activity level of a host-encoded resistance trait. Given that it is rare to find natural populations that differ only in their association with a defensive microbe, an experimental evolution approach could be used, whereby host populations are coevolved with parasites in the presence or absence of a defensive microbe. The use of lab-tractable host organisms with short generation times and well-studied innate immune systems, such as *C. elegans* [32] or *Drosophila melanogaster* [33], would facilitate such experimentation and allow us to determine whether particular immune system components have been lost or are expressed less over evolutionary time.

Defensive Microbes and Parasite Virulence Evolution

Parasite virulence is defined as the infection-induced decrease in host fitness [34] and is assumed to be an inevitable consequence of host exploitation and so parasite replication [35]. The evolution of virulence can be shaped by interactions between parasites and other microbes within a host; most research to date has focused on the effects of parasite coinfections on virulence evolution (for a full review, see [36]). Critically, as mechanisms of interaction that occur between co-infecting parasites (e.g., resource competition, interference competition, and immune mediation) are similar to those between a defensive microbe and a parasite (Box 1), predictions for virulence evolution based on coinfection might be applied to microbe-mediated defence. For example, it is predicted that if a parasite competes over resources under coinfection by evolving increased host exploitation, it will become more virulent [36]. Similarly, this prediction could be made for parasites resisting defensive microbes that function via resource competition. Consistent with this, recent theory on

defensive microbes has hypothesised that if parasite resistance to microbe-mediated defence is correlated with parasite virulence, then defensive microbes will alter the evolution of parasite virulence [11,37,38]. It follows that the direction of this correlation will determine the direction of virulence evolution. For example, if parasite resistance measures are also virulent to the host, defensive microbes will select for increased virulence [38]. Alternatively, if parasite resistance and virulence traits trade off against each other, defensive microbes will select for decreased virulence.

The extent to which defensive microbes might affect parasite virulence evolution is of concern in the application of microbe-mediated disease control strategies (Box 3). If microbe-mediated defence via resource competition indeed selects for more virulent parasites, this potential outcome could influence the choice of the defensive microbe being applied (Table 2). This example thus illustrates the significance of linking the mechanism of microbial protection to parasite virulence evolution in the application of defensive microbes

Box 3. Case Examples of the Application of Defensive Microbes

Wolbachia and vector-borne parasites

Wolbachia are bacteria that live within the cells of 60%–70% of insect species and can spread vertically via manipulating host reproduction [60,61]. Two *Wolbachia* strains isolated from *Drosophila*, *w*MelPop and *w*Mel, have been found to reduce the infection load of a range of human parasites, including dengue virus, malaria, yellow fever, chikungunya, West Nile virus, and filarial nematodes, when introduced into the novel hosts *Aedes aegypti* and *Anopheles gambiae* [62–68]. Consequently, there is great interest in using *Wolbachia* to halt disease transmission [53,61]. It has been suggested that host immune mediation may be the protective mechanism of *Wolbachia* within mosquitoes [62,69–71]; however, more research is

required to assess the role of other mechanisms (Box 1) across different host–*Wolbachia* combinations and over evolutionary time [72]. Trial introductions of *Wolbachia*-infected mosquitoes into natural populations have so far resulted in the successful spread of the *w*Mel strain [73,74].

More information will be critical to predicting the long-term efficacy of *Wolbachia* as a disease control strategy [75]. Specifically, determining whether the intracellular existence and vertical transmission of *Wolbachia* could constrain its evolution will indicate the potential for *Wolbachia*–parasite coevolution and so persistent disease control. Secondly, identifying the mechanism of protection will be vital in predicting whether *Wolbachia* will drive the evolution of mosquito dependence on microbe-mediated defence; if protection is indeed dominated by host immune mediation, *Wolbachia* may not remove the selection pressure for mosquito defences. Finally, characterising the mechanism(s) of *Wolbachia*-mediated defence will be important in predicting whether *Wolbachia* will select for changes in parasite virulence [75].

Human microbiome

Research into the human microbiome has uncovered information regarding microbe-mediated host defence and has highlighted promising routes for disease control (Table 2). Treatments aimed at manipulating the human microbiome commonly rely on the introduction of defensive microbes that suppress parasites by various means (Box 1). Faecal transplantation [6,76] and the introduction of probiotics [77] are two ways to use microbes to restore an unhealthy gut microbiota back to a healthy state. These methods likely rely on microbial competition to eliminate infections. Phage therapy, on the other hand, is a technique that kills targeted bacteria with hyperparasitic lytic phage viruses [78] and is another promising route to manipulate the composition of our microbiota [79]. Importantly, these methods all involve applying defensive microbes that have great evolutionary potential and so could act as persistent disease control agents in the face of parasite evolution.

Despite growing interest in the manipulation of the human microbiome to prevent or cure infectious disease, the evolutionary implications have been largely overlooked. Recent theory has shown that microbiome engineering could lead to the evolution of increased parasite virulence [38]. This prediction is based on the assumption that parasite resistance traits are also virulence traits [38]. This may often be the case, e.g., when the parasite's response to microbe-mediated defence is to elicit host inflammation or to produce a toxin against the defensive microbe that also harms the host. Such mechanisms might be common in the use of faecal transplantation and probiotics, as microbial competition is likely dominant [38]. In direct contrast to this, studies of hyperparasite–parasite interactions have shown that bacteria become less virulent as they resist phage attack (for full review see [37]), suggesting that phage therapy in the human microbiome may select for decreased, rather than increased, virulence. Here, parasite resistance and virulence traits may instead trade off against each other. Further investigations of the generality of these predictions will be necessary to better understand how different approaches to manipulate the microbiome affect parasite virulence evolution.

Conclusions

Defensive microbes are abundant in nature and have important fitness consequences for hosts and their parasites. By considering defensive microbes in the light of evolution, we can identify traits that enable their coevolution with parasites and reveal their impacts on the evolution of host-based resistance and parasite virulence. Such information will help us to choose defensive microbes for optimal disease control. Ultimately, by combining our understanding of defensive microbes at the mechanistic level with current evolutionary theory, we will be better able to predict the role of defensive microbes in driving host and parasite evolution in nature and in the context of disease control.

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Thesis introduction

This thesis focusses on the evolutionary implications of defensive microbe-pathogen interactions outlined in the general introduction that directly affect microbial evolution:

1. *Can defensive microbes drive the evolution of pathogen virulence?*
2. *Do defensive microbes and pathogens have the potential to undergo coevolution?*
3. *Can defensive microbe-pathogen coevolution maintain microbe-mediated defence?*

To study these questions, I utilised the *Caenorhabditis elegans* model host system. *C. elegans* is a well-established model host for investigating microbial colonisation and pathogenesis [1-3]. Individuals ingest microbes as a food source and have a number of defences to protect them from infection, such as their pharyngeal grinder, which breaks up ingested matter and an innate immune system which shares many conserved features with other animals [1, 4]. Despite these barriers, *C. elegans* can become colonised and infected by a variety of commensals and pathogens [2, 5-8].

Within *C. elegans*, I studied the interactions of the two bacterial species *Enterococcus faecalis* and *Staphylococcus aureus.* In animal microbiota, *E. faecalis* and *S. aureus* are both known commensals, but can become opportunistic pathogens [9]. It is currently unknown whether strains of *S. aureus* and *E. faecalis* infect *C. elegans* in nature; however, both bacteria are capable of colonising *C. elegans* in the lab [4, 5, 10]. We used *S. aureus* strain MSSA 476 and *E. faecalis* strain OG1RF, both of which were isolated from humans [11, 12]. Their interaction within a nematode host is therefore novel, allowing us to explore the evolution of defensive symbioses early in their formation. It has been previously found that *S. aureus* acts as a virulent pathogen within *C. elegans* hosts and that *E. faecalis* acts as a defensive microbe against this pathogen, suppressing the fitness and virulence of *S. aureus* [10]. It was shown that *E. faecalis* was able to suppress *S. aureus* fitness in-part by producing antimicrobial superoxide anions as a form of interference competition [10]. The same study found that evolving *E. faecalis* as a resident gut microbe against a single invading genotype of *S. aureus*

selected for more protective *E. faecalis* populations that produced significantly more superoxides.

To answer questions 1-3 in my thesis, I performed an evolution experiment whereby I co-passaged *S. aureus* and *E. faecalis* within genetically homogenous and non-evolving *C. elegans* populations.

Chapter one: *Can defensive microbes drive the evolution of pathogen virulence?*

In chapter one, I ask if and how interactions between defensive microbes and pathogens within host populations can affect the evolution of pathogen virulence. I predict that defensive microbes will drive the evolution of pathogen virulence when the mechanism of pathogen resistance to microbe-mediated defence is associated with harm to the host [13]. Furthermore, I predict that the direction of virulence evolution will depend on the shape of the relationship between pathogen resistance to microbe-mediated protection and its virulence [13, 14]. This chapter has been accepted by Nature Communications.

Chapter two: *Do defensive microbes and pathogens have the potential to undergo coevolution?*

In chapter two, I ask whether defensive microbes and pathogens are able to undergo coevolution within non-evolving host populations. It has been theoretically predicted that defensive microbes and pathogens have the potential to coevolve via fluctuating selection dynamics [15]. I test this prediction by looking for phenotypic and genotypic evidence of reciprocal adaptation and local adaptation. This chapter has been accepted by Molecular Ecology.

Chapter three: *Can defensive microbe-pathogen coevolution maintain selection for microbe-mediated defence?*

In chapter three, I ask if and how coevolution with a pathogen can be sufficient to maintain the selection favouring defensive microbes. It has been hypothesised that the defensive properties of microbes can evolve as a by-product of competition with pathogens [16], however it is still unclear under what conditions this is favoured and how persistent it is over evolutionary time. I propose here that when the cost and the benefit of a defensive microbe are correlated we can make predictions of the stability of defensive microbes in nature. Specifically, I predict that the shape of the relationship between the cost and the benefit of a defensive microbe can be important in predicting transitions between mutualism and parasitism and thus the maintenance of defensive microbes. This chapter is in preparation for submission for publication.

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Chapter One

Microbe-mediated host defence drives the evolution of reduced pathogen virulence

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Abstract

Microbes that protect their hosts from pathogens are widespread in nature and are attractive disease control agents. Given that pathogen adaptation to barriers against infection can drive changes in pathogen virulence, 'defensive microbes' may shape disease severity. Here we show that coevolving a microbe with host-protective properties (*Enterococcus faecalis*) and a pathogen (*Staphylococcus aureus*) within *Caenorhabditis elegans* hosts drives the evolution of reduced pathogen virulence as a by-product of adaptation to the defensive microbe. Using both genomic and phenotypic analyses, we discover that the production of fewer iron-scavenging siderophores by the pathogen reduces the fitness of the defensive microbe and underpins the decline in pathogen virulence. These data show that defensive microbes can shape the evolution of pathogen virulence and that the mechanism of pathogen resistance can determine the direction of virulence evolution.

Introduction

Microbes can defend their hosts against pathogenic infection [1]. This phenomenon has been widely observed in nature across plant and animal species, including humans, to provide a strong line of defence beyond the host response [2]. As a result, microbes with protective traits are considered promising disease control agents and alternatives to antibiotics to combat devastating human and crop pathogens [3-6]. Importantly, pathogen adaptation to barriers against infection can drive the evolution of virulence [7], as observed against immune-priming vaccines [8] and competing, co-infecting pathogens [9]. Given that defensive microbes likely impose strong selection on pathogens too [2], they may also play a pivotal role in determining why some infections are more harmful than others. Thus, understanding the effect of defensive microbes on pathogen evolution could ultimately enable us to explain variation in disease severity in nature and to use defensive microbes for optimal disease control.

Within this study, pathogen 'resistance' will be used to describe their ability to resist the defensive microbe. We propose that defensive microbes will drive the evolution of pathogen virulence when the mechanism of pathogen resistance to microbe-mediated defence is associated with harm to the host [2, 10]. The direction of virulence evolution will depend on the shape of the relationship between resistance and virulence [2, 10]. Specifically, we predict that defensive microbes will result in the evolution of increased pathogen virulence if pathogen resistance and virulence are positively associated (fig.1a), e.g. pathogens could increase in competitive ability against the defensive microbe via a faster growth rate or inducing inflammation, both of which are also detrimental to the host [10-13]. Alternatively, if a trade-off exists between pathogen resistance and virulence traits, e.g. resistance is costly

Figure 1

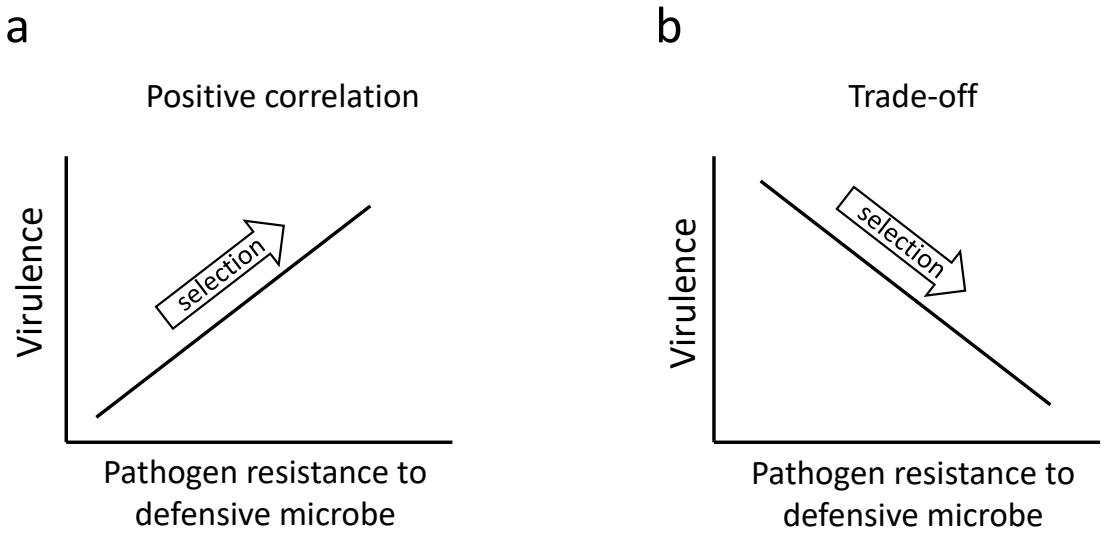


Figure 1. Proposed associations between mechanisms of pathogen virulence and resistance to microbe-mediated defence.

a, Pathogen virulence is hypothesised to increase when it correlates positively with the pathogen's ability to resist microbe-mediated defence. **b,** Pathogen virulence is hypothesised to decrease when traded off against the pathogen's ability to resist microbe-mediated defence.

and slows pathogen growth rate [14-16], then defensive microbes will drive lower levels of virulence (fig.1b). Although the evolution of pathogen resistance to defensive microbes has been theoretically illustrated [10, 17] and empirically evidenced [18], studies are few. Our data will thus provide valuable information on the evolution of pathogen resistance in addition to virulence.

Here, we have studied the interactions between *Staphylococcus aureus* and *Enterococcus faecalis* within *Caenorhabditis elegans* hosts which emulate the interactions between a pathogen and a defensive microbe, respectively [19]. Both *E. faecalis* and *S. aureus* co-occur in animal microbiomes and can colonise the gut of *C. elegans* [20]. This nematode species is a model animal system for investigating microbial pathogenesis as well as natural and lab-based host-microbiota associations [21-25]. We used *S. aureus* strain MSSA 476 and *E. faecalis* strain OG1RF, both of which were isolated from humans. Their interaction within a nematode host is therefore novel, allowing us to explore the evolutionary consequences of defensive symbioses early in their formation. We have previously illustrated that *S. aureus* acts as a virulent pathogen within the nematode host [19]. Furthermore, we have shown that although *E. faecalis* imposes a small cost to host longevity while colonising alone (un-colonised hosts experience no mortality under otherwise the same conditions [19]), this bacterium provides a net benefit to hosts during *S. aureus* infection [19]. By having context-dependent fitness effects on its host, *E. faecalis* reflects symbionts found in nature. In particular, many defensive microbes have been found to impose costs on their hosts in the absence of the enemy [26-31]. Moreover, we know that *E. faecalis* significantly reduces the virulence of *S. aureus* by producing superoxide anions that decrease pathogen fitness [19]. This mechanism of defence is a form of interference competition, whereby defensive microbes produce toxic compounds that may either kill the pathogen or slow its growth rate [2]. Interference competition is currently the most commonly detected mechanism of microbe-mediated defence in nature [2].

To test the hypothesis that defensive microbes can affect pathogen virulence evolution, we co-passaged *S. aureus* and *E. faecalis* (Coevolution treatment) and independently passaged *S. aureus* (Single evolution treatment) within non-evolving, genetically homogeneous *C. elegans* populations for 10 passages. Given that defensive microbes and pathogens likely evolve on similar timescales in nature, co-passaging allowed for the realistic possibility of coevolutionary interactions to shape pathogen evolution. Throughout our experiments, we assay host mortality after 24 hours of exposure to ancestral and evolved pathogen populations as a measure of virulence [32]. We use this measure of virulence for several reasons. Host mortality is a widespread measure in theoretical and experimental investigations on the evolution of virulence across a diversity of host-pathogen systems [9, 32-37]. Moreover, host mortality is commonly used in *C. elegans*-pathogen experimental evolution studies [38-40] and our previous work shows its relevance in our system [19]. Specifically, we measure host mortality within 24 hours to observe adaptation relevant to the exposure window in the evolution experiment.

We find that experimental coevolution between a defensive microbe and a pathogen within a host population drives the evolution of reduced pathogen virulence as a by-product of adaptation to the defensive microbe. Using both genomic and phenotypic analyses, we discover that pathogen populations evolved to produce fewer iron-scavenging siderophores and that this change lowered the fitness of defensive microbes and underpinned the decline in pathogen virulence. These data show that microbe-mediated defence can shape pathogen virulence evolution and that the mechanism of pathogen resistance can determine the direction of evolution.

Results

Microbe-mediated defence

Prior to experimental evolution, we show that *E. faecalis* protects the host from virulent infection after 24 hours of simultaneous exposure with *S. aureus* (fig. 2a, Quasibinomial GLM: F=41.96, df=2, P= 5.193e-09, Supplementary Table 1) by significantly reducing the infection load (fig.2b, Welch two-sample t-test: t=-4.5, df=16.2, P=0.0003). We find that the benefit *E. faecalis* provides the nematode host (12% reduction in host mortality after 24 hours of exposure to the pathogen, fig.2a) is much greater than its cost (0.69% mortality after 24 hours of exposure to the defensive microbe, fig.2a).

Interestingly, we discover that *E. faecalis* reached significantly higher population sizes in the host when it is infected with *S. aureus* (fig.2c, Two sample t test: t=-2.8, df=24, P=0.009) suggesting that, although *E. faecalis* harms *S. aureus,* the pathogen conversely alters the environment in a way that facilitates defensive microbe growth. These data therefore reveal a strong antagonistic relationship between the defensive microbe and the pathogen, whereby one species benefits and the other is harmed.

Pathogen virulence

To test whether defensive microbes can affect pathogen virulence evolution, we co-passaged *S. aureus* and *E. faecalis* (Coevolution treatment) and independently passaged *S. aureus* (Single evolution treatment) within non-evolving, genetically homogeneous *C. elegans* populations for 10 passages (fig.3). Both treatments consisted of five replicate populations started from the same clone of each bacterial species such that evolution was *de novo.* Every passage, hosts were exposed to bacterial treatments for 24 hours, and 10 colonies of *S. aureus* and *E. faecalis*

Figure 2

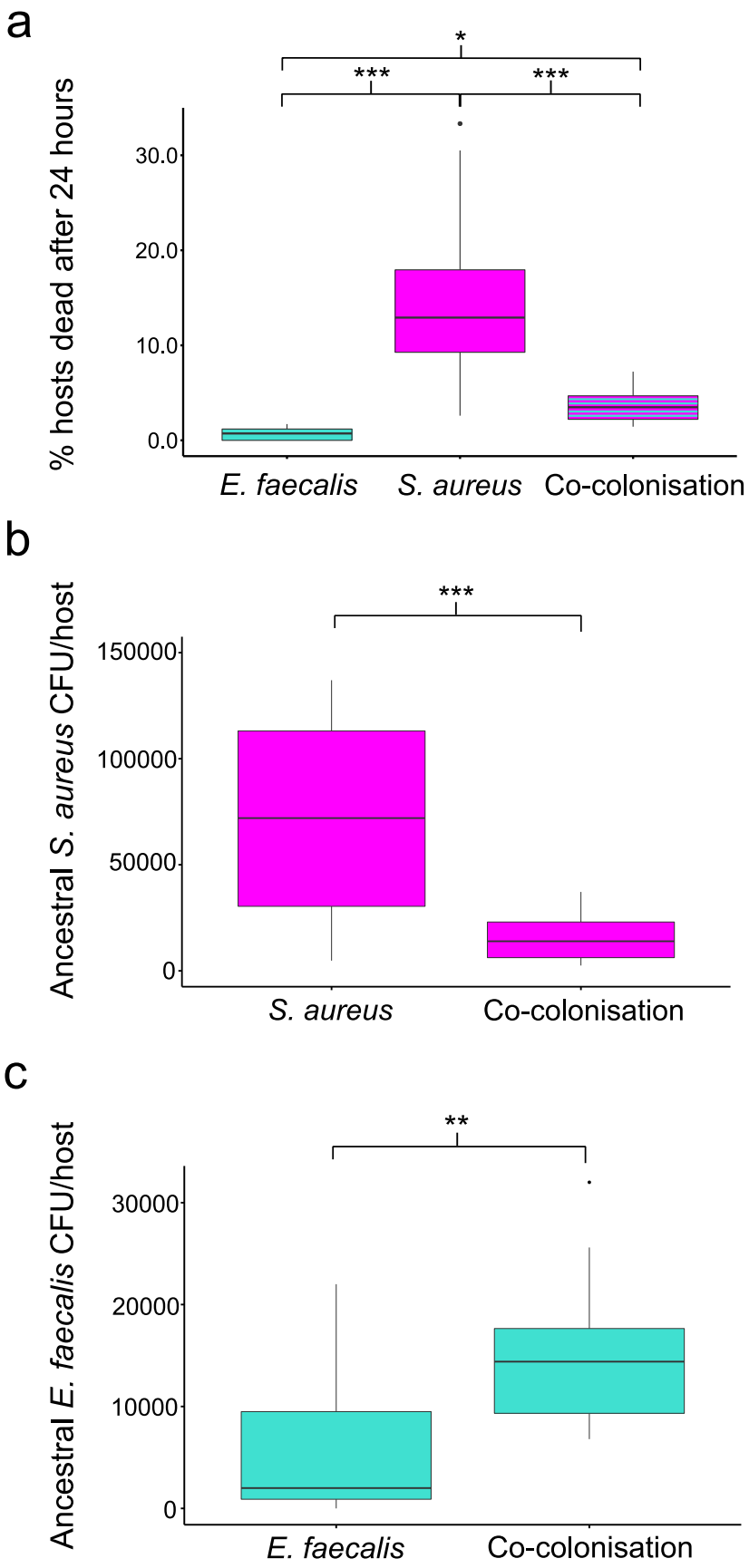

a
*
***
***
% hosts dead after 24 hours
30.0
20.0
10.0
0.0
E. faecalis
S. aureus
Co-colonisation
b
***
Ancestral S. aureus CFU/host
150000
100000
50000
0
S. aureus
Co-colonisation
c
**
Ancestral E. faecalis CFU/host
30000
20000
10000
0
E. faecalis
Co-colonisation

Figure 2. Ancestral bacterial virulence and within-host bacterial population sizes

a, Virulence (% hosts dead after 24hrs exposure) of *E. faecalis, S. aureus* and both under co-colonisation. **b**, Bacterial fitness (colony forming units, CFU/host) of ancestral *S. aureus* under single and co-colonisation after 24hrs exposure. **c**, Bacterial fitness (CFU/host) of ancestral *E. faecalis* under single and co-colonisation after 24hrs exposure. *P<0.05, **P<0.01, ***P<0.001. Colours indicate the bacterial species being measured: magenta, pathogen (*S. aureus*)*;* turquoise, defensive microbe (*E. faecalis*)*.*

Figure 3

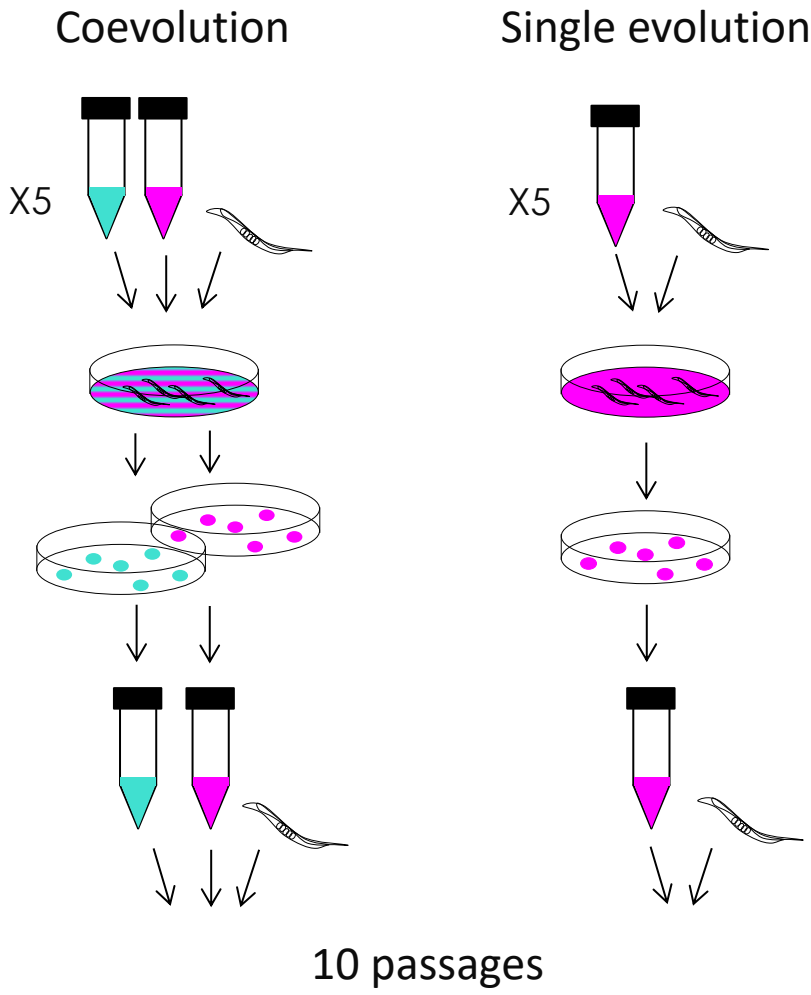


Figure 3. Experimental coevolution

S. aureus and *E. faecalis* were co-passaged (Coevolution treatment) and *S. aureus* was passaged alone (Single evolution treatment) within non-evolving, genetically homogeneous *C. elegans* host populations. At each generation, the same *C. elegans* host populations were exposed to mixed bacterial lawns of *S. aureus* and *E. faecalis* or single species lawns of *S. aureus.* After 24 hours of exposure, 10 colonies of *S. aureus* and/or *E. faecalis* were collected from the pooled guts of 10 dead hosts using selective media and passaged to the next generation. Each treatment had five replicate populations and was conducted for 10 passages. Colours: magenta, pathogen (*S. aureus*)*;* turquoise, defensive microbe (*E. faecalis*).

were collected from the pooled gut contents of 10 dead hosts and used to start the next generation (fig.3).

We found that experimental coevolution with the defensive microbe drove reductions in pathogen virulence across all replicate populations, beyond those seen in the Single evolution treatment (fig.4a, Quasibinomial GLM, F=25.7, df=2, P=4.627e-05, Supplementary Table 1). This reduction in pathogen virulence correlated with a reduction in *in vitro* pathogen growth rate (fig.4b, Pearson's product-moment correlation: t = 3.38, df = 9, p= 0.008. R^2=0.56), supporting a link between virulence and growth rate, as is commonly assumed [32]. Despite a decrease in growth rate, coevolved S*. aureus* reached significantly higher infection loads than either the ancestor or Single evolution populations in hosts protected by ancestral *E. faecalis* (fig.4c, ANOVA, F=6.55, df=2, P=0.012, Supplementary Table 1). This result indicates that the pathogens have adapted to interact with the defensive microbe. Together, these data suggest a trade-off between pathogen virulence and resistance to microbe-mediated defence, supporting our second hypothesis in figure 1b.

Siderophore production

We proposed that the mechanism underpinning changes in *S. aureus* virulence and growth rate involved the production of siderophores, compounds produced by microbes to bind and uptake iron [41]. In iron-limited host environments, siderophores can affect pathogen growth rate and so contribute to virulence, as evidenced in *S. aureus* infections [42]. Crucially, siderophores are also a 'public good' and so can be exploited by neighbouring microbes [41, 43]. Theory posits that competition for host resources between unrelated pathogens selects for reduced public good production, and thus reduced virulence [41]. This has been supported by studies of both experimental and natural infections [44, 45]. Importantly, when a pathogen

Figure 4

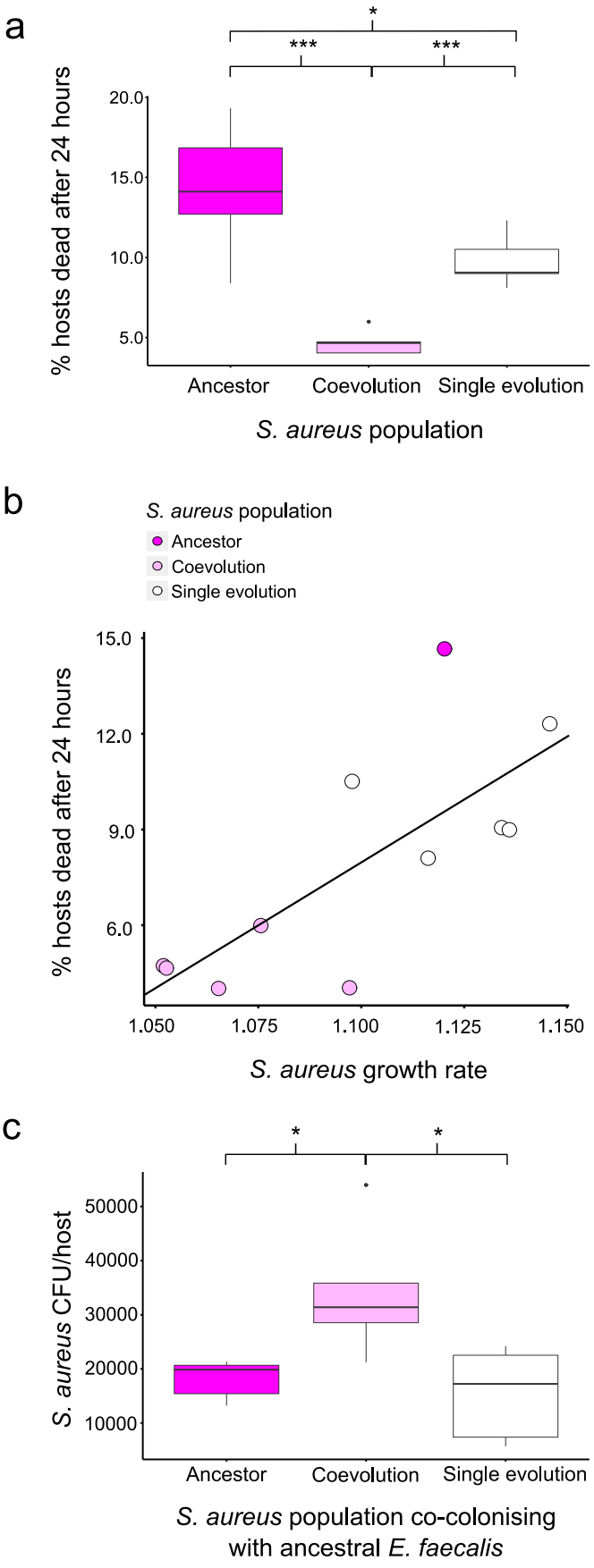

a
*
***
***
20.0
15.0
10.0
5.0
% hosts dead after 24 hours
Ancestor
Coevolution
Single evolution
S. aureus population
b
S. aureus population
Ancestor
Coevolution
Single evolution
15.0
12.0
9.0
6.0
% hosts dead after 24 hours
1.050
1.075
1.100
1.125
1.150
S. aureus growth rate
c
*
*
50000
40000
30000
20000
10000
S. aureus CFU/host
Ancestor
Coevolution
Single evolution
S. aureus population co-colonising
with ancestral E. faecalis

Figure 4. Pathogen virulence and fitness

a, Virulence of ancestral and evolved *S. aureus* populations in *C. elegans* hosts after 24 hours of exposure. **b**, Plot of virulence against the *in vitro* growth rate of ancestral and evolved *S. aureus* populations. **c**, Bacterial fitness (CFU/host) of ancestral and evolved *S. aureus* populations under co-colonisation with ancestral *E. faecalis*. *P<0.05, **P<0.01, ***P<0.001.

is exploited by a defensive microbe, we hypothesise that there would be even stronger selection against public good production, and so virulence, since the exploiting microbe also harms the producer. This extension of current theory would be consistent with our findings that pathogen virulence and growth rate decrease over time in microbe-protected hosts, and would further explain the observed benefit ancestral *S. aureus* confers to *E. faecalis*.

To determine whether the defensive microbe drove a reduction in pathogen virulence as a by-product of selecting for decreased siderophore production, we measured siderophore activity and correlated it with pathogen virulence. By measuring the siderophore concentration produced by *S. aureus* populations *in vitro,* we found that *S. aureus* populations from the Coevolution treatment produced the fewest (fig.5a, ANOVA, $F=6.681$, $df=2$, $P=0.01$, Supplementary Table 1). We then discovered that siderophore production was positively correlated with pathogen virulence (fig.5b, Pearson's product-moment correlation: $t = 2.8$, $df = 9$, $P=0.02$. $R^2=0.5$). This pattern can be seen both within and between treatments.

Molecular evolution

Given that the production of fewer siderophores by coevolved *S. aureus* was detected in the absence of *E. faecalis*, we concluded that this change was constitutive. To confirm that it was a result of evolutionary change, we conducted whole-genome resequencing of ancestral and *S. aureus* populations at the end of the evolution experiment. No single mutation was shared between any replicate populations, suggesting the independent evolution of the same phenotype multiple times within each treatment (Supplementary Table 2). Importantly, mutations from both evolution treatments can be linked to siderophore production pathways [46] (the putative functions of each mutation is listed in Supplementary Table 2); however SNPs in the Coevolution treatment were present at much higher frequencies compared to those found in the Single evolution treatment, consistent with the distinct phenotypes (fig.6). Specifically, at least one non-synonymous mutation in each coevolved pathogen population

Figure 5

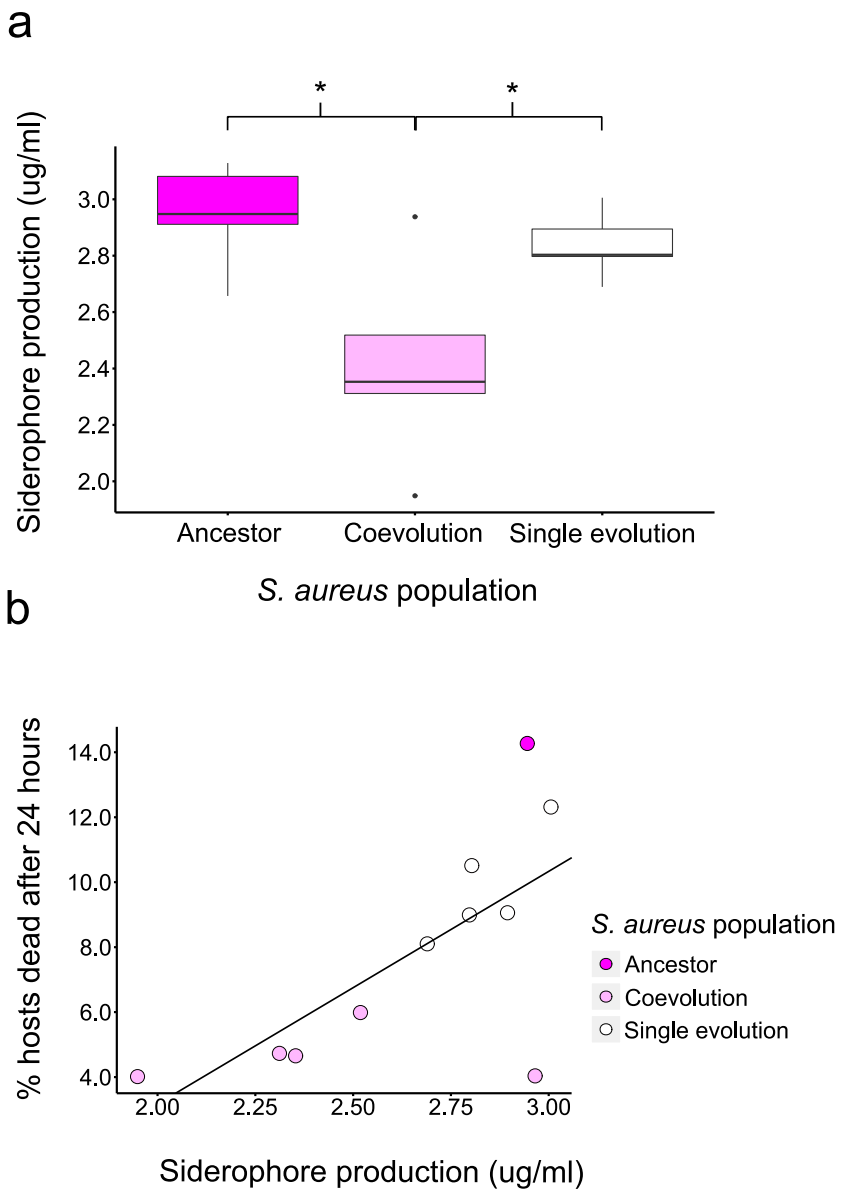


Figure 5. Siderophore production

a, Concentration (ug ml^{-1}) of siderophores produced by ancestral and evolved *S. aureus* populations *in vitro.* **b**, Plot of virulence against *in vitro* siderophore production (ug ml^{-1}) by ancestral and evolved *S. aureus* populations. *P<0.05, **P<0.01, ***P<0.001.

Figure 6

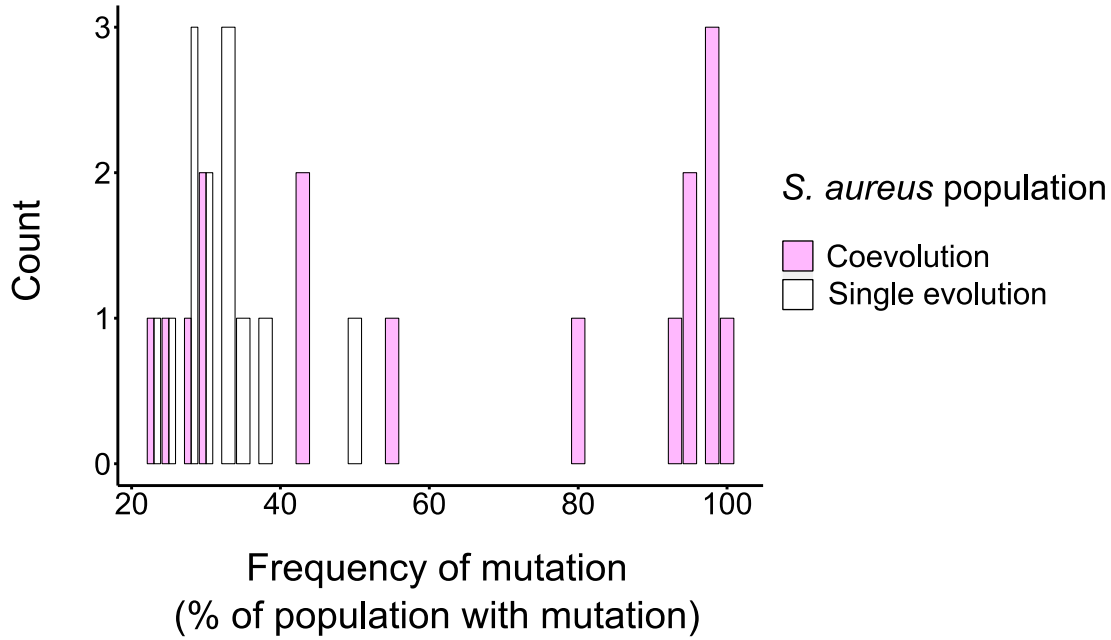


Figure 6. Molecular evolution

Count of non-synonymous mutations in the Coevolution and Single evolution replicate populations at a given frequency in the population (the % of 40 randomly-sampled clones).

that had spread to >80% frequency could be linked to siderophore production (Supplementary Table 2). In contrast, only one mutation in one Single evolution replicate population reached 50%. This distribution of mutations suggests that defensive microbes imposed strong selection on *S. aureus* populations which resulted in selective sweeps.

Effect of siderophores on defensive microbe fitness

To determine whether siderophores were public goods exploitable by the defensive microbe, we measured the *in vitro* growth rate of ancestral *E. faecalis* across a range of exogenous siderophore concentrations. We found that siderophore concentration correlated positively with *E. faecalis* growth rate over the natural range of *S. aureus* siderophore production (fig.7a, Pearson's product-moment correlation: t=6.1, df=2, P=0.026. R^2=0.95). We then found that defensive microbe fitness was directly affected by evolutionary changes in *S. aureus* siderophore production. Here, the fitness benefit *E. faecalis* gained from interacting with the ancestral pathogen (compared to growing independently) disappeared when co-cultured with coevolved *S. aureus* populations, which produced the fewest siderophores (fig.7b, ANOVA, F=9.2, df=3, P=0.0009 Supplementary Table 1).

To test whether changes in siderophore production were sufficient to explain these differences, we measured the effect of surplus iron on *E. faecalis* fitness when co-cultured with ancestral and evolved *S. aureus* populations. Under surplus iron, siderophore production should be down-regulated [47]. We found that surplus iron removed the differential effects of *S. aureus* treatment on *E. faecalis* fitness (fig.7c, Control ANOVA, F=8.1, df=2, fdr corrected P=0.019; Supplementary Table 1; +Fe^{3+} ANOVA, F=3.05, df=2, fdr corrected P=0.092). This result confirms public good siderophores as the mechanism underlying pathogen adaptation to microbe-mediated defence in this system.

Figure 7

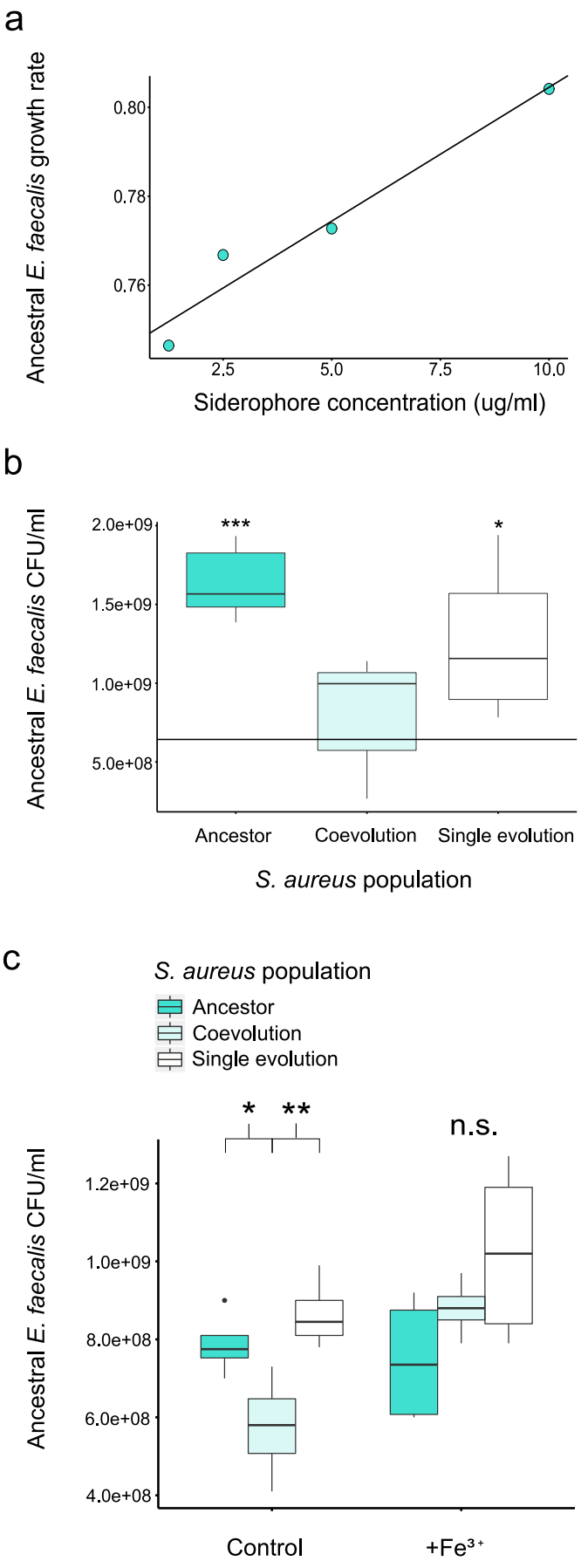

a
Ancestral E. faecalis growth rate
0.80
0.78
0.76
2.5
5.0
7.5
10.0
Siderophore concentration (ug/ml)
b
Ancestral E. faecalis CFU/ml
2.0e+09
1.5e+09
1.0e+09
5.0e+08
***
*
Ancestor
Coevolution
Single evolution
S. aureus population
c
S. aureus population
Ancestor
Coevolution
Single evolution
Ancestral E. faecalis CFU/ml
1.2e+09
1.0e+09
8.0e+08
6.0e+08
4.0e+08
* **
n.s.
Control
+Fe3+

Figure 7. Effect of siderophores on defensive microbe fitness

a, Exogenous siderophore concentration (ug ml^{-1}) against growth rate of ancestral *E. faecalis in vitro.* **b**, Fitness of ancestral *E. faecalis* (CFU ml^{-1}) after 24hrs growth *in vitro* with ancestral or evolved *S. aureus.* The black line indicates the average fitness of *E. faecalis* in the absence of *S. aureus.* Statistical comparisons are made between this black line and each treatment. **c**, Fitness of ancestral *E. faecalis* (CFU ml^{-1}) after 24hrs growth *in vitro* with ancestral or evolved *S. aureus* with ($+Fe^{3+}$) and without (control) surplus iron. *P<0.05, **P<0.01, ***P<0.001.

Discussion

We sought to establish how within-host interactions between defensive microbes and pathogens would affect the evolution of pathogen virulence. We found that the coevolution between a defensive microbe and a pathogen drove the evolution of reduced pathogen virulence as a trade-off against pathogen resistance to microbe-mediated defence.

We have previously shown that *S. aureus* is a virulent pathogen within *C. elegans* and that *E. faecalis* acts as a defensive microbe by producing superoxide anions that reduce *S. aureus* fitness [19]. In addition to reducing the fitness of the pathogen, we found here that the defensive microbe could exploit the iron-scavenging siderophores produced by *S. aureus,* gaining a fitness benefit under co-colonisation. Together, these data reveal a strong antagonistic interaction between the two species (fig.8). By experimentally coevolving *S. aureus* and *E. faecalis* within nematode populations, we discovered that the pathogens from all replicate populations evolved to produce fewer siderophores. These data correspond with the spread of mutations in genes putatively linked with the siderophore pathway. We confirmed that the decrease in siderophore production resulted in the significant reduction of defensive microbe fitness and was associated with a dramatic increase in pathogen infection load within protected hosts. As a by-product of this adaptation, all coevolved pathogen populations were significantly less virulent than the ancestral and Single evolution populations since decreased siderophore production slowed their growth rate in the absence of the defensive microbe. Given that siderophores are widely used by pathogens [44], reduced siderophore production may be a common adaptation to interactions with members of a host's microbiota and may contribute in explaining the presence of siderophore mutants in pathogen populations in nature.

Figure 8

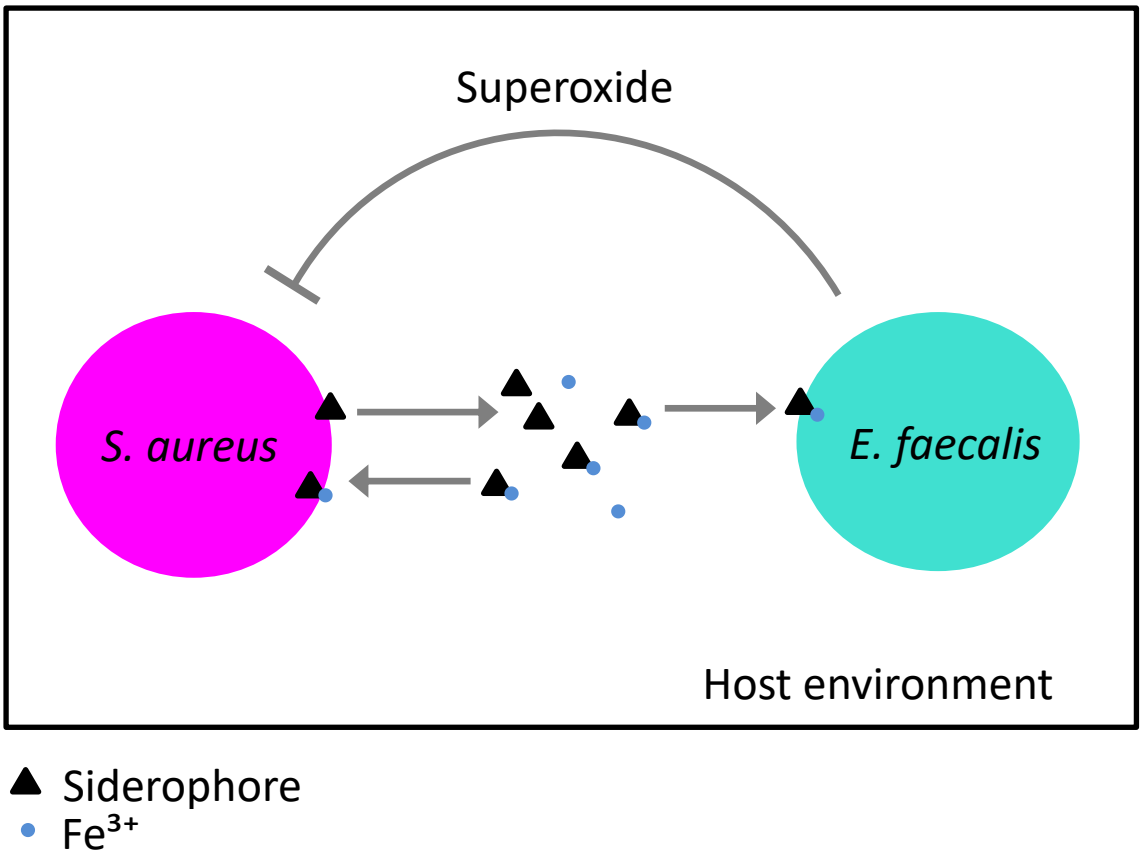


Figure 8. Model of *S. aureus* (pathogen) – *E. faecalis* (defensive microbe) interactions within the nematode host

S. aureus produces iron-scavenging siderophores that *E. faecalis* can exploit. In turn *E. faecalis* produces superoxide anions that suppress pathogen growth.

More generally, our results illustrate that just a single component of a host's microbiota could be a major source of selection acting to shape the virulence of pathogens in nature today. As in our experiment, there are many observations in nature of one defensive microbe species protecting against one enemy species [2]. However, defensive microbe-pathogen interactions may often be more complicated [2], for example complex microbiomes are known to protect species as diverse as bumblebees and humans from infection [3, 48], and a single defensive microbe species can defend hosts against multiple enemies [49, 50]. We have demonstrated the potential for evolutionary interactions between defensive microbes and pathogens and we have highlighted important implications for virulence. Future research will need to establish how these evolutionary interactions can play out in more complex communities.

The trade-off we observe between pathogen virulence and resistance to microbe-mediated defence is an important consideration for disease control. Host-associated microbes that cause pathogens to make this trade-off, either by similar or different mechanisms to that presented here, could be useful tools to tackle infectious disease. This is not only because they protect hosts in the short-term but they would also select for less virulent pathogens in the long-term. For example, lytic phage that act as defensive microbes by killing pathogenic bacteria can select for decreased pathogen virulence when they infect via bacterial receptors necessary for pathogen growth rate or virulence [16]. By altering these receptors, the pathogen trades-off resistance to phage with virulence mechanisms [16]. In this way, microbe-mediated therapy could provide a more powerful strategy to treat disease than drugs designed to block the production of virulence factors [46] by favouring the evolution of pathogens that are less virulent in the first place. Thus, as an alternative to anti-virulence strategies, which can have unforeseen evolutionary implications [51, 52], we could design disease control strategies that aim to direct pathogen evolution in a favourable direction.

Microbes that protect hosts from infection can shape the evolution of pathogen virulence. Moreover, it is becoming clear that understanding the mechanisms underlying pathogen resistance to microbe-mediated defence may be crucial in explaining the variation in disease severity in nature and in predicting the direction of virulence evolution under applied disease control.

Methods

Nematode host and bacteria

Caenorhabditis elegans is a nematode that constantly interacts with microbes in its natural habitat [53], and it can act as a predator or host for numerous microbial species [22, 54, 55]. These animals are thus an established model for microbial colonisation and pathogenesis [21] and their gut can be co-colonised by multiple pathogens and commensals [38, 56-58]. We used the simultaneous hermaphroditic N2 wild-type *Caenorhabditis elegans* strain from the *Caenorhabditis* Genetics Centre (University of Minnesota, Minneapolis, MN) [56]. A genetically homogenous line was generated by selfing a single hermaphrodite for 5 generations. Populations of these nematodes were frozen in 50% M9 solution and 50% liquid freezing solution in cryotubes at -80°C [56]. Populations were regularly resurrected throughout experimentation to prevent the accumulation of *de novo* mutations in host populations. Worms were maintained at 20°C on nematode growth medium (NGM) plates seeded with *Escherichia coli* OP50 from the *Caenorhabditis* Genetics Centre (University of Minnesota, Minneapolis, MN). *E. coli* OP50 is grown at 30°C shaking at 200rpm overnight in LB and 100µl of this is spread onto NGM plates and incubated overnight at 30°C [56]. To ensure clean stocks and to synchronise the life stages of populations for experimentation, worms were treated with bleach (NaClO) and sodium hydroxide (NaOH) solution which kills everything except unhatched worm eggs [56].

We used *Staphylococcus aureus* strain MSSA 476 (GenBank: BX571857.1), an invasive community-acquired methicillin-susceptible isolate and *Enterococcus faecalis* strain OG1RF (GenBank: CP002621.1), both isolated from humans. Both isolates were sourced from the University of Liverpool. A single ancestral population of each species was grown from a single colony overnight in 6ml Todd Hewitt Broth (THB) shaking at 200rpm at 30°C. Bacteria were frozen in a 1:1 ratio of sample to 50% glycerol solution in cryotubes at -80°C.

Experimental coevolution

The coevolution experiment consisted of two treatments: (i) *S. aureus* and *E. faecalis* were co-passaged under co-colonisation (Coevolution treatment), and (ii) *S. aureus* evolution was passaged on its own (Single evolution treatment) within *C. elegans* hosts (fig.3a). Each treatment had 5 replicate populations and was conducted for 10 passages. Every passage, bacteria were cultured overnight in 6ml THB shaking 200rpm at 30°C. They were then standardised to an OD600 reading of 1.00, and 120µl of each species was spread onto Tryptic Soy Broth (TSB) agar plates and kept overnight at 30°C to make the exposure lawns. Co-culture exposure lawns for the Coevolution treatment were made by mixing 120µl of each species together such that their population sizes were kept the same across treatments. Approximately 1000 young adult worms from the genetically homogenous stock were placed onto each exposure lawn and incubated at 25°C for 24 hours.

After 24 hours, 10 dead worms were picked from each lawn to ensure we took hosts colonised with bacteria. Under co-colonisation dead worms did not differ significantly from live worms in the proportion of pathogen collected from the infection (Supplementary fig.1. Two sample t-test. $t = 1.22$, $df = 10$, $P = 0.25$). Worms were considered dead when they did not respond to touch with a platinum wire [20]. Worms were washed to remove external bacteria by moving them between five 5µl drops of M9 buffer on fresh TSB plates using a platinum wire, an adapted method [20, 38]. Worms were then crushed in 20µl of M9 in a 1ml Eppendorf tube

with a pestle to release internal bacteria. An inoculation loop was used to streak each sample onto selective media (TSB plates with 100µg ml^{-1} rifampicin were used to isolate *E. faecalis* from the Coevolution treatment, and Mannitol Salt Agar (MSA) plates isolated *S. aureus* from both treatments) and the plates were grown overnight at 30°C. 10 colonies of *S. aureus* and *E. faecalis* were picked from each replicate and grown in THB overnight, shaking at 200rpm at 30°C, and were used for the next generation of exposures.

Virulence assays

Exposure plates were made in the same way as for the evolution experiment (see above). Approximately 150 young adult worms from the stock *C. elegans* population were placed onto exposure plates and incubated at 25°C for 24 hours. Exposure plates were labelled with a random code so that the treatments were unknown during measurement. The total numbers of worms were counted and the proportion dead calculated as a measure of virulence. Worms were considered dead when they did not respond to touch with a platinum wire [20].

In vivo *bacterial population sizes*

Exposure plates were made in the same way as for the evolution experiment (see above). Approximately 1000 young adult worms from the stock *C. elegans* population were placed onto exposure plates and incubated for 24 hours at 25°C. Exposure plates were labelled with a random code so that the treatments were unknown during experimentation. After 24 hours of exposure, 3-5 worms were picked from each plate. For Supplementary fig. 1, live worms were picked for each replicate of the live treatment and dead worms were picked for each replicate of the dead treatment. For all other infection load assays dead worms were picked, reflecting the evolution experiment. Worms were washed to remove external bacteria by moving them between five 5µl drops of M9 buffer on fresh TSB plates using a platinum wire. Worms were then crushed in 20µl of M9 in a 1ml Eppendorf tube with a pestle to release

internal bacteria. After crushing, serial dilutions were plated onto selective media and grown overnight at 30°C. MSA and rifampicin TSB selective media were used to count *S. aureus* and *E. faecalis* colony-forming units (CFUs) per worm host, respectively.

In vitro *bacterial growth rate*

Bacterial growth rate was measured in 96-well plates. Bacteria were cultured overnight in 6ml THB shaking 200rpm at 30°C. A volume of 2µl of bacteria was added to 198µl THB and kept for 10 hours in a plate reader set to shake intermittently at 30°C. OD630 Readings were taken every 1 minute and 15 seconds with Gen5 software and Curve fitter software was used to calculate growth rates of the bacteria in the exponential growth phase.

Siderophore production

Ancestral and evolved *S. aureus* populations were grown in 6ml THB in falcon tubes with loose cap lids allowing for unlimited oxygen, and so growth, and were shaken at 200rpm overnight at 30°C. Overnights were standardised by OD630 and then filtered to remove bacterial cells. 100µl of each sample was added to 100µl reaction mixture from the SideroTech (EmergenBio) kit in a 96-well plate and incubated at room temperature for 5 minutes. OD630 was then measured in a plate reader at room temperature. The concentration of siderophore was estimated from a reference curve of the relationship between OD630 and desferoxamine siderophore (EmergenBio) concentration (Supplementary fig.2). This reference curve was made by measuring the OD630 of samples containing desferoxamine siderophore at known concentrations increasing from 0 to 100µg ml^{-1}. The SideroTech assay works by measuring the changes in OD630 when siderophore removes ferric iron from the reagent complex (composed of a dye, iron, and a detergent).

Siderophores and in vitro E. faecalis *growth rate*

The growth rate of ancestral *E. faecalis* was measured in the presence of increasing desferoxamine (EmergenBio) siderophore concentrations in THB. Bacteria were cultured overnight in 6ml THB shaking 200rpm at 30°C. A volume of 2µl of overnight ancestral *E. faecalis* culture was added to a mixture of 160µl THB and 40µl siderophore-diluent solution at final siderophore concentrations of 1.25, 2.5, 5 and 10µg ml^{-1}. The OD630 was measured every 1 minute and 15 seconds for 10 hours in a plate reader set to shake intermittently at 30°C. OD630 Readings were taken every 1 minute and 15 seconds with Gen5 software and Curve fitter software was used to calculate growth rates of the bacteria in the exponential growth phase.

In vitro E. faecalis *fitness with and without* S. aureus

Bacteria were cultured overnight in 6ml THB shaking 200rpm at 30°C and standardised to an OD630 of 1.00. A volume of 3ul of ancestral *E. faecalis* and either ancestral, Coevolution or Single evolution *S. aureus* populations were added to 194ul of THB in a 96-well plate. As a control, a volume of 3ul of ancestral *E. faecalis* was also added to 194ul of THB without *S. aureus*. The 96-well plate was shaken at 175rpm for 24 hours at 30°C. Resulting samples were diluted and plated onto rifampicin TSB selective media to count E. *faecalis* CFU per ml as a fitness measure. Plates were labelled with a random code so that the treatments were unknown during quantification.

Surplus iron and in vitro E. faecalis *fitness with* S. aureus

THB containing 15µg ml^{-1} iron (III) sulphate was made by mixing THB and iron (III) sulphate dissolved in H_2O. Control THB was made by mixing the same volume of THB and H_2O without iron. Bacteria were cultured overnight in 6ml THB shaking 200rpm at 30°C and standardised to an OD630 of 1.00. A volume of 3µl of ancestral *E. faecalis* and either ancestral, Coevolution or

Single evolution *S. aureus* populations were added to 194µl of surplus-iron THB or control THB in a 96-well plate. The 96-well plate was shaken at 175rpm for 24 hours at 30°C. Resulting samples were diluted and plated onto rifampicin TSB selective media to count E*. faecalis* CFU per ml. Plates were labelled with a random code so that the treatments were unknown during quantification.

Genomic extraction

40 clones were picked from streaked cultures of *S. aureus* replicate populations from both evolved treatments and grown independently overnight in 200µl THB in a 96-well plate shaking at 175rpm at 30°C. Subsequently, each set of 40 clones was checked for equal OD630 and pooled in equal volumes. A single clone of ancestral *S. aureus* was similarly grown.

DNA was extracted from *S. aureus* by taking 1ml of culture and centrifuging at 7500rpm, removing the supernatant and re-suspending the pellet in 160µl enzymatic lysis buffer (Qiagen), 40µl lysostaphin (200µg ml^{-1}, Sigma-Aldrich), 40µl lysozyme (100mg ml^{-1}, Sigma-Aldrich) and 8µl RNAse A (10mg ml^{-1}, Sigma-Aldrich). This mixture was incubated for 1 hour at 37°C. 25µl proteinase K (Qiagen) was then added and 200µl Buffer AL (without ethanol) was added and vortexed. This was then incubated at 56°C for 1 hour. 200µl of ethanol was added and vortexed and DNA purification followed DNeasy Blood and Tissue Spin-Column Protocol (Qiagen). The extracted DNA was sequenced using the HiSeq4000 platform with 100bp paired end at the Wellcome Trust Centre for Human Genetics, Oxford.

Genomic analysis

The project accession number at the European Nucleotide Archive for the raw read data is PRJEB13385. Sequenced read data in fastq files were trimmed for the presence of Illumina adapter sequences using Cutadapt version v1.9.1 (revision e960cc1 from github.com/marcelm/cutadapt) [59]. The reads were further trimmed using Sickle version 1.33

(revision f3d6ae3 from github.com/najoshi/sickle) [60] with a minimum window quality score of 25 and retaining only reads longer than 50 bp after trimming. Single reads remaining from pairs were retained.

Reference genomes for short read mapping were obtained from the NCBI Assembly database (www.ncbi.nlm.nih.gov/assembly). Short reads of the DNA extractions from the *Staphylococcus aureus* strain MSSA476 isolates were mapped to assembly GCF_000011525.1 using BWA MEM v0.7.12 (revision cc9eef2 from github.com/lh3/bwa) [61] using option -M to flag shorter split hits as secondary ensuring the single best alignment was used. Alignments were manipulated using SAMTools v1.2 (revision ac5b8e7 from github.com/samtools/samtools) [62].

The Genome Analysis Toolkit v3.4 (GATK) [63, 64] Indel Realigner module was used to realign reads around putative insertions and deletions after which duplicate reads were identified and removed with Picard v1.135 (*https://github.com/broadinstitute/picard/releases/tag/1.135*). Single nucleotide polymorphism, insertion and deletion discovery was performed with GATK's Haplotype caller module with sample ploidy n = 40 [65]. Default parameter values were used except the priors "--heterozygosity 0.0001 –indel_heterozygosity 0.00001" were used because diversity was expected to be relatively low given the experiment duration (these values are ten-fold lower than the defaults). Owing to the difficulties in distinguishing called variants at low frequencies from noise caused by sequencing errors, only variants at frequencies greater than 20% were included in this analysis.

Statistical analysis

Data analysis was carried out in R v 3.2.0 (http://www.r-project.org/). Parametric tests were used for all data which met the required assumptions. These assumptions were checked using the Shapiro test to detect whether data was normally distributed and F-tests to compare the

variances of two samples from normal populations. Non-parametric equivalents were used if the data did not meet those assumptions. Outlying data points were detected and removed by using the Dixon test. For statistical analyses on passaged bacteria, each replicate population was treated as an independent biological replicate. Thus, there always existed five biological replicates for evolved bacterial population. Quasibinomial GLMs were used for virulence assays to correct for over-dispersion of data, and Tukey contrasts were used for *post-hoc* comparisons. T-tests were used for fitness assays of ancestral bacteria within the host. The Welch two sample t-test was used for assays which did not have equal variance and the independent two-sample t-test was used when they did have equal variances.

ANOVAs were used for assays comparing ancestral and evolved *S. aureus* treatments measuring within-host bacterial fitness, siderophore production, growth rate and *in vitro* competition of bacteria. Plots of each ANOVA were checked by eye for model quality. For the analysis of ancestral *E. faecalis* in the presence and absence of ancestral and evolved *S. aureus* (fig.4b) we performed Dunnett contrasts between treatments with *S. aureus* and the treatment without *S. aureus* as the control. All remaining ANOVAs used Tukey contrasts for *post-hoc* comparisons. For the *in vitro* competition assay where a single ANOVA was used for the treatments with surplus iron and a separate ANOVA for the treatments without surplus iron we corrected the treatment p-values for multiple comparisons using the fdr correction.

Pearson's product-moment correlation was used to test for correlations between *S. aureus* growth rate and siderophore production against virulence, as well as the correlation between siderophore concentration and *E. faecalis* growth rate. The coefficients and R^2 value for the lines of best fit were obtained from a linear regression model. To model the relationship between siderophore concentration and optical density a non-linear curve was fitted by estimating the parameters and standard errors of the parameters as starting estimates. The parameters we estimated included the asymptote, intercept and steepest point in curve, 0.2, -

0.53 and 0.039, respectively. All these parameters appeared to be significant in explaining the model and were not extraneous.

Data availability

Raw read data for the bacterial genomic sequences were deposited in the European Nucleotide Archive under project accession number PRJEB13385. The CSV files containing data for each figure are available in the Dryad repository (http://dx.doi.org/10.5061/dryad.cb7ns) [66]. The authors declare that all other data supporting the findings of this study are available within the article and its supplementary information files, or from the corresponding authors upon request.

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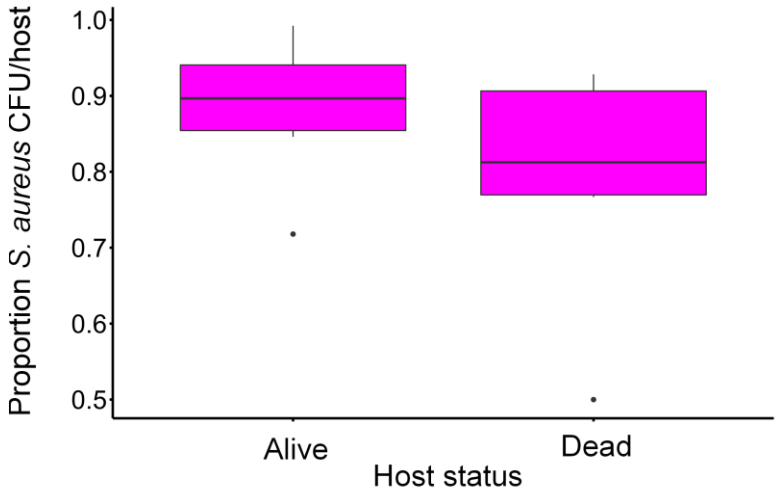
Author contributions

S.A.F. and K.C.K. conceived and designed the project; S.A.F. conducted the experiments and analysed the data; S.A.F, D.K. and D.W. conducted the bioinformatics analysis; S.A.F. and K.C.K. wrote the paper.

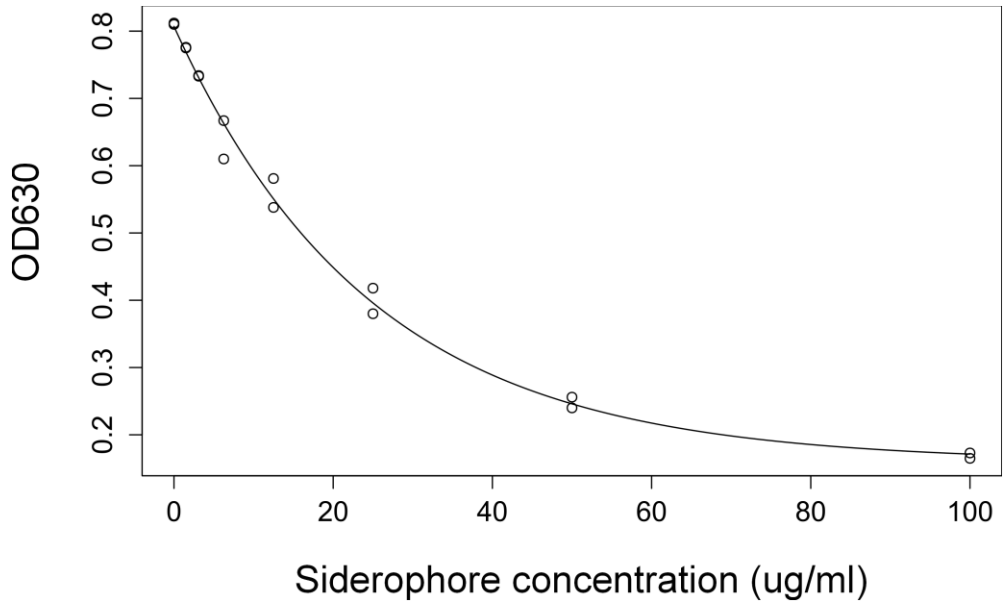
Competing financial interests

The authors declare no competing financial interests.

Supplementary information



Supplementary Figure 1. The proportion *S. aureus* CFU of the total CFU (*S. aureus* + *E. faecalis* CFU) per host in worms alive or dead at 24 hours of exposure. Two sample t-test: t = 1.22, df = 10, P= 0.25. Sample size for each treatment: 6 biological replicates.



Supplementary Figure 2. The relationship between OD630 and siderophore concentration (ug ml^{-1}) *in vitro.* Nonlinear (weighted) least-squares estimates of the parameters of a nonlinear model: OD = 0.1599 - -0.6471*exp(-0.04*concentration). Sample size: 2 biological replicates for each concentration.

Supplementary Table 1. Statistical results listed by figure

Figure	Statistical results
2a	**Quasibinomial GLM:** F=41.96, df=2, P= 5.193e-09 Tukey contrasts: *S. aureus* vs. *E. faecalis*: P<1e-04 *S. aureus* vs. co-colonisation: P<1e-04 *E. faecalis* vs. co-colonisation*:* P=0.025
2b	**Welch two sample t test:** t=-4.5, df=16.2, P=0.0003 95 percent confidence interval: -81755.14 -29842.08
2c	**Two sample t test:** t=-2.8, df=24, P=0.009. 95 percent confidence interval: -14421.823 -2292.925
4a	**Quasibinomial GLM:** F=25.7, df=2, P=4.627e-05 Tukey contrasts: Coevolution vs. Ancestor: P<0.001 Coevolution vs. Single evolution: P<0.001 Ancestor vs. Single evolution: P=0.035
4b	**Pearson's product-moment correlation:** t = 3.38, df = 9, p= 0.008. R^2=0.56 95 percent confidence interval: 0.2682905 0.9303416
4c	**ANOVA:** F=6.55, df=2, P=0.012 Tukey contrasts & 95% family-wise confidence level: diff lwr upr p value Ancestor vs Coevolution: -16106.2 -31087.38 -1125.017 0.035 Single evolution vs Coevolution: -18791.0 -33772.18 -3809.817 0.015 Single evolution vs. Ancestor: -2684.8 -17665.98 12296.383 0.883

5a	**ANOVA:** F=6.681, df=2, P=0.01 Tukey contrasts & 95% family-wise confidence level: diff lwr upr p value Coevolution vs. Ancestor: -0.531180 -0.94121802 -0.121142 0.01 Single Evolution vs. Ancestor: -0.107136 -0.51717402 0.302902 0.8 Single Evolution vs Coevolution: 0.424044 0.01400598 0.834082 0.04
5b	**Pearson's product-moment correlation:** t = 2.8, df = 9, P=0.02. R^2=0.5. 95 percent confidence interval: 0.1441149 0.9106005
7a	**Pearson's product-moment correlation:** t=6.1, df=2, P=0.026. R^2=0.95 95 percent confidence interval: 0.2052983 0.9994809
7b	**ANOVA**: F=9.2, df=3, P=0.0009 Dunnett contrasts: Coevolution *S. aureus* & ancestral *E. faecalis* vs. ancestral *E. faecalis:* P=0.77 Single evolution *S. aureus* & ancestral *E. faecalis* vs. ancestral *E. faecalis:* P=0.023 Ancestor *S. aureus* & ancestral *E. faecalis* vs. ancestral *E. faecalis:* P<0.001
7c	**Control ANOVA**: F=8.1, df=2, fdr corrected P=0.019 Tukey contrasts & 95% family-wise confidence level: diff lwr upr p value Ancestor vs Coevolution: 212500000 4318164 420681836 0.046 Single evolution vs Coevolution: 290000000 81818164 498181836 0.009 Single evolution vs. Ancestor: 77500000 -130681836 285681836 0.57 **+Fe^{3+} ANOVA Treatment:** F=3.05, df=2, fdr corrected P=0.092.
Supp 1	**Two sample t-test:** t = 1.22, df = 10, P= 0.25. 95 percent confidence interval: -0.07642428 0.26070806
Supp 2	**Nonlinear (weighted) least-squares estimates of the parameters of a nonlinear model:** OD = 0.1599 - -0.6471*exp(-0.04*concentration) Parameters: Estimate Std. Error t value Pr(>|t|) a 0.159890 0.014898 10.73 7.88e-08 *** b -0.647120 0.015333 -42.20 2.68e-15 *** c 0.040329 0.002729 14.78 1.66e-09 *** Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1 Residual standard error: 0.01949 on 13 degrees of freedom

	Number of iterations to convergence: 4 Achieved convergence tolerance: 2.732e-07

Supplementary Table 2. Mutations found in *S. aureus* listed by treatment and replicate population. Locus tag is given when mutation occurs in a coding region. Otherwise, the coordinate is given. Mutation abundance is the percentage of 40 sampled clones that contain the mutation in the replicate population. Only mutations present in >20% clones are listed to control false positives at the expense of low frequency true positives. Gene names and putative gene function are given where known, along with a reference. A proposed link with siderophore activity is listed where identified. Mutation type: S=synonymous. N=non-synonymous. *=stop gain. */= stop loss.

Treatment	Replicate	Locus tag/ coordinate	Mutation	abundance (%)	Gene name/annotation	Putative function	**Putative** link with siderophores	Reference (PMID)
Coevolution	1	SAS_RS09425	N	95	Protein export protein PrsA Foldase.	A facultative facilitator of protein secretion or extracellular folding. Cell wall biosynthesis.	Excretion or folding of proteins involved in iron acquisition	22526301 21765893
	2	SAS_RS00600	*	98	Hypothetical protein.	ABC transporter superfamily.	Transport of substrates involved in iron acquisition	25414356
		549806		100	Between pyrimidine nucleoside transporter NupC & transcriptional regulator CtsR.			
		SAS_RS02825	N	30	Glycosyl transferase family 1.	Cell wall biosynthesis.		17237164
		SAS_RS02905	S	100	Uracil-DNA glycosylase.	DNA base-excision repair.		2836397
		SAS_RS04210	N	23	Cysteine desulfurase.	iron-sulphur cluster biosynthesis.		11577100
		SAS_RS13205	N	98	Holin-like protein CidB.	Cytolysis. Cellular suicide in response to cellular stress or damage.		12670989 14617136
	3	629811		95	Between octanoyl-[GcvH]:protein N-octanoyltransferase and mevalonate kinase.			
		SAS_RS05930	N	43	Penicillin-binding protein.	Cell wall biosynthesis.		3882429

Treatment	Replicate	Locus tag/ coordinate	Mutation	abundance (%)	Gene name/annotation	Putative function	**Putative** link with siderophores	Reference (PMID)
		1267927		30	Between DNA topoisomerase 1 and TrmFO.	TrmFO: oxidoreductase flavoprotein.	TRmFO: Siderophore-iron reductase	
		SAS_RS07345	N	98	Sulphite reductase subunit alpha.	Sulphite to sulphide reduction. Iron ion binding. Oxidoreductase. ferrisiderophore reduction. Hydrogen sulphide biosynthesis. Sulphate assimilation.	Siderophore-iron reductase	7657631
		1839866		28	Between MarR family transcriptional regulator and lysophospholipase.			
		SAS_RS11185	N	43	Methicillin resistance protein FmtB.	Cell wall biosynthesis.		17951380 10896508
		SAS_RS13665	N	55	Hypothetical protein.	No conserved domains identified.		
	4	SAS_RS13620	*/	80	Anaerobic ribonucleoside-triphosphate reductase activating protein.	Oxidoreductase. Iron-sulfur cluster binding.	Siderophore-iron reductase	10821845
	5	SAS_RS03320	N	93	Membrane protein.	Solute carrier families 5 and 6-like. Transports inorganic ions, sugars, amino acids.	Transport of substrates involved in iron acquisition	25414356

Treatment	Replicate	Locus tag/ coordinate	Mutation	abundance (%)	Gene name/annotation	Putative function	**Putative** link with siderophores	Reference (PMID)
		SAS_RS12635	N	25	Glycerate kinase.	Glycine metabolism.	Ferrichrome siderophore is made of glycine residues	
Single Evolution	1	SAS_RS06355	N	28	RIP metalloprotease RseP.	Coordinating gene transcription during extracytoplasmic stress response.		12073657
		SAS_RS09685	N	50	2-hydroxyacid dehydrogenase.	Oxidoreductase.		17049749
	2	10884		33	Between carbohydrate kinase and histidine ammonia-lyase.			
		SAS_RS01275	N	35	Ribose transporter RbsU.	Sugar transport across bacterial membrane. Uptake of ribose.		10941799
		SAS_RS03290	N	28	Ferrichrome ABC transporter permease.	Part of the ABC transporter involved in iron-siderophore import.	Transport of substrates involved in iron acquisition	3020380
		SAS_RS03845	*	30	Helicase.	ATP-dependent RNA or DNA unwinding.		
		SAS_RS04065	S	25	Thermonuclease.	Catalyses the hydrolysis of both DNA and RNA.		6307819
	3	626196		25	Between hypothetical protein and heme-binding protein.		Heme-binding protein; iron acquisition	
		SAS_RS05650	N	28	Heme uptake system protein IsdE.	ABC transport of heme, siderophores and metal ions.	Transport of substrates involved in iron acquisition	15240116

Treatment	Replicate	Locus tag/ coordinate	Mutation	abundance (%)	Gene name/annotation	Putative function	**Putative** link with siderophores	Reference (PMID)
		1985301		33	Between aspartyl/ glutamyl-tRNA(Asn/Gln) amidotransferase subunit C and sodium:proline symporter.			
		SAS_RS11115	N	38	Membrane protein.	No conserved domains identified.		
		SAS_RS12235	N	33	DNA-binding response regulator.	Contains a singal receiver domain, an effector domain and a DNA-binding response regulator.		
		SAS_RS13800	N	30	Surface anchored protein.	No conserved domains identified.		
	4	No mutations >20%						
	5	SAS_RS12000	N	23	RpiR family transcriptional regulator.	Transcription factor.		

Chapter Two

Coevolutionary dynamics between a defensive microbe and a pathogen driven by fluctuating selection

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Abstract

Microbes that protect their hosts from pathogenic infection are widespread components of the microbiota of both plants and animals. It has been found that interactions between 'defensive' microbes and pathogens can be genotype-specific and even underlie the variation in host resistance to pathogenic infection. These observations suggest a dynamic coevolutionary association between pathogens and defensive microbes, but direct evidence of coevolution is lacking. We tested the hypothesis that defensive microbes and pathogens could coevolve within host populations by co-passaging a microbe with host-defensive properties (*Enterococcus faecalis*) and a pathogen (*Staphylococcus aureus)* within *Caenorhabditis elegans* nematodes. Using both phenotypic and genomic analyses across evolutionary time, we found patterns of pathogen local adaptation and defensive microbe-pathogen coevolution via fluctuating selection dynamics. Moreover, coevolution with defensive microbes resulted in more rapid and divergent pathogen evolution compared to pathogens evolved independently in host populations. Taken together, our results indicate the potential for defensive microbes and pathogens to coevolve, driving interaction specificity and pathogen evolutionary divergence in the absence of host evolution.

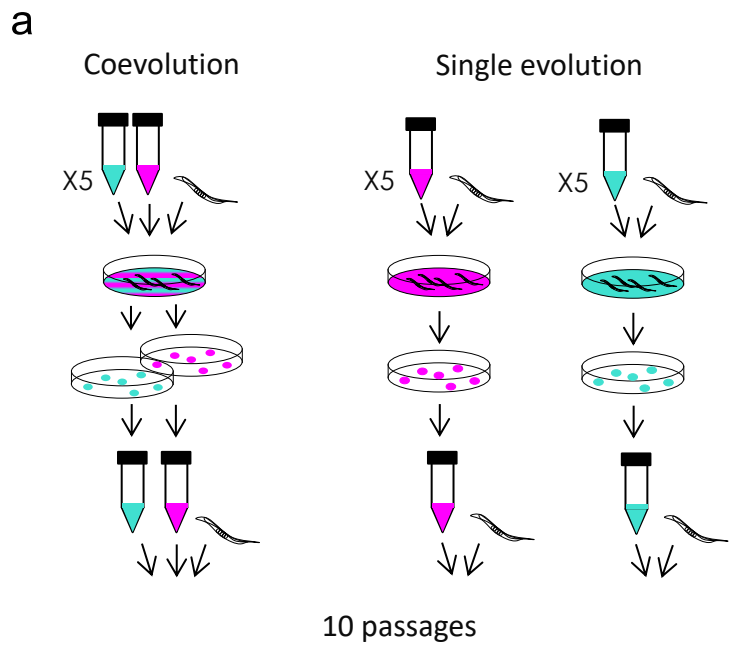
Introduction

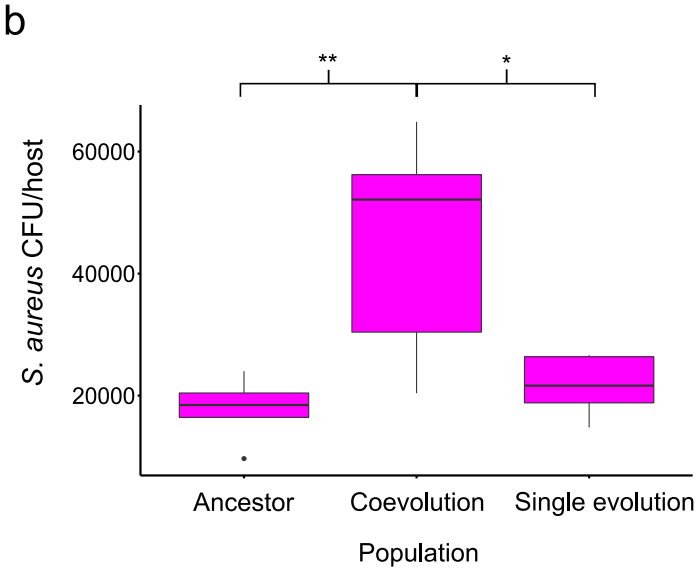
Many microbes can determine host health by defending against pathogenic infection [1]. Such 'defensive' microbes are a valuable addition to the host immune system [2] and are found in a diversity of plants and animals, including humans [1]. It is theoretically predicted that defensive microbes and pathogens could undergo coevolutionary interactions within a host via fluctuating selection dynamics [3]. Consistent with this prediction, interactions between defensive microbes and pathogens in nature (including parasites and parasitoids) can show a high degree of specificity and determine the variation in host resistance to pathogenic infection [4-9]. These observations suggest the potential for a dynamic coevolutionary association between defensive microbes and pathogens within host populations. However, direct tests of defensive microbe-pathogen coevolution are currently lacking [1].

Coevolutionary pattern and process can vary across interacting species [10, 11], with coevolution occurring via arms race dynamics or fluctuating selection dynamics. Arms race dynamics can occur when interacting species accumulate generally-beneficial traits that can become increasingly exaggerated [12]. Alternatively, fluctuating selection dynamics occur when traits are advantageous only when rare in the population, resulting in cycles of genotypes through time [12]. Importantly, this rare advantage only results when there is a high degree of specificity in species interactions. As such, the observed specificity in defensive microbe-pathogen interactions in nature [1] indicates the possibility of coevolution via fluctuating selection dynamics, consistent with theory [3].

In this study, we experimentally coevolved a microbe with host-protective properties (*Enterococcus faecalis*) and a pathogen (*Staphylococcus aureus*) within non-evolving *Caenorhabditis elegans* host populations (Coevolution treatment, fig.1a). We also passaged the defensive microbe and pathogen independently within *C. elegans* populations (Single

Figure 1


a
Coevolution
Single evolution
X5
X5
X5
10 passages


b
**
*
S. aureus CFU/host
60000
40000
20000
Ancestor
Coevolution
Single evolution
Population

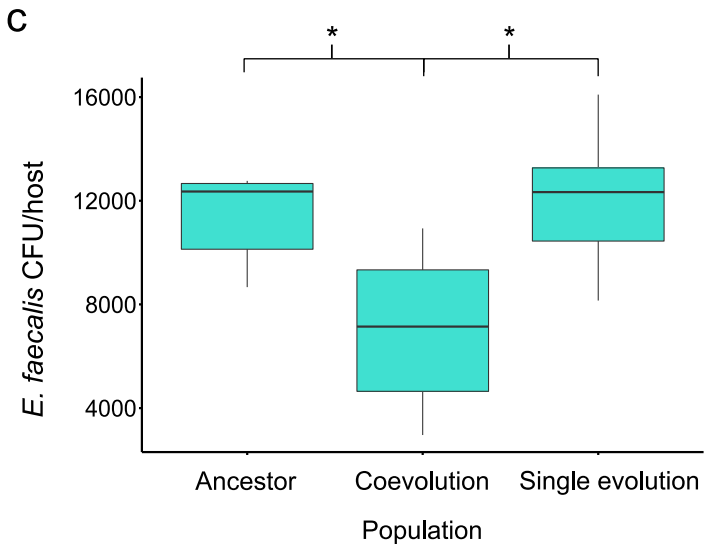

c
*
*
E. faecalis CFU/host
16000
12000
8000
4000
Ancestor
Coevolution
Single evolution
Population

Figure 1 Experimental coevolution

a, Design of evolution experiment: *S. aureus (magenta)* and *E. faecalis* (turquoise) were co-passaged (Coevolution) or passaged independently (Single evolution). At each passage, the same genetically homogeneous population of *C. elegans* hosts was exposed to *S. aureus* and/or *E. faecalis.* After 24 hours of exposure, 10 colonies of *S. aureus* and/or *E. faecalis* were collected from the pooled guts of 10 hosts using selective media and passaged to the next generation. Each treatment had 5 replicate populations and was conducted for 10 passages. **b,** Fitness of *S. aureus* (CFU/host) under co-colonisation with *E. faecalis.* Populations of *E. faecalis* and *S. aureus* were co-colonised from the same replicate populations in each treatment. **c,** Fitness of *E. faecalis* (CFU/host) under co-colonisation with *S. aureus.* Populations of *E. faecalis* and *S. aureus* were co-colonised from the same replicate populations in each treatment. *P<0.05, **P<0.01, ***P<0.001.

evolution treatment, fig.1a). Each treatment consisted of five replicate populations originating from the same clone of each bacterial species such that any evolutionary change was *de novo.* We conducted the evolution experiment for 10 passages, by which time considerable evolutionary change is known to occur in this system [13]. *C. elegans* is used as a model for investigating microbial pathogenesis and host-microbiota interactions [14-18]. Both *E. faecalis* and *S. aureus* are found in the animal microbiota and can colonise the gut of *C. elegans* [19]. In this experiment, we used bacteria isolated from humans. As a result, their interaction within a nematode host is novel such that we can explore the evolutionary dynamics of defensive symbioses early in their formation. We have previously shown that within-nematode interactions between *E. faecalis* and *S. aureus* mimic those of a defensive microbe and pathogen, respectively [13]. *E. faecalis* can suppress *S. aureus* fitness and virulence by producing antimicrobial superoxides [13]. At the same time, *E. faecalis* also exploits *S. aureus* by thieving the iron-scavenging siderophores it produces [20]. Importantly, both superoxide production by *E. faecalis* and siderophore production by *S. aureus* have been shown to evolve under co-colonisation, indicating the possibility of coevolutionary interactions [13, 20].

By experimentally co-passaging *E. faecalis* and *S. aureus* within *C. elegans* hosts, we found direct evidence of defensive microbe-pathogen coevolution. We revealed that the defensive microbe and the pathogen underwent reciprocal adaptation via fluctuating selection dynamics and produced patterns of pathogen local adaptation and genetic diversification. Our results support theoretical predictions and illustrate the potential for defensive microbes to drive patterns of pathogen adaptation in the absence of host evolution.

Results

Co-colonisation dynamics:

We found a significant increase in pathogen fitness (Fig.1b, ANOVA F=7.98, df=2, P=0.0063, supplementary Table 1) and decrease in defensive microbe fitness (Fig.1c, ANOVA F=4.86, df=2, P=0.028, supplementary Table 1) over time in the Coevolution treatment that did not occur in the Single evolution treatment. These data suggest that *S. aureus* was 'winning' at passage 10 of experimental coevolution with *E. faecalis*.

Local adaptation:

We found that the high pathogen fitness observed at passage 10 in the Coevolution treatment under co-colonisation was significantly reduced under allopatric conditions (fig.2b Mixed-effect model: Chisq=5.82, df=1, P=0.016). Conversely, the low defensive microbe fitness recorded at passage 10 in the Coevolution treatment under co-colonisation significantly increased under allopatric conditions (fig.2c Mixed-effect model: Chisq=5.79, df=1, P=0.016). These results thus indicate that the co-colonisation dynamics observed at passage 10 were a result of interaction specificity due to pathogen local adaptation-in-time and defensive microbe local maladaptation-in-time.

As expected, there was no significant differences in *E. faecalis* or *S. aureus* fitness between the allopatric and pseudo-sympatric pairings from the Single evolution treatment (supplementary fig.1a Mixed-effect model: Chisq=1.8, df=1, P-0.18. supplementary fig.1b Mixed-effect model: Chisq=0.8, df=1, P-0.36). Thus, the patterns of local adaptation we observe in the Coevolution treatment cannot be reproduced by evolving the bacteria independently within the host.

Figure 2

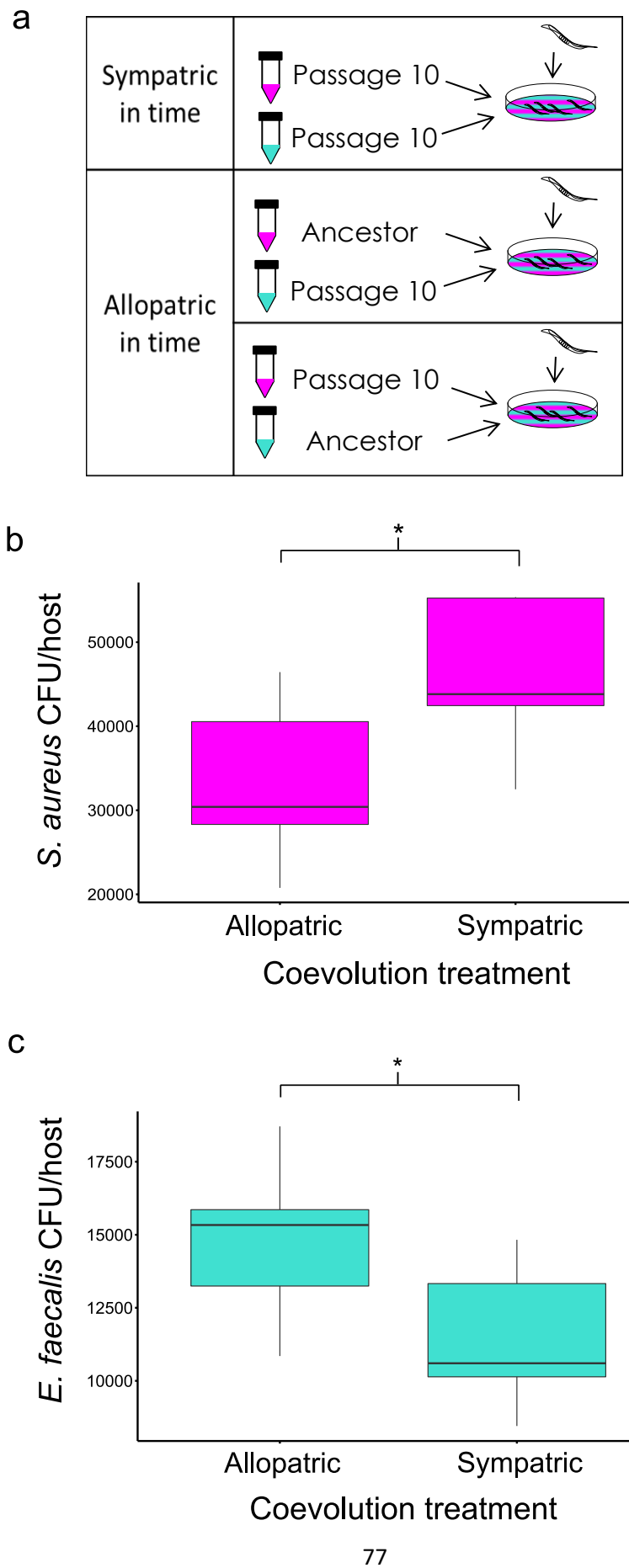

a
Sympatric in time
Passage 10
Passage 10
Allopatric in time
Ancestor
Passage 10
Passage 10
Ancestor
b
*
S. aureus CFU/host
50000
40000
30000
20000
Allopatric
Sympatric
Coevolution treatment
c
*
E. faecalis CFU/host
17500
15000
12500
10000
Allopatric
Sympatric
Coevolution treatment

Figure 2 Local adaptation in time

a, Sympatric and allopatric-in-time conditions. Sympatric conditions involve co-colonisation of contemporary *S. aureus* (magenta) and *E. faecalis* (turquoise) from the same replicate population at passage 10. Allopatric crosses involve co-colonisation of ancestral and passage 10 populations. **b,** Fitness of *S. aureus* (CFUs/host) under allopatric and sympatric conditions (Coevolution treatment). **c,** Fitness of *E. faecalis* (CFUs/host) under allopatric and sympatric conditions (Coevolution treatment). *P<0.05, **P<0.01, ***P<0.001.

Time-shifts:

We discovered strong evidence of reciprocal adaptation between *E. faecalis* and *S. aureus* in replicate populations from the Coevolution treatment. We found that the fitness of *S. aureus* differed significantly when paired with defensive microbe populations from the past, present and future (Fig.3c Mixed-effect model: Chisq=29.1, df=6, P=5.819e-05, supplementary table 1). Specifically, defensive microbes from the future were significantly better at suppressing pathogens than defensive microbes from the past (8 vs. 5, P<0.001), indicating that the defensive microbe adapted to recently-experienced pathogen populations. Critically, as we sampled further back into the past, we found evidence that adaptation was driven by fluctuating selection dynamics. Defensive microbes from the distant past were significantly better at suppressing pathogens than defensive microbes from the recent past (4 vs. 5, P<0.001). From this pattern, we can assume that the defensive microbe genotypes common at passage 8 were also common at passage 4, and were thus fluctuating over time.

From the reciprocal time-shift, we found that the within-host fitness of *E. faecalis* differed significantly when paired with pathogen populations from the past, present and future (Fig.3d Mixed-effects model: Chisq=17.18, df=6, P=0.0086, supplementary table 1). Future pathogens were significantly better at suppressing defensive microbes than pathogens from the past (8 vs. 5 P=0.011), suggesting that pathogens were also adapting to recently-experienced defensive microbe populations. Consistent with the reciprocal time-shift above, we discovered that pathogen adaptation was driven by fluctuating selection dynamics. Here, pathogens sampled further back into the past were significantly better at suppressing defensive microbes than pathogens from the recent past (4 vs. 5 P=0.034). We can assume from this pattern that the pathogen genotypes common at passage 8 were also common in the past at passage 4, consistent with the cycle of defensive microbe genotypes we observed in fig.3c. In addition to this apparent cycle, we also discovered that pathogens sampled into the distant future were

Figure 3

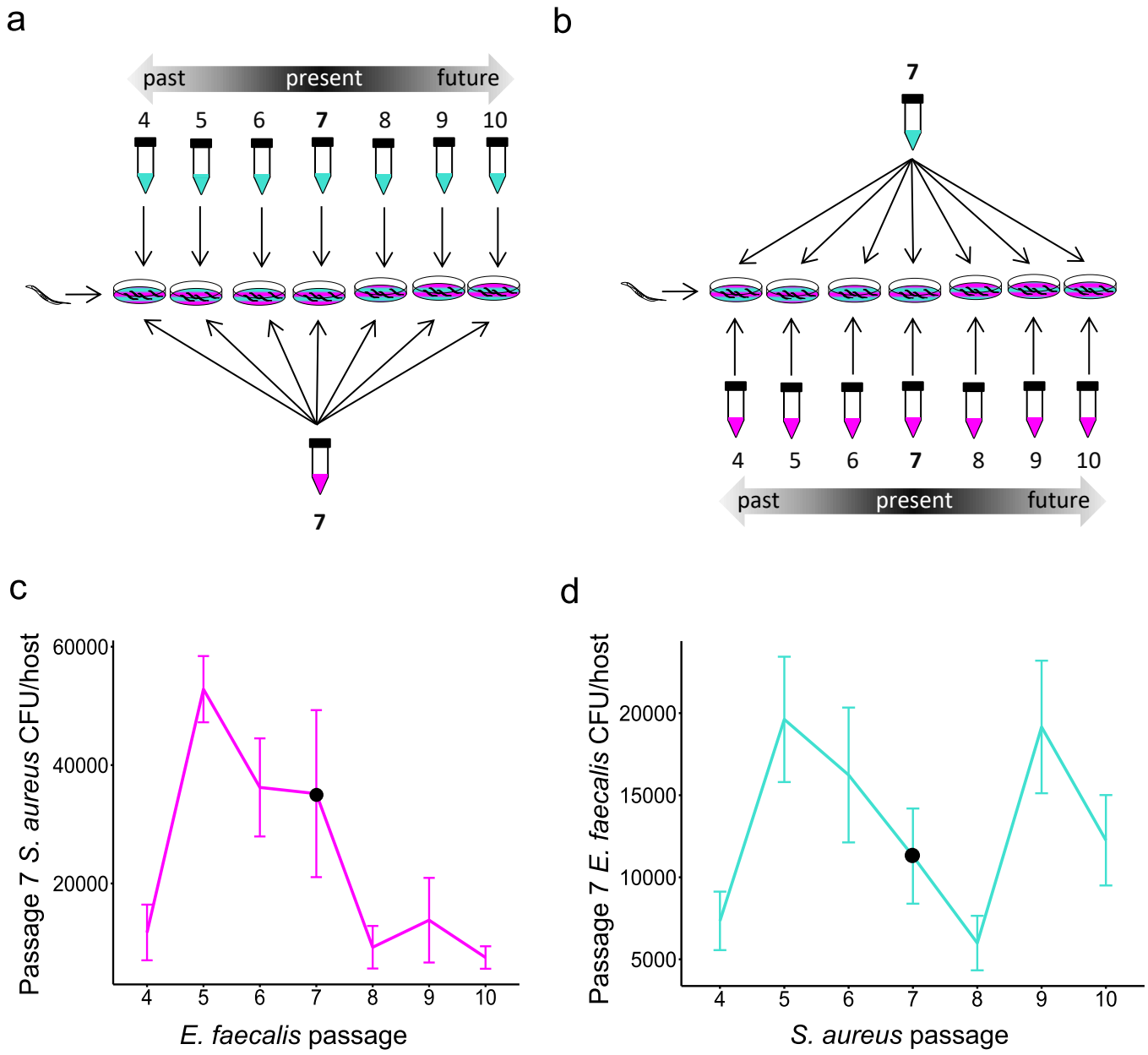

a
past
present
future
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past
present
future
c
Passage 7 S. aureus CFU/host
60000
40000
20000
4
5
6
7
8
9
10
E. faecalis passage
d
Passage 7 E. faecalis CFU/host
20000
15000
10000
5000
4
5
6
7
8
9
10
S. aureus passage

Figure 3 Time-shifts

Design of time-shift assays whereby **a,** *S. aureus* pathogen (magenta) and **b,** *E. faecalis* defensive microbe (turquoise) replicate populations were paired with the other from the past, present and future time points. **c,** Fitness of *S. aureus* (CFU/host) from passage 7 against *E. faecalis* from passages 4-10 (Coevolution treatment). **d,** Fitness of *E. faecalis* (CFU/host) from passage 7 against *S. aureus* from passage 4-10 (Coevolution treatment).

significantly worse at suppressing defensive microbes than pathogens from the near future (9 to 8 P=0.017). Interestingly, this fluctuation did not correspond with an equivalent fluctuation in *E. faecalis* genotypes in the reciprocal time-shift (fig.3c), suggesting that the pathogen was leading a new cycle of adaptation to which the defensive microbes had yet to respond. Together, these phenotypic results provide strong evidence of ongoing, reciprocal adaptation between *S. aureus* and *E. faecalis* by fluctuating selection dynamics.

Coevolutionary genomics:

We plotted the frequency of mutations for each replicate population of the Coevolution treatment over time for both *S. aureus* and *E. faecalis*. Consistent with the results indicating coevolution via fluctuating selection, we discovered that *de novo* mutations fluctuated in frequency over time in all *S. aureus* (fig.4a, Supplementary Table 2) and *E. faecalis* (fig. 4b, Supplementary Table 3) replicate populations from the Coevolution treatment. Most mutations that were common in the populations at passage 5 became rare (or extinct) by passage 10, whilst many rare (or non-existent) mutations at passage 5 increased in frequency by passage 10. A few mutations were found to increase to near fixation across both time points, indicating either slower fluctuations for these mutations or the existence of simultaneous arms race dynamics.

Many of the mutations in the coevolved *S. aureus* populations were located in genes with putative functions in the siderophore pathway (see Supplementary Table 2), a pathway important in *S. aureus-E. faecalis* interaction [20]. These included putative siderophore-iron reductases, substrate transporters and proteins involved in glycine metabolism, a key component of ferrichrome [20]. In addition to this, several mutations in the coevolved *E. faecalis* populations were located in genes with putative functions in the production of superoxides [21, 22], a known protection mechanism in this system [13] (See Supplementary Table 3). For example, mutations were found in genes involved in purine metabolism and the

Figure 4

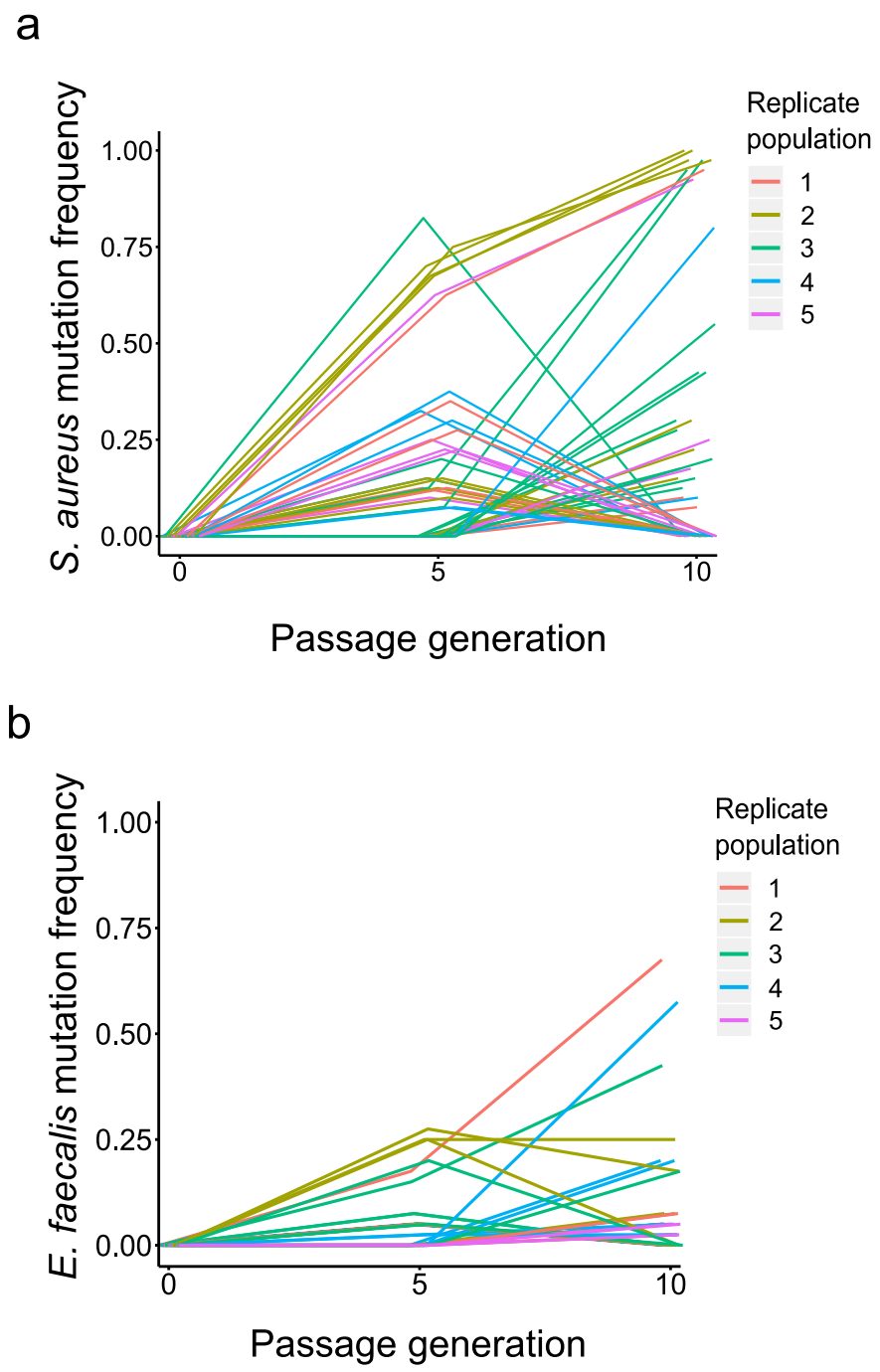


Figure 4 Dynamics of genomic evolution

The frequency of mutations in 40 sampled clones from **a,** *S. aureus* and **b,** *E. faecalis* replicate populations at passages 5 and 10 under co-passage (Coevolution Treatment). Each line represents an individual mutation, and colours correspond to replicate populations.

transport of Xanthine, both of which affect the production of superoxides [21, 22]. Interestingly, we discovered the same stop mutation in a gene involved in purine metabolism as evolved in a previous experiment whereby *E. faecalis* had evolved increased superoxide production during repeated exposure to a single *S. aureus* genotype [13].

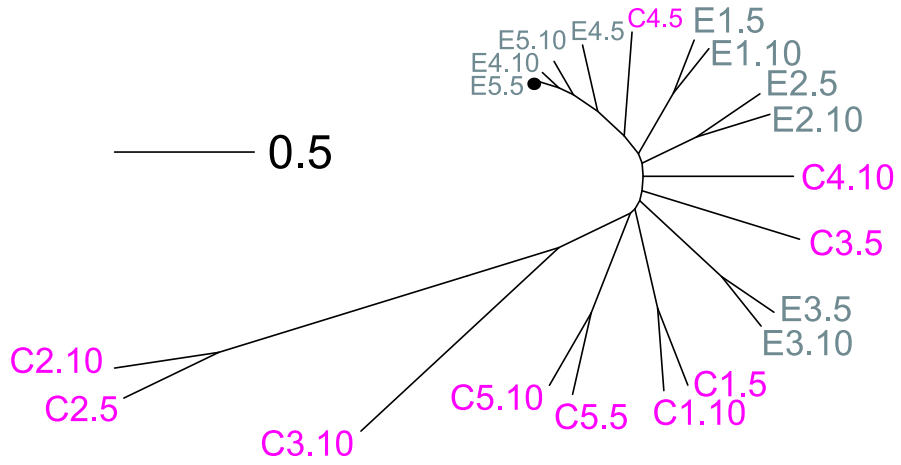
Genetic distance:

Co-passage with the defensive microbe resulted in greater pathogen genetic divergence and turnover than passaging the pathogen through hosts alone. Coevolved *S. aureus* populations were significantly more genetically distant from the ancestor, and thus experienced faster evolutionary change than independently passaged pathogen populations (fig.5a Two sample t-test, t = -2.95, df = 8, fdr-corrected P= 0.018). Furthermore, we found significantly greater pairwise genetic distances between each replicate of the coevolved pathogen populations within the independently passaged pathogen populations (fig.5a Welch two sample t-test, t=-6.97, df=12.9, fdr-corrected P= 3.108e-05), suggesting that coevolving pathogen populations followed more diverse evolutionary trajectories. Coevolved *S. aureus* populations also exhibited greater genetic distance between passage 5 and 10 than the independently passaged populations (fig.5a Wilcoxon rank sum test W = 25, fdr-corrected P= 0.012), indicating greater genetic turnover in the coevolved pathogens, consistent with fluctuating selection dynamics.

In contrast, we saw no difference in genetic divergence and turnover between *E. faecalis* treatments. Neither the genetic distance from the ancestor, nor the pairwise genetic distances among replicate defensive microbe populations differed significantly between the Single evolution and Coevolution treatments (fig.5b genetic distance from ancestor: Two sample t-test, t= 1.1, df = 8, fdr-corrected P= 0.302; among-replicate genetic distances: Two sample t-test, t= 2.36, df = 18, fdr-corrected P= 0.09). In addition, the Coevolution treatment did not

Figure 5

a



b

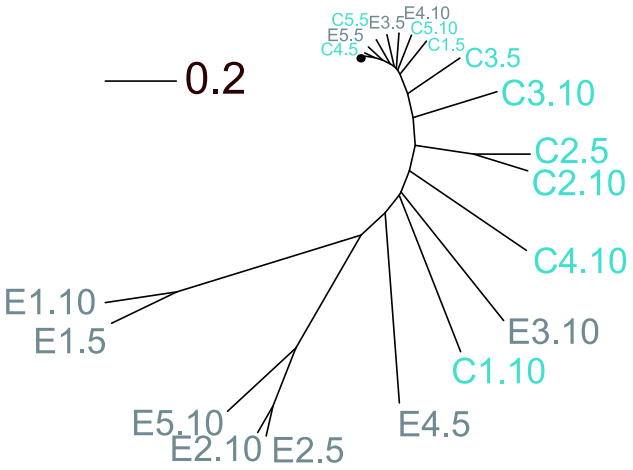


Figure 5 Genetic distances of pathogen and defensive microbe populations

a, Phylogenetic tree for *S. aureus* populations from the Single evolution treatment (E1–5 in grey) and Coevolution treatment (C1-5 in magenta) from passage 5 and 10 along with the ancestor (black circle). **b,** Phylogenetic tree for *E. faecalis* populations from the Single evolution treatment (E1–5 in grey) and Coevolution treatment (C1-5 in turquoise) from passage 5 and 10 along with the ancestor (black circle). Genetic distances based on Euclidean distances calculated from the frequency and identity of mutations in each population. Scale bar indicates Euclidean distance.

differ from the Single evolution treatment in genetic distance between passages 5 and 10 (fig.5b Two Sample t-test t = 1.55, df =8, fdr-corrected P= 0.24).

Discussion

It is theoretically predicted that defensive microbes and pathogens could undergo coevolutionary interactions within host populations via fluctuating selection dynamics [3], however direct tests are currently lacking [1, 23, 24]. Here, by experimentally co-passaging defensive microbes and pathogens through host populations, we found evidence of defensive microbe-pathogen coevolution via fluctuating selection dynamics.

We detected defensive microbe-pathogen interaction specificity as a result of experimental co-passage. Such specificity is a key assumption underlying coevolution by fluctuating selection dynamics [12]. At passage 10, all replicate pathogen populations were locally adapted in time to the defensive microbe, whilst the defensive microbe was locally maladapted, resulting in the appearance that the pathogen was 'winning'. This pattern may parallel studies of host-pathogen coevolution which have found that pathogen local adaptation is more commonly identified than host local adaptation because pathogens have faster evolutionary responses to reciprocal adaptation than hosts (i.e., due to higher rates of pathogen migration and mutation, or shorter generation times) [25, 26]. Although our experimental design did not allow for migration, we observed that all pathogen populations accumulated more mutations than defensive microbe populations, suggesting a faster pathogen evolutionary rate.

By performing time-shift experiments, we found that both pathogens and defensive microbes were constantly and reciprocally adapting to each other throughout the experiment. We show that both species peaked in fitness against antagonist populations recently experienced in evolutionary history, a pattern expected from reciprocal evolutionary change. Here, the fitness

of a focal species against their recent enemy is expected to be higher than against their current enemy since the current enemy has begun to respond to the adaptive changes of the focal species [26]. Critically, our time-shift data also indicate the dynamics of coevolution. In line with our finding of interaction specificity, we show that pathogen and defensive microbe adaptation appeared to fluctuate over time, rather than increase directionally, indicating fluctuating selection dynamics [10, 12]. For example, the pathogen and defensive microbe genotypes that were common at passage 4 became rare throughout passages 5-7 and then increased in frequency again by passage 8, pointing to a full cycle of genotype fluctuation.

Consistent with our phenotypic evidence of fluctuating selection dynamics, we found direct confirmation of rapid genotype frequency changes across the evolution experiment. We revealed that mutations that had spread rapidly in the population by passage 5 were almost all rare (or extinct) by passage 10, whilst mutations that were rare (or non-existent) at passage 5 increased in frequency by passage 10. Importantly, many of these mutations were located in genes putatively linked to *S. aureus- E. faecalis* interactions, including siderophore production by *S. aureus* [20] and superoxide production by *E. faecalis* [13]. Kwiatkowski *et al.* (2012) show that such oscillations in defensive microbe and pathogen alleles are likely to occur when the host genetic background is evolutionarily stable, as in our experiment. This outcome suggests that defensive microbes could 'take over' from the host and provide rapid coevolutionary responses to pathogens [3] which could remove or reduce parasite-mediated selective pressure on hosts [24, 27]. We speculate that possessing a defensive microbe might allow hosts with relatively limited evolutionary potential (e.g. due to asexual reproduction, low genetic diversity, or long generation times) to maintain resistance to coevolving pathogens.

An important implication of our findings is that defensive microbes have the potential to be a major source of selection on pathogens, driving fast evolutionary rates and divergent evolutionary trajectories. We observed here that pathogens coevolving with defensive

microbes had higher rates of evolution, greater genetic turnover, and more diverse evolutionary trajectories than those evolving independently in host populations. Similar patterns of molecular evolution in pathogens have been found to occur during host-pathogen coevolution [28, 29]. Unlike the pathogen, however, we found no difference in the rate of genomic evolution between the co-passaged and independently passaged defensive microbe populations. It is possible that we saw such asymmetry because *S. aureus* was ahead in the coevolutionary arms race and, according to our time shift data, was starting a new cycle to which the defensive microbe was not yet adapted to. It is therefore likely that more evolutionary changes had accumulated in coevolved *S. aureus* populations relative to independently passaged populations, whilst this was not yet the case for *E. faecalis*. This explanation is consistent with our observations of pathogen local adaptation and the higher total number of mutations in the pathogen populations compared to the defensive microbe populations. Given that coevolution started *de novo*, we would expect that more evolutionary time, and so more cycles of reciprocal adaptation, would eventually result in greater evolutionary change in both species relative to the independently passaged populations.

Together, our data show the potential for defensive microbes and pathogens to engage in coevolution driven by fluctuating selection. Our findings suggest that coevolution may underlie the interaction specificity observed between pathogens and defensive microbes in nature [4-9]. Given that studies investigating coevolution have largely focussed on host-parasite interactions [12, 25, 30-32], it is an implied assumption that the host is the strongest source of selection acting upon parasites and pathogens. Although we do not account for additional effects of host immunity on *E. faecalis-S. aureus* interactions in this study, it is nevertheless clear that defensive microbes can be as powerful as hosts in imposing strong and divergent selection on pathogens. Identifying that pathogens are able to undergo coevolution with defensive microbes, instead of the host, has important implications for how we understand the factors shaping patterns of pathogen evolution in nature. Moreover, the ability of

defensive microbes to coevolve with pathogens may have great consequences for their role in disease control. For example, if defensive microbes are able to undergo continual counter-adaptation to pathogen resistance, they could be powerful tools to sustainably control infectious diseases of animals and plants [1, 33]. In an era of emerging antibiotic resistance, the allure of evolution-proof disease control is at an all-time high [34, 35].

It is being increasingly revealed that defensive microbes are common within host populations and across numerous host species. As a result, we are only just beginning to realise their immense value to hosts. In this study, we further our understanding of defensive microbes by illustrating their role as an evolutionarily dynamic arm of host immunity and their role in driving pathogen divergence.

Materials and Methods

Nematode host and bacteria:

C. elegans nematodes constantly interact with microbes in their natural habitats [36], wherein they can act as predators or hosts for numerous microbial species [15, 37]. These animals are thus an established model for microbial colonisation and pathogenesis [14, 17] and their gut can be co-colonised by multiple pathogens and commensals [38-40].

We used the simultaneous hermaphroditic N2 wild-type *C. elegans* strain from the *Caenorhabditis* Genetics Centre (University of Minnesota, Minneapolis, MN). A genetically homogeneous line was generated by selfing a single hermaphrodite for five generations. Populations of these nematodes were frozen in 50% M9 solution and 50% liquid freezing solution in cryotubes at -80°C [41]. Populations were regularly resurrected to prevent the accumulation of *de novo* mutations in hosts. Nematodes were maintained at 20°C on nematode growth medium (NGM) plates seeded with *Escherichia coli* OP50. Seeded NGM was

made by growing *E. coli* OP50 at 30°C shaking at 200rpm overnight in Luria-Bertani (LB) broth and then incubating 100µl of this culture on NGM plates at 30°C overnight [41]. To ensure clean stocks for experimentation and to synchronise the age of individuals in the population, nematodes were regularly treated with a bleach (NaClO) and sodium hydroxide (NaOH) solution which kills both microbes and worms but not worm eggs [41].

We used *Staphylococcus aureus* strain MSSA 476 (GenBank: BX571857.1), an invasive community-acquired methicillin-susceptible isolate, and *Enterococcus faecalis* strain OG1RF (GenBank: CP002621.1), an isolate from a human digestive tract. A single ancestral population of each species was grown from a single colony overnight in Todd Hewitt Broth (THB) shaking at 200rpm at 30°C. Bacteria were frozen in a 1:1 ratio of sample to 50% glycerol solution in cryotubes at -80°C.

Experimental evolution:

The experiment consisted of two treatments: (1) *S. aureus* and *E. faecalis* were co-passaged under co-colonisation within host populations (Coevolution); and (2) *S. aureus* and *E. faecalis* were passaged separately in host populations (Single evolution) (fig.1a). Each treatment consisted of five replicate populations and 10 passages. To make exposure lawns, bacteria were cultured overnight in THB shaking at 200rpm at 30°C. After standardising the cultures to an OD600 reading of 1.00, the bacteria were spread onto Tryptic Soy Broth (TSB) plates. We spread a volume of 120µl of either *S. aureus* or *E. faecalis* for the Single evolution treatment and we spread a mixture of 120µl of *S. aureus* and 120µl of *E. faecalis* on the same exposure plate for the Coevolution treatment. Exposure plates were kept overnight at 30°C.

Approximately 1000 young adult nematodes from the stock *C. elegans* population were placed onto each exposure lawn and incubated at 25°C. After 24 hours of incubation, bacteria from 10 dead nematodes from each lawn were picked to ensure that we took hosts successfully

colonised with bacteria. Under co-colonisation, the proportion of pathogen collected from the infection did not differ significantly between dead and live worms [20]. Nematodes were considered dead when they did not respond to touch with a platinum wire. Nematodes were washed to remove external bacteria by transferring them between five 5µl drops of M9 buffer on fresh TSB plates using a platinum wire. Nematodes were then crushed in 20µl of M9 in a 1ml Eppendorf tube with a pestle to release internal bacteria. An inoculation loop was used to streak each sample onto selective media (TSB plates with 100µg/mL rifampicin were used to isolate *E. faecalis* and Mannitol Salt Agar (MSA) plates were used to isolate *S. aureus*), and the plates were incubated overnight at 30°C. Ten colonies of *S. aureus* and/or *E. faecalis* were subsequently picked from each replicate and grown in THB overnight, shaking at 200rpm at 30°C. These cultures were used to make the exposure plates for the next passage.

Co-colonisation dynamics:

To determine whether co-passage affected the fitness of the defensive microbe and pathogen under co-colonisation, we compared the fitness of the defensive microbe and pathogen from the Coevolution treatment under co-colonisation with the ancestral and Single evolution treatment populations under co-colonisation. We performed this experiment using populations from passage 10 of the evolution experiment. Exposure plates were made as above. Approximately 1000 young adult nematodes from the stock *C. elegans* population were placed onto exposure plates and incubated for 24 hours at 25°C. After 24 hours of exposure, 3-5 dead nematodes were picked from each plate. Nematodes were washed as in the evolution experiment, and then crushed in 20µl of M9 with a pestle to release internal bacteria. After crushing, serial dilutions were plated onto selective media and grown overnight at 30°C. *E. faecalis* and *S. aureus* colony-forming units (CFUs) per host were counted.

Local adaptation:

To test whether the defensive microbe or pathogen became locally adapted-in-time as a result of co-passage, we measured the number of CFUs per nematode of each bacterial species under sympatric-in-time and two allopatric-in-time co-colonisation conditions (see fig.2a). Exposure plates were made as above. The sympatric condition paired defensive microbe and pathogen populations from the same replicates at passage 10. The two allopatric conditions paired defensive microbe and pathogen ancestors with passage 10 populations. Our assays of local adaptation in time are conducted in a similar fashion to the 'local vs. foreign' and 'home vs. away' comparisons commonly used to measure local adaptation in space [42]. Using two allopatric conditions in a single comparison is a conservative method of measuring local adaptation because it allows us to tease apart the evolutionary effects of both interacting species. We also performed the same comparisons using the Single evolution treatment populations. Given that both bacterial species evolved independently in the Single evolution treatment, the sympatric pairings were 'pseudo-sympatric', and we did not expect to find a signal of local adaptation.

Time-shift assays:

We looked for evidence of reciprocal adaptation in both pathogen and defensive microbe populations over time by performing time-shift assays [10]. Here, *S. aureus* populations from passage 7 were allowed to co-colonise the host with *E. faecalis* from past, present and future replicate populations (passages 4-10, see fig.3a), and their respective CFUs/host were counted. The reciprocal was done for *E. faecalis* (see fig.3b). Exposure plates were made and CFU per host for each species was counted as above.

Genome extraction and analysis:

Forty clones were randomly selected from each *S. aureus* and *E. faecalis* replicate population from both selection treatments at passages 5 and 10. Each clone was grown independently

overnight in 200µl THB in a 96-well plate shaking at 175rpm at 30°C. Subsequently, each set of 40 clones was checked for equal OD600 and pooled in equal volumes, resulting in one sample per 40 clones. A single clone of ancestral *S. aureus* and *E. faecalis* was also grown.

S. aureus DNA was extracted by centrifuging 1ml of culture at 7500rpm, removing the supernatant and re-suspending the pellet in 160µl enzymatic lysis buffer (Qiagen), 40µl lysostaphin (200µg/ml, Sigma-Aldrich), 40µl lysozyme (100mg/ml, Sigma-Aldrich) and 8µl RNAse A (10mg/ml, Sigma-Aldrich). This mixture was incubated for 1 hour at 37°C. A volume of 25µl proteinase K (Qiagen) was then added and 200µl Buffer AL (without ethanol) was added and vortexed. This mixture was then incubated at 56°C for 1 hour. A volume of 200µl of ethanol was added and vortexed and DNA purification followed DNeasy Blood and Tissue Spin-Column Protocol (Qiagen). From *E. faecalis*, DNA was extracted by centrifuging 2ml of culture at 7500rpm, removing the supernatant and re-suspending the pellet in 160ul buffer B1 (Qiagen), 6µl of 25KU/ml mutanolysin (Sigma-Aldrich), 40µl of 200µg/ml lysostaphin (Sigma-Aldrich) and 40µln 100mg/ml lysozyme (Sigma-Aldrich). This mixture was incubated for 1 hour at 37°C. A volume of 25µl proteinase K (Qiagen) was then added and 200µl Buffer AL (without ethanol) was added and vortexed. This mixture was then incubated at 56°C for an hour. Ethanol (200ul) was added and DNA purification followed the DNeasy Blood and Tissue Spin-Column Protocol (Qiagen).

DNA was sequenced using the HiSeq4000 platform with 100bp paired end at the Wellcome Trust Centre for Human Genetics. The project accession number at the European Nucleotide Archive for the raw read data is PRJEB13385. Sequenced read data in fastq files were trimmed for the presence of Illumina adapter sequences using Cutadapt version v1.9.1 (revision e960cc1 from github.com/marcelm/cutadapt)[43]. The reads were further trimmed using Sickle version 1.33 (revision f3d6ae3 from github.com/najoshi/sickle)[44] with a minimum

window quality score of 25 and retaining only reads longer than 50bp after trimming. Single reads remaining from pairs were retained.

Reference genomes for short read mapping were obtained from the NCBI Assembly database (www.ncbi.nlm.nih.gov/assembly). Short reads of the DNA extractions from the *Staphylococcus aureus* strain MSSA476 isolates were mapped to assembly GCF_000011525.1 using BWA MEM v0.7.12 (revision cc9eef2 from github.com/lh3/bwa) [45] using option -M to flag shorter split hits as secondary ensuring the single best alignment was used. Evolved populations of *E. faecalis* OG1RF were mapped to assembly GCF_000172575.2 using the same aligner and options. Alignments were manipulated using SAMTools v1.2 (revision ac5b8e7 from github.com/samtools/samtools)[46].

The Genome Analysis Toolkit v3.4 (GATK) [47-49] Indel Realigner module was used to realign reads around putative insertions and deletions after which duplicate reads were identified and removed with Picard v1.135 [50]. Single nucleotide polymorphism, insertion and deletion discovery was performed with GATK's Haplotype caller module with sample ploidy n = 40 as described in [51]. Default parameter values were used, however given diversity was expected to be relatively low due to the experimental duration the priors "--heterozygosity 0.0001 –indel_heterozygosity 0.00001" were used. One replicate population of *E. faecalis* from the Coevolution treatment at passage 10 (C3.10) had high read coverage depth over one region that caused excessive memory usage. This coverage difference was remedied by setting "--downsample_to_fraction 0.8" to decrease the reads used and "--minPruning 2" to simplify the de Bruijn k-mer graph used in variant calling. In order to distinguish low frequency mutations from sites prone to sequencing error, the ancestral clone was sequenced and variants called with ploidy of 40, as above, and any sites exhibiting apparent polymorphism were filtered out of further analyses. Any sites appearing as polymorphic in more than one population were checked by manual inspection in IGV2.

Genetic distance:

A phylogenetic tree was constructed using Euclidean genetic distances (the square root of pairwise differences), which is suggested as an appropriate metric for molecular variation data [29, 52]. It is important to note that a few observed mutations are likely to have arisen in the overnight culture of the ancestral populations used to initiate all populations of all treatments, resulting in some shared mutations from the start of the evolution experiment. Pairwise genetic distances were taken from the distance matrix for statistical analysis. We compared the genetic distances of replicate populations to the ancestor to assess differences in the rate of evolutionary change between treatments. We compared the genetic distances between replicate populations of the same treatments to contrast the diversity in evolutionary trajectories. Finally, to assess differences in rates of genetic turnover of each treatment, we compared the genetic distances of replicate populations between passages 5 and 10.

Statistical analyses:

Data analysis was carried out in R v 3.2.0 (http://www.r-project.org/). Parametric tests were used for all data which met the required assumptions. These assumptions were checked using the Shapiro test to detect whether data was normally distributed and F-tests to compare the variances of two samples from normal populations. Non-parametric equivalents were used if the data did not meet those assumptions. Outlying data points were detected and removed by using the Dixon test. ANOVAs were used to compare bacterial fitness across treatments under co-colonisation and Tukey contrasts were used for *post hoc* comparisons. Plots of each ANOVA were checked by eye for model quality. A mixed-effect model was used to measure local adaptation, including replicate population as a random effect and the co-colonisation condition as a fixed effect. Another mixed-effect model was used to measure reciprocal adaptation across time-shifts, including replicate population as a random effect and time as a fixed effect. Tukey contrasts were used for *post-hoc* comparisons. Two-sample t-tests were

used to compare pairwise genetic distances of bacterial populations. The Welch two sample t-test was used when the assumption of equal variance was broken. The Wilcoxon rank sum test was used when the assumption of normality was broken. P-values generated by comparing pairwise genetic distances were corrected for multiple comparisons for each bacterial species using the FDR method.

Data availability:

The data will be deposited in DRYAD repository. The project accession number at the European Nucleotide Archive for the raw read data is PRJEB13385.

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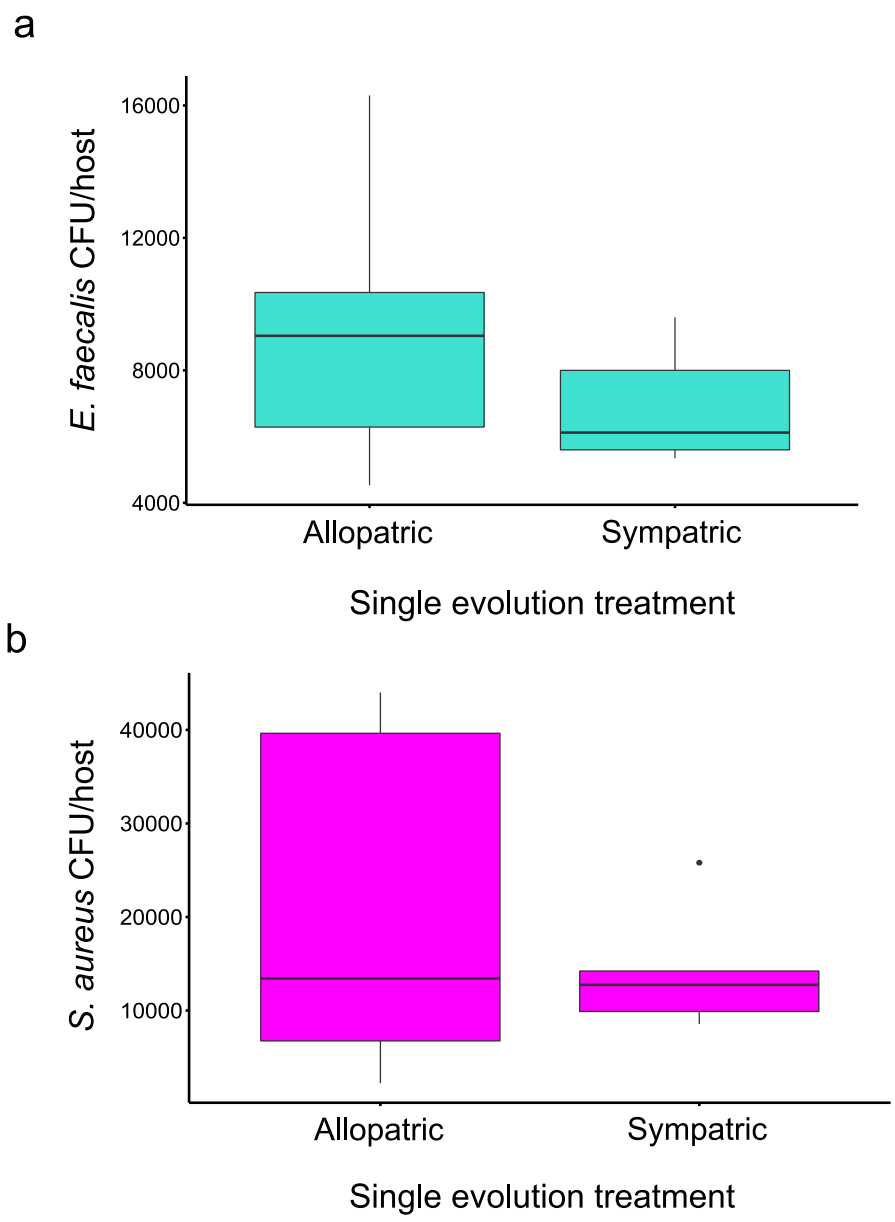
Author contributions

SAF and KCK designed the experiments. SAF conducted the experiments and analysed the data. SAF, DW, and SP conducted the bioinformatics. SAF and KCK wrote the paper.

Competing financial interests

The authors declare no competing financial interests.

Supplementary information



Supplementary figure 1

a, Fitness of *E. faecalis* (CFU/host) and **b,** *S. aureus* (CFU/host) from the Single evolution treatment under sympatric and allopatric-in-time conditions.

Supplementary Table 1. Statistical results listed by figure.

Figure	Statistic
1b	**ANOVA:** F=7.98, df=2, P=0.0063 **Tukey Contrasts:** Coevolution-Ancestor: P=0.008 Single evolution-Ancestor: P=0.86 Single evolution-Coevolution: P=0.02
1c	**ANOVA**: F=4.86, df=2, P=0.028 **Tukey Contrasts:** Coevolution-Ancestor: P=0.071 Single evolution-Ancestor: P=0.91 Single evolution-Coevolution: P=0.034
2b	**Mixed-effect model** with replicate population as random effect and sympatric/allopatric treatment as fixed effect. Chisq=5.82, df=1, P=0.016
2c	**Mixed-effect model** with replicate population as random effect and sympatric/allopatric treatment as fixed effect. Chisq=5.79, df=1, P=0.016
3c	**Mixed-effect model** with replicate population as random effect and *E. faecalis* passage as fixed effect. Chisq=29.1, df=6, P=5.819e-05 **Tukey Contrasts:** 5-8: P<0.001 4-8: P=0.99 9-8: P=0.99 7-8: P=0.31 6-8: P=0.001 10-8: P=0.99 4-5: P<0.001 9-5: P<0.001 7-5: P=0.28 6-5: P=29 10-5: P<0.001 9-4: P=1 7-4: P=0.079 6-4: P=0.005 10-4: P=0.99 7-9: P=0.14 6-9: P=0.017 10-9: P=0.99 6-7: P=0.99 10-7: P=0.025 10-6: P=0.0028

Supplementary Table 1. Statistical results listed by figure.

Figure	Statistic
3d	**Mixed-effects model** with replicate population as random effect and *S. aureus* passage as fixed effect. Chisq=17.18, df=6, P=0.0086 **Tukey Contrasts:** 5-8: P=0.011 4-8: P=0.99 9-8: P=0.017 7-8: P=0.84 6-8: P=0.14 10-8: P=0.70 4-5: P=0.034 9-5: P=1 7-5: P=0.36 6-5: P=0.98 10-5: P=0.52 9-4: P=0.048 7-4: P=0.95 6-4: P=0.28 10-4: P=0.88 7-9: P=0.43 6-9: P=0.99 10-9: P=0.60 6-7: P=0.88 10-7: P=1 10-6: P=0.95
5a	Pairwise distances of passage 10 from ancestor, comparing Coevolution and Single evolution treatments: **Two Sample t-test** t = -2.95, df = 8, P= 0.018 **fdr-corrected P=** 0.018 Within-treatment pairwise distances of passage 10, comparing Coevolution and Single evolution treatment: **Welch two Sample t-test** t = -6.97, df = 12.9, P=1.036e-05 **fdr-corrected P=** 3.108e-05 Pairwise distances of passage 5 to passage 10 replicate populations, comparing Coevolution and Single evolution treatments: **Wilcoxon rank sum test** W = 25 , P= 0.0079 **fdr-corrected P=** 0.012
5b	Pairwise distances of passage 10 from ancestor, comparing Coevolution and Single evolution treatments: **Two Sample t-test** t = 1.1, df = 8, P= 0.302 **fdr-corrected P=** 0.302 Within-treatment pairwise distances of passage 10, comparing Coevolution and Single evolution treatments: **Two Sample t-test** t = 2.36, df = 18, P= 0.03 **fdr-corrected P=** 0.09

Supplementary Table 1. Statistical results listed by figure.

Figure	Statistic
	Pairwise distances of passage 5 to passage 10 replicate populations, comparing Coevolution and Single evolution treatments: **Two Sample t-test** t = 1.55, df =8, P= 0.16 **fdr-corrected P=** 0.24
Supp 1a	**Mixed-effect model** with replicate population as random effect and sympatric/allopatric treatment as fixed effect. Chisq=1.8, df=1, P=0.18
Supp 1b	**Mixed-effect model** with replicate population as random effect and sympatric/allopatric treatment as fixed effect. Chisq=0.8, df=1, P=0.36

Supplementary Table 2. Mutations found in *S. aureus* listed by passage, treatment and replicate population. The position in the chromosome is given and the locus tag when the mutation occurs in a coding region. Mutation frequency is the proportion of 40 sampled clones that contain the mutation in the replicate population. Gene product is given where known. Mutation type: S=synonymous. N=non-synonymous. *=stop gain. */= stop loss.

Position	Locus tag	Gene product	Mutation	Mutation frequency																	
				Passage 10										Passage 5							
				C1	C2	C3	C4	C5	E1	E2	E3	E4	E5	C1	C2	C3	C4	C5	E1	E2	E3	E4	E5
10884				0	0	0	0	0	0	0.325	0	0	0	0	0	0	0	0	0	0.65	0	0	0
59573	SAS_RS00235	transposase	N	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.1	0	0	0	0	0
135427	SAS_RS00600	hypothetical protein	*	0	0.975	0	0	0	0	0	0	0	0	0	0.675	0	0	0	0	0	0	0	0
203056				0	0	0.175	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
292191	SAS_RS01275	ribose transporter RbsU	N	0	0	0	0	0	0	0.35	0	0	0	0	0	0	0	0	0	0.2	0	0	0
307388	SAS_RS01340	protein EsaA	N	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.25	0	0	0	0	0
423924				0	0	0	0	0	0.075	0	0	0	0	0	0	0	0	0	0	0	0	0.425	0
425660	SAS_RS01965	nuclease	N	0	0	0	0	0	0.125	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Position	Locus tag	Gene product	Mutation	Mutation frequency																	
				Passage 10										Passage 5							
				C1	C2	C3	C4	C5	E1	E2	E3	E4	E5	C1	C2	C3	C4	C5	E1	E2	E3	E4	E5
549806				0	1	0	0	0	0	0	0	0	0	0	0.7	0	0	0	0	0	0	0	0
574305	SAS_RS02720	DNA-directed RNA polymerase subunit beta	N	0	0	0	0	0.175	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
606165	SAS_RS02825	glycosyl transferase family 1	N	0	0.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
613323				0	0	0	0	0	0	0	0	0	0	0	0	0	0.15	0	0	0	0	0	0
621623	SAS_RS02905	uracil-DNA glycosylase	S	0	1	0	0	0	0	0	0	0	0	0	0.675	0	0	0	0	0	0	0	0
626196				0	0	0	0	0	0	0	0.25	0	0	0	0	0	0	0	0	0	0.4	0	0
627445				0	0	0	0	0	0	0	0	0	0	0	0.15	0	0	0	0	0	0	0	0
629811				0	0	0.95	0	0	0	0	0	0	0	0	0	0.125	0	0	0	0	0	0	0
692784	SAS_RS03290	ferrichrome ABC transporter permease	N	0	0	0	0	0	0	0.275	0	0	0	0	0	0	0	0	0	0	0	0	0

Position	Locus tag	Gene product	Mutation	Mutation frequency																	
				Passage 10										Passage 5							
				C1	C2	C3	C4	C5	E1	E2	E3	E4	E5	C1	C2	C3	C4	C5	E1	E2	E3	E4	E5
697673	SAS_RS03320	membrane protein	N	0	0	0	0	0.925	0	0	0	0	0	0	0	0	0	0.625	0	0	0	0	0
733955	SAS_RS03515	membrane protein	N	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.075	0	0	0
739478	SAS_RS03545	uncharacterized protein	S	0	0	0	0	0	0	0	0	0.125	0	0	0	0	0	0	0	0	0	0	0
797809	SAS_RS03845	helicase	*	0	0	0	0	0	0	0.3	0	0	0	0	0	0	0	0	0	0	0	0	0
848832	SAS_RS04065	thermonuclease	S	0	0	0	0	0	0	0.25	0	0	0	0	0	0	0	0	0	0.2	0	0	0
862180	SAS_RS04175	methionine import ATP-binding protein MetN 2	N	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.075	0
868946	SAS_RS04210	cysteine desulfurase	N	0	0.225	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
875238	SAS_RS04245	hypothetical protein	N	0	0	0.15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1034531	SAS_RS05095	hypothetical protein	N	0	0	0	0	0	0	0	0	0	0	0	0.125	0	0	0	0	0	0	0	0

Position	Locus tag	Gene product	Mutation	Mutation frequency																	
				Passage 10										Passage 5							
				C1	C2	C3	C4	C5	E1	E2	E3	E4	E5	C1	C2	C3	C4	C5	E1	E2	E3	E4	E5
1101366	SAS_RS05445	pyruvate dehydrogenase E1 component subunit alpha	S	0	0	0	0	0	0	0	0.1	0	0	0	0	0	0	0	0	0	0	0	0
1136694	SAS_RS05630	iron-regulated surface determinant protein B	S	0.075	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1140755	SAS_RS05650	heme uptake system protein IsdE	N	0	0	0	0	0	0	0	0.275	0	0	0	0	0	0	0	0	0	0.4	0	0
1148196				0	0	0	0	0	0	0	0.175	0	0	0	0	0	0	0	0	0	0	0	0
1169390	SAS_RS05825	alpha-hemolysin	N	0	0	0	0.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1187071	SAS_RS05930	penicillin-binding protein	N	0	0	0.425	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1222841	SAS_RS06085	primosomal protein N	N	0	0	0	0	0	0	0	0	0	0	0	0	0.2	0	0	0	0	0	0	0
1267927				0	0	0.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1280122	SAS_RS06355	RIP metalloprotease RseP	N	0	0	0	0	0	0.275	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Position	Locus tag	Gene product	Mutation	Mutation frequency																	
				Passage 10										Passage 5							
				C1	C2	C3	C4	C5	E1	E2	E3	E4	E5	C1	C2	C3	C4	C5	E1	E2	E3	E4	E5
1332868	SAS_RS06570	GTPase HflX	N	0	0	0	0	0	0	0	0.075	0	0	0	0	0	0	0	0	0	0.075	0	0
1380051	SAS_RS06840	aconitate hydratase	N	0	0	0	0	0	0	0	0	0	0	0	0.15	0	0	0	0	0	0	0	0
1477356	SAS_RS07310	matrix-binding protein	N	0	0	0	0	0	0	0	0	0	0	0	0	0	0.075	0	0	0	0	0	0
1508098	SAS_RS07345	sulfite reductase subunit alpha	N	0	0	0.975	0	0	0	0	0	0	0	0	0	0.075	0	0	0	0	0	0	0
1512778	SAS_RS07380	hypothetical protein	N	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.225	0	0	0	0	0
1839866				0	0	0.275	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1886279				0	0	0	0	0	0.125	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1892242				0	0.15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1914095	SAS_RS09425	foldase	N	0.95	0	0	0	0	0	0	0	0	0	0.625	0	0	0	0	0	0	0	0	0

Position	Locus tag	Gene product	Mutation	Mutation frequency																	
				Passage 10										Passage 5							
				C1	C2	C3	C4	C5	E1	E2	E3	E4	E5	C1	C2	C3	C4	C5	E1	E2	E3	E4	E5
1923186	SAS_RS09460	transcriptional regulator	S	0	0	0	0	0	0	0	0	0	0	0.125	0	0	0	0	0	0	0	0	0
1945482	SAS_RS09685	2-hydroxyacid dehydrogenase	N	0	0	0	0	0	0.5	0	0	0	0	0	0	0	0	0	0	0.675	0	0	0	0
1985301				0	0	0	0	0	0	0	0.325	0	0	0	0	0	0	0	0	0	0	0	0	0
2004992				0	0	0	0	0	0	0	0	0	0	0	0	0	0.325	0	0	0	0	0	0	0
2200113	SAS_RS11115	uncharacterized protein	N	0	0	0	0	0	0	0	0.375	0	0	0	0	0	0	0	0	0	0.4	0	0
2208902				0	0	0	0	0	0	0	0	0	0	0.125	0	0	0	0	0	0	0	0	0
2223288	SAS_RS11185	methicillin resistance protein FmtB	N	0	0	0.425	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2232539				0	0	0	0	0	0	0	0	0	0	0	0	0.825	0	0	0	0	0	0	0
2338045	SAS_RS11875	urease accessory protein	N	0	0	0	0	0	0	0	0	0.1	0	0	0	0	0	0	0	0	0	0	0

Position	Locus tag	Gene product	Mutation	Mutation frequency																	
				Passage 10										Passage 5							
				C1	C2	C3	C4	C5	E1	E2	E3	E4	E5	C1	C2	C3	C4	C5	E1	E2	E3	E4	E5
2362203	SAS_RS12000	RpiR family transcriptional regulator	N	0	0	0	0	0	0	0	0	0	0.225	0	0	0	0	0	0	0	0	0	0
2379210	SAS_RS12080	urocanate hydratase	N	0	0	0	0	0	0	0	0	0	0	0	0.1	0	0	0	0	0	0	0	0
2407915	SAS_RS12235	DNA-binding response regulator	N	0	0	0	0	0	0	0	0.325	0	0	0	0	0	0	0	0	0	0.45	0	0
2412116	SAS_RS12255	malate:quinone oxidoreductase	N	0	0	0	0	0	0	0	0	0	0	0	0	0	0.375	0	0	0	0	0	0
2474326	SAS_RS12570	hypothetical protein	S	0	0	0	0	0	0	0	0	0	0	0.35	0	0	0	0	0	0	0	0	0
2485521	SAS_RS12635	glycerate kinase	N	0	0	0	0	0.25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2553454	SAS_RS12975	accumulation-associated protein	S	0	0	0	0	0	0	0	0	0	0	0	0	0	0.3	0	0	0	0	0	0
2597224				0	0	0	0	0	0.15	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2602946	SAS_RS13205	holin-like protein CidB	N	0	0.975	0	0	0	0	0	0	0	0	0	0.75	0	0	0	0	0	0	0	0

Position	Locus tag	Gene product	Mutation	Mutation frequency																	
				Passage 10										Passage 5							
				C1	C2	C3	C4	C5	E1	E2	E3	E4	E5	C1	C2	C3	C4	C5	E1	E2	E3	E4	E5
2623997				0	0	0.125	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2632830	SAS_RS13340	CHAP domain-containing protein	N	0	0	0.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2641524	SAS_RS13390	glyoxalase	N	0	0	0	0	0	0	0	0	0	0	0	0	0	0.075	0	0	0	0	0	0
2667682				0.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2682764	SAS_RS13620	anaerobic ribonucleoside-triphosphate reductase activating protein	*/	0	0	0	0.8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2691066	SAS_RS13665	hypothetical protein	N	0	0	0.55	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2701801	SAS_RS13715	clumping factor B	S	0	0	0	0	0	0	0	0	0	0	0.275	0	0	0	0	0	0	0	0	0
2725891	SAS_RS13800	hypothetical protein	N	0	0	0	0	0	0	0	0.3	0	0	0	0	0	0	0	0	0	0.375	0	0
2727708	SAS_RS13810	accessory Sec system glycosyltransferase GtfA	N	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.225	0	0	0	0	0

Supplementary Table 3. Mutations found in *E. faecalis* listed by passage, treatment and replicate population. The position in the chromosome is given and the locus tag when the mutation occurs in a coding region. Mutation frequency is the proportion of 40 sampled clones that contain the mutation in the replicate population. Gene product is given where known. Mutation type: S=synonymous. N=non-synonymous. *=stop gain. */= stop loss.

Position	Locus Tag	Gene product	Mutation	Mutation frequency																	
				Passage 10										Passage 5							
				C1	C2	C3	C4	C5	E1	E2	E3	E4	E5	C1	C2	C3	C4	C5	E1	E2	E3	E4	E5
12611	OG1RF_RS00060	DHH family phosphoesterase	N	0	0	0	0.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
27611	OG1RF_RS00125	hypothetical protein	N	0	0	0	0	0	0	0.125	0	0	0	0	0	0	0	0	0	0	0	0	0	0
100222	OG1RF_RS00440	cell surface protein	S	0	0.05	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
144619	OG1RF_RS00655	hypothetical protein	S	0	0	0	0	0	0	0	0	0	0.1	0	0	0	0	0	0	0	0	0	0	0
321843	OG1RF_RS01685	multidrug transporter	N	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.05	0	0	0
396903	OG1RF_RS02080	LacI family transcriptional regulator	N	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.275	0	0	0
495652	OG1RF_RS02520	nicotinamide mononucleotide transporter PnuC	S	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.05	0	0	0	0	0	0	0
665142	OG1RF_RS03265	bifunctional acetaldehyde-CoA/alcohol dehydrogenase	N	0	0	0	0	0	0	0.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Position	Locus Tag	Gene product	Mutation	Mutation frequency																	
				Passage 10										Passage 5							
				C1	C2	C3	C4	C5	E1	E2	E3	E4	E5	C1	C2	C3	C4	C5	E1	E2	E3	E4	E5
687035	OG1RF_RS03385	hypothetical protein	N	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.05	0
708115	OG1RF_RS03485	peptidase M23	N	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.05
716744	OG1RF_RS03535	galactose mutarotase	N	0	0	0	0	0	0	0	0	0.05	0	0	0	0	0	0	0	0	0	0	0
763202	OG1RF_RS03810	ileS	N	0.675	0	0	0	0	0	0	0	0	0	0.175	0	0	0	0	0	0	0	0	0
769006	OG1RF_RS03840	RNA helicase	N	0	0	0.425	0	0	0	0	0	0	0	0	0	0.15	0	0	0	0	0	0	0
822615				0	0	0	0.05	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
822619				0	0	0	0.05	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
822626				0	0	0	0.05	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
822630				0	0	0	0.05	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
823050				0	0.075	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Position	Locus Tag	Gene product	Mutation	Mutation frequency																	
				Passage 10										Passage 5							
				C1	C2	C3	C4	C5	E1	E2	E3	E4	E5	C1	C2	C3	C4	C5	E1	E2	E3	E4	E5
823132				0	0	0	0	0	0	0	0	0	0	0	0	0.075	0	0	0	0	0	0	0
823133				0	0	0	0	0	0	0	0	0	0	0	0	0.075	0	0	0	0	0	0	0
840921	OG1RF_RS04210	membrane protein	N	0	0.05	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
866204				0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.05	0	0	0	0	0
866205				0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.05	0	0	0	0	0
866209				0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.05	0	0	0	0	0
963095				0	0	0	0	0	0	0	0	0	0	0	0	0.05	0	0	0	0	0	0	0
963102				0	0	0	0	0	0	0	0	0	0	0	0	0.05	0	0	0	0	0	0	0
963103				0	0	0	0	0	0	0	0	0	0	0	0	0.05	0	0	0	0	0	0	0
1063726	OG1RF_RS05300	hypothetical protein	N	0	0	0	0	0	0	0	0	0	0	0	0	0.05	0	0	0	0.025	0	0	0

Position	Locus Tag	Gene product	Mutation	Mutation frequency																	
				Passage 10										Passage 5							
				C1	C2	C3	C4	C5	E1	E2	E3	E4	E5	C1	C2	C3	C4	C5	E1	E2	E3	E4	E5
1063735	OG1RF_RS05300	hypothetical protein	N	0	0	0	0	0	0	0.025	0	0	0	0	0	0.05	0	0	0	0	0	0	0
1063737	OG1RF_RS05300	hypothetical protein	N	0	0	0	0	0	0	0	0	0	0	0	0	0.05	0	0	0	0	0	0	0
1108593	OG1RF_RS05535	PadR family transcriptional regulator	N	0	0	0	0	0	0	0	0	0	0	0	0	0	0.05	0	0.025	0	0	0	0
1120269	OG1RF_RS05590	dnaK	N	0	0	0	0	0	0	0	0	0.1	0	0	0	0	0	0	0	0	0	0	0
1142731	OG1RF_RS05700	2-hydroxyglutaryl-CoA dehydratase	S	0	0	0	0	0	0	0	0.05	0	0	0	0	0	0	0	0	0	0	0	0
1190499				0	0	0	0	0	0	0.05	0	0	0	0	0	0	0	0	0	0	0	0	0
1194063	OG1RF_RS05930	dihydrolipoamide acetyltransferase	N	0	0	0	0	0.05	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1210254				0	0	0	0	0	0	0	0	0	0	0	0.05	0	0	0	0	0	0.025	0	0
1210263				0	0	0	0	0	0	0	0	0	0	0	0.05	0	0	0	0	0	0.025	0	0

Position	Locus Tag	Gene product	Mutation	Mutation frequency																	
				Passage 10										Passage 5							
				C1	C2	C3	C4	C5	E1	E2	E3	E4	E5	C1	C2	C3	C4	C5	E1	E2	E3	E4	E5
1245088	OG1RF_RS06175	colicin V production protein CvpA	N	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.1	0	0	0
1310130				0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.05	0
1317832				0	0	0	0	0	0	0.925	0	0	0.475	0	0	0	0	0	0	0.45	0	0	0
1345266	OG1RF_RS06655	Cro/Cl family transcriptional regulator	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.05	0	0	0	0	0
1383178	OG1RF_RS06830	6-phospho-beta-glucosidase	S	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.05	0	0	0	0	0
1399022				0.025	0	0	0.05	0	0	0.025	0	0.025	0	0	0	0.05	0.025	0	0.025	0	0.025	0	0.025
1400745	OG1RF_RS06895	ethanolamine utilization protein EutN	S	0	0	0	0	0	0	0	0	0	0	0.05	0	0	0	0	0	0	0	0	0
1402371	OG1RF_RS06910	hypothetical protein	N	0	0	0	0	0	0	0	0	0	0	0	0.05	0	0	0	0	0	0	0	0
1420644	OG1RF_RS07005	transcriptional repressor CodY	S	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.05	0	0	0	0	0

Position	Locus Tag	Gene product	Mutation	Mutation frequency																	
				Passage 10										Passage 5							
				C1	C2	C3	C4	C5	E1	E2	E3	E4	E5	C1	C2	C3	C4	C5	E1	E2	E3	E4	E5
1465495	OG1RF_RS07240	hypothetical protein	N	0	0	0	0	0.05	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1547103	OG1RF_RS07655	purH	*/	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.075
1573630	OG1RF_RS07775	beta-galactosidase	N	0	0	0	0	0	0	0	0	0	0.025	0	0	0	0	0	0	0	0	0	0.05
1617044	OG1RF_RS07935	1-(5-phosphoribosyl)-5-amino-4-imidazole-carboxylate carboxylase	*	0	0	0	0.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1712344	OG1RF_RS08425	hypothetical protein	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.55	0
1717072	OG1RF_RS08465	competence protein ComG	N	0	0.075	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1717074	OG1RF_RS08465	competence protein ComG	N	0	0	0	0	0	0	0	0	0	0.05	0	0	0	0	0	0	0	0	0	0
1717074	OG1RF_RS08465	competence protein ComG	N	0	0	0	0	0	0	0	0.05	0	0	0	0	0	0	0	0	0	0	0	0.05
1717074	OG1RF_RS08465	competence protein ComG	N	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.05	0	0	0	0

Position	Locus Tag	Gene product	Mutation	Mutation frequency																	
				Passage 10										Passage 5							
				C1	C2	C3	C4	C5	E1	E2	E3	E4	E5	C1	C2	C3	C4	C5	E1	E2	E3	E4	E5
1717076	OG1RF_RS08465	competence protein ComG	N	0	0	0	0	0	0	0.05	0	0	0	0	0	0	0	0	0	0	0	0	0
1750854	OG1RF_RS08605	membrane protein	N	0	0	0	0	0	0	0	0.2	0	0	0	0	0	0	0	0	0	0	0.125	0	0
1787496				0	0.25	0	0	0	0	0	0	0	0	0	0.25	0	0	0	0	0	0	0	0	0
1796656				0	0	0	0	0	0	0	0	0	0.05	0	0	0	0	0	0	0	0	0	0	0
1807271	OG1RF_RS08830	glycosyl transferase family 2	N	0	0	0	0	0	0	0	0	0	0	0	0.05	0	0	0	0	0	0	0	0	0
1807272	OG1RF_RS08830	glycosyl transferase family 2	N	0	0	0	0	0	0	0	0	0	0	0	0.05	0	0	0	0	0	0	0	0	0
1848356	OG1RF_RS13215	cell wall surface anchor family protein	N	0	0	0	0	0	0	0	0	0	0	0	0	0.05	0	0	0	0	0	0	0	0
1848743	OG1RF_RS13215	cell wall surface anchor family protein	N	0.025	0	0	0.025	0	0	0.025	0.05	0.025	0.025	0	0	0	0.025	0	0	0	0	0	0	0
1872619	OG1RF_RS09105	hypothetical protein	S	0	0	0	0	0	0	0	0.625	0	0	0	0	0	0	0	0	0	0	0	0	0

Position	Locus Tag	Gene product	Mutation	Mutation frequency																	
				Passage 10										Passage 5							
				C1	C2	C3	C4	C5	E1	E2	E3	E4	E5	C1	C2	C3	C4	C5	E1	E2	E3	E4	E5
1895280	OG1RF_RS09200	xanthine permease	*/	0	0	0	0.575	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1945378	OG1RF_RS09430	era	S	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0.65	0	0	0	0
1952046	OG1RF_RS09470	phosphotransferase	*/	0.075	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2033084				0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.05
2069087	OG1RF_RS10020	PLP-dependent lyase/thiolase	S	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.175	0
2084379	OG1RF_RS10085	multidrug ABC transporter ATP-binding protein	N	0	0	0	0	0	0	0	0	0	0	0	0.25	0	0	0	0	0	0	0	0
2152729	OG1RF_RS10415	50S rRNA methyltransferase	N	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.05	0	0
2353107	OG1RF_RS11435	valS	N	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.525	0
2458960	OG1RF_RS11930	protein-tyrosine-phosphatase	S	0	0.175	0	0	0	0	0	0	0	0	0	0.275	0	0	0	0	0	0	0	0
2501430				0	0	0	0	0	0	0	0	0.1	0	0	0	0	0	0	0	0	0	0	0

Position	Locus Tag	Gene product	Mutation	Mutation frequency																	
				Passage 10										Passage 5							
				C1	C2	C3	C4	C5	E1	E2	E3	E4	E5	C1	C2	C3	C4	C5	E1	E2	E3	E4	E5
2502191				0	0	0	0	0.025	0	0	0	0	0.05	0	0	0	0	0	0	0	0	0	0
2506615	OG1RF_RS12140	peptide ABC transporter ATP-binding protein	N	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.05	0
2519898	OG1RF_RS12200	thiamine pyrophosphokinase	S	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.2	0	0	0	0
2576425	OG1RF_RS12470	damage-inducible protein A	S	0	0	0	0	0	0	0	0	0	0	0	0	0.2	0	0	0	0	0	0	0
2595770	OG1RF_RS12560	isochorismatase	N	0	0	0	0	0	0	0.075	0	0	0	0	0	0	0	0	0	0	0	0	0
2603433				0	0	0.175	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2691574				0	0	0	0	0.05	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Chapter Three

Evolutionary interactions between a pathogen and a defensive microbe favour the maintenance of microbe-mediated host defence

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Abstract

Microbes that defend their hosts against pathogens (including parasites and parasitoids) are valuable weapons against infection for a wide variety of plant and animal hosts. It has been hypothesised that the defensive properties of such microbes can evolve as a by-product of competition with pathogens, however it is still unknown what conditions favour the net benefit of defensive microbes and so their persistence as mutualists over evolutionary time. In this study we co-passaged a microbe with host-protective traits (*Enterococcus* faecalis) and a virulent pathogen (*Staphylococcus aureus*) within a non-evolving *C. elegans* host in order to test how evolutionary interactions between pathogens and defensive microbes could be sufficient to favour the maintenance of defensive microbes. We reveal a positive correlation between the cost and the benefit of harbouring the defensive microbe in this system and show that the shape of this correlation determined the maintenance of the net benefit of the defensive microbe to the host. As a result, protection as a by-product of microbial interactions can persist over evolutionary time in the absence of host selection; however it is likely that the mechanisms of pathogen-defensive microbe-host interaction will determine how selection for protection will affect the position of the defensive microbe along the mutualism-parasitism continuum.

Introduction

Microbes that defend their hosts against infection by pathogens (including parasites and parasitoids) have been found across a large diversity of both plants and animals [2, 3]. It has been hypothesised that many defensive microbes in nature are likely to have evolved as by-products of competitive interactions with pathogens [4]. This hypothesis posits that microbes can be 'unlikely mutualists'; inadvertently providing protective benefits to their hosts when they compete for host space and resources with virulent pathogens [4]. Empirical support for this hypothesis has been recently demonstrated [1], however it is still unknown what conditions favour the net benefit of the defensive microbe to the host and so prevent transitions across the mutualism-parasitism continuum. This has important implications for how we understand the evolution of defensive microbes in nature and predicting their maintenance in applied disease control.

In this study we investigate how evolutionary interactions with pathogens affect the maintenance of defensive microbes over evolutionary time. Defensive microbes are characterised by providing a net benefit to the host, i.e. when their cost is outweighed by their protective benefit [3]. Their protective benefit will be determined not only by the increase in host fitness that they confer (e.g. the reduction in host mortality) but also by the frequency of the pathogen in the environment. Costs of defensive microbes can either be due to: the exploitation of host resources; the induction of host immunity (e.g. inflammation); the production of toxins against the pathogen that can also harm the host; or even the manipulation of the host's reproduction or behaviour [5-12]. Interestingly, the costs of defensive microbes and their benefits often correlate [8, 13, 14]. This may occur because either: 1) the cost and benefit are outcomes of the same mechanism(s); or 2) the genes conferring costly and beneficial mechanisms are controlled by the same regulatory pathway.

It follows that mutualism breakdown can occur when the cost begins to exceed the benefit, resulting in the transition across the mutualism-parasitism continuum [15]. One barrier against transition to parasitism is host selection, e.g. 'partner choice' or 'sanctions' whereby defensive microbes that lose their net benefit are actively selected against [3, 4, 15]. Alternatively, such 'cheating' genotypes can be prevented from spreading if the host's fitness is necessary for microbial transmission (i.e. vertical transmission) [16, 17]. Importantly, how defensive microbes that arose as by-products of competition persist in nature is currently unclear.

We propose here that when the cost and the benefit of a defensive microbe are correlated we can make specific predictions of the stability of defensive microbes in nature. When cost positively correlates with protection, we predict that selection for increasing protection can lead to parasitism (c>b) if the slope of the correlation is greater than 1 (fig.1a). Alternatively, if the slope is less than 1 selection for increasing protection can lead to mutualism (c<b). When the cost negatively correlates with protection, we predict that selection for increasing protection can lead to mutualism (c<b) regardless of the slope (fig.1b). Of course, these predictions become more complicated if the correlation between cost and protection is not linear. In this study, we test our predictions by experimentally coevolving a defensive microbe (*Enterococcus* faecalis) and a pathogen (*Staphylococcus aureus*) within a non-evolving host (*Caenorhabditis elegans*) (fig.2). It has been previously shown that the bacterium *E. faecalis* acts as a defensive microbe against *S. aureus* pathogens within the *C. elegans* model host by producing antimicrobial superoxides [1].

We found that coevolutionary interactions between a pathogen and a defensive microbe revealed a positive correlation between cost and protective ability in our system. Consistent with our predictions, we found that this correlation was <1 and as such, selection for protection maintained the net benefit of defensive microbes. In addition to this, we identified that there were likely multiple mechanisms underlying this correlation. Our data illustrate that

coevolutionary interactions with a pathogen can maintain the net benefit of microbe-mediated defence; supporting the hypothesis that protection as a by-product of microbial interactions can persist enough over evolutionary time to form defensive symbioses. At the same time they indicate the importance of the mechanisms of pathogen-defensive microbe-host interaction in determining the position of the defensive microbe along the mutualism-parasitism continuum.

Figure 1

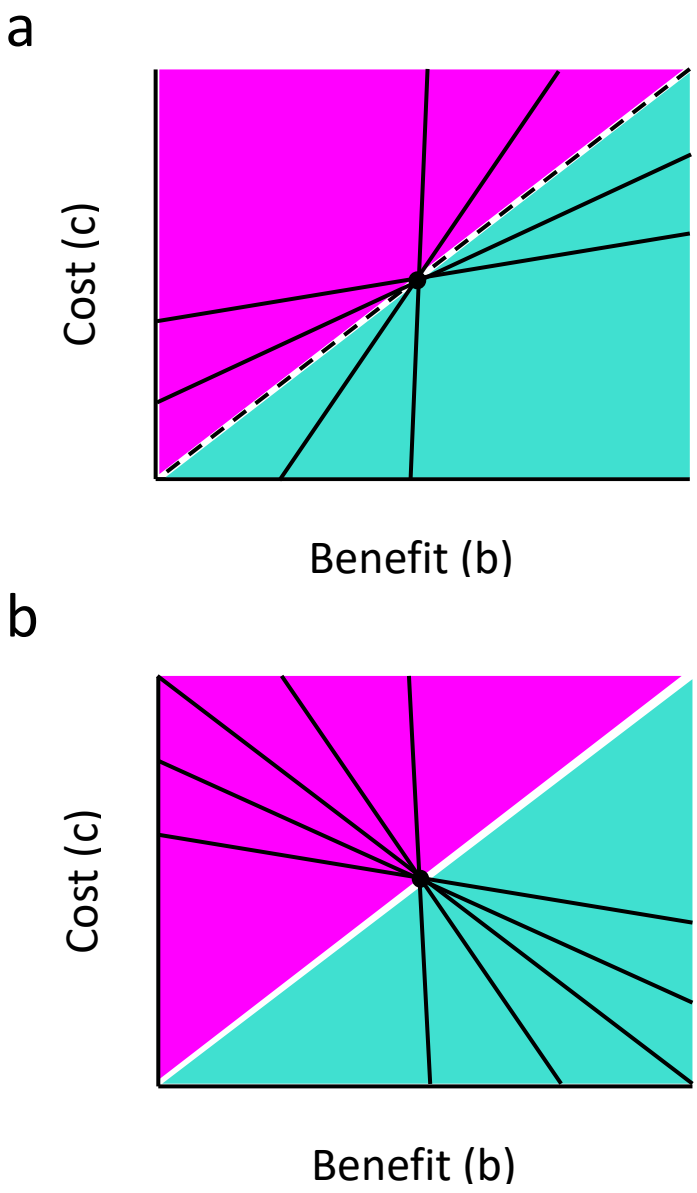


Figure 1 Transitions between mutualism and parasitism

Graphs depicting evolutionary trajectories through phenotypic space given **a,** positive correlations and **b,** negative correlations between the cost of a defensive microbe and their protective ability (benefit). The area shaded in turquoise denotes the phenotypic space where microbes confer a net benefit to the host (c<b) and so they can be considered defensive mutualists. The area shaded in magenta is where c>b and so microbes are considered parasitic. **a,** If the slope of a positive correlation is greater than 1 (slope of 1 is indicated by dashed line), then selection for increasing protection can lead to parasitism (c>b). If the slope is less than 1, then selection for increasing protection can lead to mutualism (c<b). **b,** Regardless of the slope of a negative correlation, selection for increasing protection can lead to c<b and so mutualism.

Figure 2

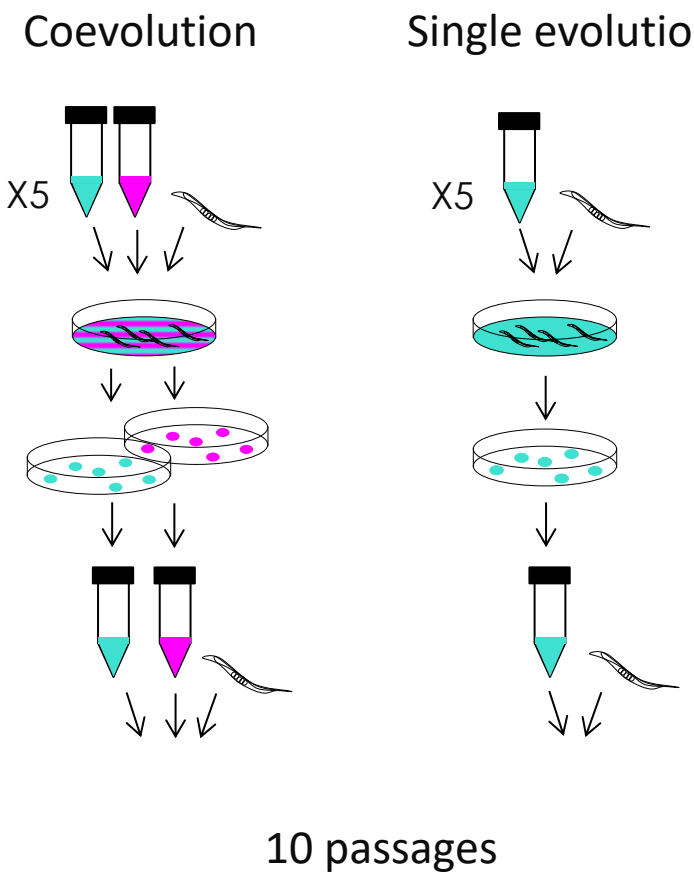


Figure 2 Experimental coevolution

S. aureus and *E. faecalis* were co-passaged (Coevolution) and *E. faecalis* was passaged alone (Single evolution) through *C. elegans* host populations. At each generation the same genetically homogeneous population of *C. elegans* hosts was exposed to *S. aureus* and/or *E. faecalis.* After 24 hours of exposure, 10 colonies of *S. aureus* and/or *E. faecalis* were collected from the pooled guts of 10 dead hosts using selective media and then passaged to the next generation. Each treatment had 5 replicate populations and was conducted for 10 passage generations.

Results

E. faecalis *protection against* S. aureus

By measuring the virulence (% hosts dead after 24 hours of exposure) of ancestral *S. aureus* infection in the absence of *E. faecalis* and the presence of ancestral and evolved *E. faecalis* we found that all *E. faecalis* populations remained protective (fig. 3a, Quasibinomial GLM: F=106.63, df=3, P=8.662e-11. *Post Hoc* Tukey comparisons: Coevolution vs. S. aureus: P<0.001; Ancestor vs. S. aureus: P<0.001; Single evolution vs. S. aureus: P<0.001, Supplementary Table 1). In addition to this, however, we also found that the *E. faecalis* populations passaged through uninfected hosts were significantly less protective than the ancestral population and the populations co-passaged with the pathogens (*Post Hoc* Tukey comparisons: Single evolution vs. Coevolution: P=0.0123; Single evolution vs. Ancestor: P<0.001). These data therefore indicate that coevolution with a pathogen selects for the maintenance of protection in defensive microbes, whilst defensive microbes evolved in their absence become less protective.

We found that experimental coevolution with a pathogen significantly affected the production of superoxides by defensive microbes (fig. 3b, Mixed effects model with biological replicate and experiment as random factors: χ^2=6.45, df=2, P=0.0398, supplementary Table 1). We show that *E. faecalis* populations that were passaged through uninfected hosts produced significantly less superoxides than the defensive microbes that were co-passaged with the pathogen (Single evolution vs. Coevolution: P=0.0279). Importantly, neither the Coevolution or Single evolution *E. faecalis* populations were significantly different from the ancestor (Coevolution vs. Ancestor: P=0.363; Single evolution vs. Ancestor: P=0.802). These data indicate that coevolution with a pathogen selects for *E. faecalis* genotypes that produce more superoxide anions than evolution in their absence.

Figure 3

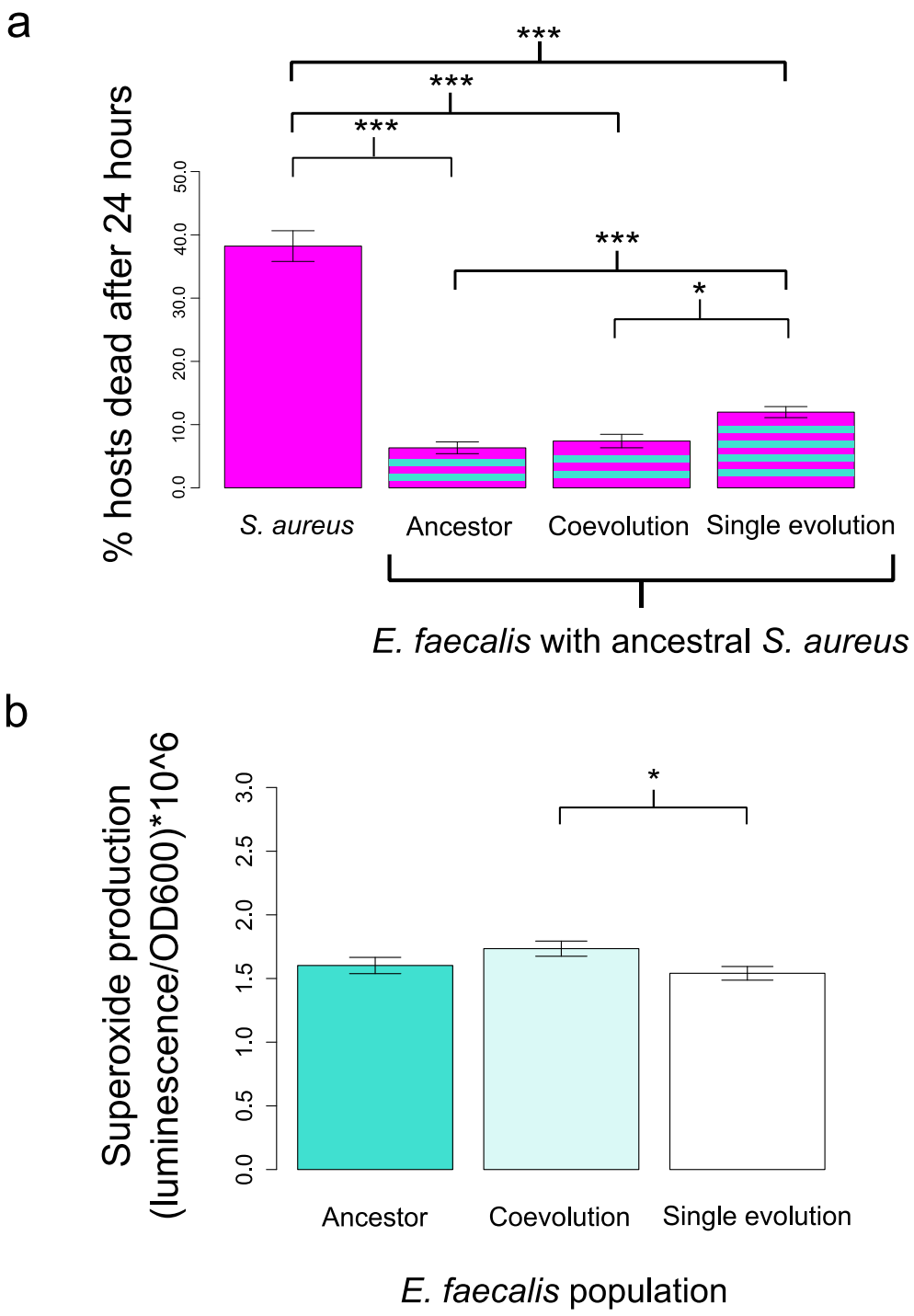

a
***
***
***
***
*
% hosts dead after 24 hours
0.0
10.0
20.0
30.0
40.0
50.0
S. aureus
Ancestor
Coevolution
Single evolution
E. faecalis with ancestral S. aureus
b
*
Superoxide production
(luminescence/OD600)*10^6
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Ancestor
Coevolution
Single evolution
E. faecalis population

Figure 3 *E. faecalis* protective ability

a, The virulence of ancestral *S. aureus* after 24 hours of exposure to the *C. elegans* host in the presence and absence of *E. faecalis* populations. **b,** The production of superoxide anions from ancestral *E. faecalis* and *E. faecalis* populations from the Coevolution and the Single evolution treatments. *P<0.05, **P<0.01, ***P<0.001. Bar plots show ±1SE.

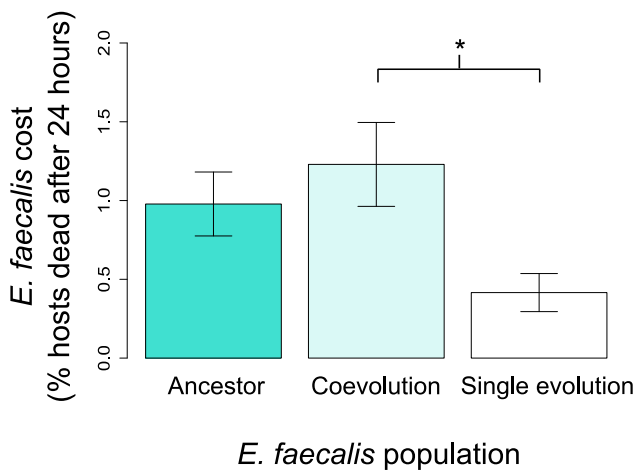
E. faecalis *cost*

We found that experimental coevolution with a pathogen significantly affected the evolution of defensive microbe cost (fig. 4a, Binomial GLM: df=2, P=0.022, supplementary Table 1). Neither the Coevolution or Single evolution *E. faecalis* populations were significantly different from the ancestor (Coevolution vs. Ancestor: P=0.71; Single evolution vs. Ancestor: P=0.17). However we found that the coevolved *E. faecalis* populations were significantly more costly than *E. faecalis* populations passaged through uninfected hosts (Single evolution vs. Coevolution: P=0.0298). Thus coevolution with a pathogen selects for more costly defensive microbes than evolution in their absence.

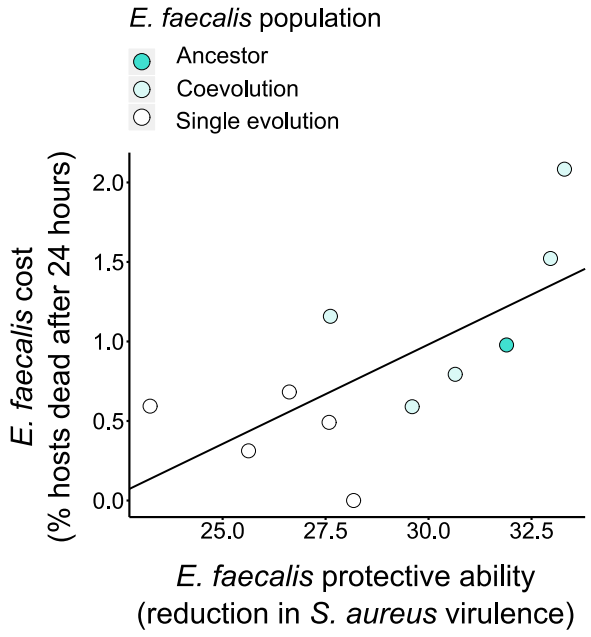
We found that the cost of harbouring *E. faecalis* correlated positively with the ability to protect the host against *S. aureus* virulence and that this correlation was less than 1 (fig.4b, Pearson's product-moment correlation. Cor = 0.68, t = 2.7842, df = 9, P = 0.02125 R^2: 0.46, supplementary Table 1). Protective ability is measured here by the reduction in *S. aureus* virulence (i.e. subtracting the average ancestral *S. aureus* virulence by the virulence under co-colonisation with each *E. faecalis* treatment in fig.3a). This measure of protective ability is the benefit (b) of the defensive microbe. In figure 4c we show where all our *E. faecalis* populations sit in the cost-benefit phenotypic space presented in figure 1. We can see from this figure that due to the slope of the correlation between the cost and benefit selection for protection maintained the net benefit of the defensive microbe. The cost of harbouring *E. faecalis* did not exhibit a significant correlation with superoxide production (supplementary fig.1, Pearson's product-moment correlation. t = 0.80655, df = 9, p-value = 0.4407, supplementary Table 1).

Figure 4

a


E. faecalis cost
(% hosts dead after 24 hours)
0.0
0.5
1.0
1.5
2.0
*
Ancestor
Coevolution
Single evolution
E. faecalis population

b


E. faecalis population
Ancestor
Coevolution
Single evolution
E. faecalis cost
(% hosts dead after 24 hours)
0.0
0.5
1.0
1.5
2.0
25.0
27.5
30.0
32.5
E. faecalis protective ability
(reduction in S. aureus virulence)

c

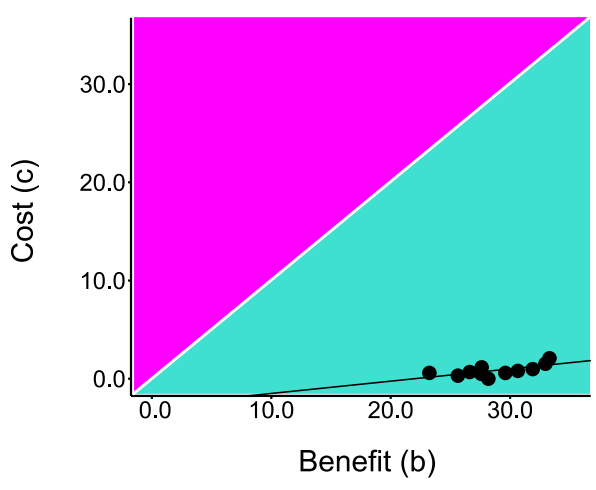

Cost (c)
0.0
10.0
20.0
30.0
0.0
10.0
20.0
30.0
Benefit (b)

Figure 4 *E. faecalis* cost

a, The cost of *E. faecalis* populations measured as host mortality (% hosts dead) after 24 hours of exposure. Given that un-colonised hosts experience no mortality under otherwise the same conditions [1], the cost of *E. faecalis* was measured as the host mortality following 24 hours of exposure to *E. faecalis* populations. **b,** The correlation between the cost of *E. faecalis* populations and their protective ability (measured as the reduction in ancestral *S. aureus* host mortality). **c,** Graph depicting the location of *E. faecalis* populations in the phenotypic space of cost and benefit. We calculated the benefit of *E. faecalis* populations by subtracting the virulence of ancestral *S. aureus* in the presence of *E. faecalis* populations from the virulence of ancestral *S. aureus* in the absence of *E. faecalis.* The area shaded in turquoise denotes the phenotypic space where microbes confer a net benefit to the host (c<b) and so they can be considered defensive mutualists. The area shaded in magenta is where c>b and so microbes are considered parasitic. *P<0.05, **P<0.01, ***P<0.001. Bar plots show ±1SE.

Discussion

It is hypothesised that many defensive microbes have evolved as a by-product of competition with pathogens [4], however it is still unknown under what conditions this is favoured and how persistent it is over evolutionary time. In this study we outlined specific predictions about the evolution of defensive microbes when their cost and benefit correlate.

We found that coevolutionary interactions between a pathogen and a defensive microbe selected for defensive microbes that were significantly more protective to their hosts and produced significantly more antimicrobial superoxide anions than those that were passaged through uninfected hosts. We also found that the coevolved defensive microbes were significantly more costly to harbour than those independently passaged. As a result, we show a significant correlation between the cost and the protective ability of the defensive microbes such that the most protective were the most costly to harbour. Consistent with our predictions, the slope of this correlation was less than 1 and so the net benefit of the defensive microbe was maintained (fig.4c). Furthermore, as coevolutionary interactions would progress over time, the maintenance of the defensive microbe is predicted to persist. Together, these data supporting the hypothesis that protection as a by-product of microbial interactions can persist over evolutionary time to form defensive symbioses.

The positive correlation we observe between cost and protection in our system is consistent with observations from natural systems. For example, the protective ability of differing *Wolbachia* strains against *Drosophila* viral infection increases with the cost of harbouring *Wolbachia* [8]. This may be because the *Wolbachia* bacteria compete directly for host cells with the viruses [14]. Importantly, our data demonstrate that the slope of this correlation could be informative in predicting the long-term stability of such protection. In contrast to this positive correlation, there exist other cases in nature where the most protective defensive

microbes are actually the least costly [13]. Here, our predictions indicate that selection for increasing protection will maintain the net benefit of the defensive microbe regardless of the slope of the negative correlation.

In addition to these findings, we identified that there were likely multiple mechanisms underlying the correlation between cost and benefit of the defensive microbe in our system. Superoxide is known as a virulence factor and so is a likely candidate to link the cost and protective ability of the defensive microbe [18, 19], however we found no significant correlation between superoxide production and the cost of the defensive microbe. This is consistent with previous findings [1]. As a result, it is likely that the cost and protective ability of *E. faecalis* within *C. elegans* is determined by multiple factors. One possible mechanism is the competition over host resources, as is found in other systems [4]. In addition to superoxide production, resource competition between *E. faecalis* and *S. aureus* could link both the harm *E. faecalis* can cause to the host and the ability to compete and so reduce the fitness of the pathogen. Consistent with this idea, we have previously shown the ability of *E. faecalis* to utilise *S. aureus* siderophores to acquire iron from the host environment [20].

Finally, our results have important implications for the application of defensive microbes as disease control agents. Given that many defensive microbes are being researched for this purpose [21-24], it is important that we understand how they're protective traits are maintained over evolutionary time, particularly when these defensive microbes are placed in novel host organisms [25]. It has been previously discussed that selecting for less costly defensive microbes for disease control purposes may result in less effective protection [11]. Our findings demonstrate how understanding the shape of the relationship between cost and benefit can be used to more accurately assess the likelihood of this outcome.

In this project we shed light on the evolutionary trajectories favouring defensive microbes in nature and propose how linking mechanism with evolutionary theory could be a powerful tool in predicting transitions between mutualism and parasitism.

Materials and Methods

Nematode host and bacteria:

Caenorhabditis elegans is a nematode that ingests microbes for nutrients [26-29] and is a well-established model for studying microbial colonisation and pathogenesis [30-34]. We used the simultaneous hermaphroditic N2 wild-type *C. elegans* strain from the Caenorhabditis Genetics Centre (University of Minnesota, Minneapolis, MN). A genetically homogenous line was generated by selfing a single hermaphrodite for 5 generations. Populations of these nematodes were frozen in 50% M9 solution and 50% liquid freezing solution in cryotubes at -80°C [31]. Populations were regularly resurrected throughout experimentation to prevent the accumulation of *de novo* mutations in host populations. Worms were maintained at 20°C on nematode growth medium (NGM) plates seeded with *Escherichia coli* OP50. *E. coli* OP50 is grown at 30°C shaking at 200rpm overnight in LB and 100µl of this is spread onto NGM plates and incubated overnight at 30°C[31]. To ensure clean stocks and to synchronise the life stages of populations for experimentation, worms were treated with bleach (NaClO) and sodium hydroxide (NaOH) solution which kills everything except unhatched worm eggs [31].

We used *Staphylococcus aureus* strain MSSA 476 (GenBank: BX571857.1), an invasive community-acquired methicillin-susceptible isolate and *Enterococcus faecalis* strain OG1RF (GenBank: CP002621.1), both isolated from humans. A single ancestral population of each species was grown from a single colony overnight in 6ml Todd Hewitt Broth (THB) shaking at 200rpm at 30°C. Bacteria were frozen in a 1:1 ratio of sample to 50% glycerol solution in cryotubes at -80°C.

Experimental coevolution:

The coevolution experiment consisted of two treatments: (i) *S. aureus* and *E. faecalis* were co-passaged (Coevolution), and (ii) *E. faecalis* was passaged indepedently (Single evolution) within non-evolving *C. elegans* host populations (fig.2a). Each treatment had five replicate populations and was conducted for 10 passages, over which time sufficient evolution has been previously demonstrated to occur [1]. To make exposure lawns, bacteria were cultured overnight in 6ml THB shaking at 200rpm at 30°C. After standardising the cultures to an OD600 reading of 1.00, the bacteria were spread onto Tryptic Soy Broth (TSB) plates. For the Single evolution treatment, we spread a volume of 120µl of *E. faecalis*. For the Coevolution treatment, we spread a mixture of 120µl of *S. aureus* and 120µl of *E. faecalis* on the same exposure plate. This was done to keep the population size of *E. faecalis* identical across treatments. Exposure plates were kept overnight at 30°C.

Approximately 1000 young adult nematodes from the stock *C. elegans* population were placed onto each exposure lawn and incubated at 25°C. For the Coevolution treatment, worms were placed on the mixed exposure plates to allow simultaneous co-colonisation. This was done to avoid giving advantage to one species during coevolution. This experimental design therefore differs to the one-sided adaptation experiment presented in [1] which used a resident-invader protocol. After 24 hours of incubation, 10 dead nematodes from each lawn were picked to ensure that we took hosts colonised with bacteria. Under co-colonisation, the proportion of pathogen/defensive microbe collected from the infection did not differ significantly between dead and live worms [35]. Nematodes were considered dead when they did not respond to touch with a platinum wire. Nematodes were washed to remove external bacteria by transferring them between five 5µl drops of M9 buffer on fresh TSB plates using a platinum wire. Nematodes were then crushed in 20µl of M9 in a 1ml Eppendorf tube with a pestle to release internal bacteria. An inoculation loop was used to streak each sample onto selective

media (TSB plates with 100µg/mL rifampicin were used to isolate *E. faecalis* and Mannitol Salt Agar (MSA) plates isolated *S. aureus*), and the plate was grown overnight at 30°C. Ten colonies of *S. aureus* and/or *E. faecalis* were picked from each replicate and grown in THB overnight, shaking at 200rpm at 30°C, and were then used to make the exposure plates for the next passage.

Defensive microbe protective ability:

The protective ability of each *E. faecalis* population was measured by recording the host mortality rate after 24 hours of exposure to *S. aureus* in the presence and absence of *E. faecalis* populations. Exposure plates were made in the same way as for the evolution experiment (see above). Approximately 150 young adult worms from the stock *C. elegans* population were placed onto exposure plates and incubated at 25°C for 24 hours. Exposure plates were labelled with a random code so that the treatments were unknown during measurement. The total numbers of worms were counted and the proportion dead calculated as a measure of virulence. To correlate these data against *E. faecalis* cost we calculated the benefit of *E. faecalis* populations by subtracting the virulence of ancestral *S. aureus* in the presence of *E. faecalis* populations from the virulence of ancestral *S. aureus* in the absence of *E. faecalis.*

Superoxide production:

Superoxide is a reactive oxygen species usually produced by eukaryotic hosts in their immune response against pathogens [36]. *E. faecalis* is uniquely able to produce superoxide anions and this has been found to have antimicrobial activity against *S. aureus* [1, 18, 37]. The superoxide anion assay kit (Sigma-Aldrich) was used to measure the production of superoxide anions by *E. faecalis* cells, following published protocols [1]. Ancestral *E. faecalis* and each population of *E. faecalis* from both the evolution treatments were cultured overnight in 6ml THB shaking

200rpm at 30°C. They were then standardised to an OD600 reading of 1.00. A volume of 5ul of either ancestral, Coevolution or Single evolution *E. faecalis* populations were added to 95µl TSB and 100µl reaction mixture (containing assay buffer, luminol solution and enhancer solution) in an opaque, black 96-well plate with a transparent bottom.

Luminol luminesces following oxidation by superoxide, allowing the quantitative and relativistic measure of superoxide production. The inoculated reaction mixtures were monitored over 10 h and measured every 2.5 min for both bacterial density (OD600) and luminescence in a Synergy 2 plate reader (BioTek, Winooski, VT, USA). The actual luminescence produced by a sample was divided by bacterial density.

Defensive microbe cost:

Given that un-colonised hosts experience no mortality under otherwise the same conditions [1], the cost of *E. faecalis* was measured as the host mortality following 24 hours of exposure to *E. faecalis* populations. Exposure plates were made in the same way as for the evolution experiment (see above). Approximately 150 young adult worms from the stock *C. elegans* population were placed onto exposure plates and incubated at 25°C for 24 hours. Exposure plates were labelled with a random code so that the treatments were unknown during measurement. The total numbers of worms were counted and the proportion dead calculated as a measure of virulence.

Statistical analysis:

Data analysis was carried out in R v 3.2.0 (http://www.r-project.org/). Parametric tests were used for all data which met the required assumptions. The Shapiro test was used to detect whether data was normally distributed and F-tests to compare the variances of two samples from normal populations. Outlying data points were detected and removed using the Dixon test. A binomial GLM was used to compare the virulence of *E. faecalis* treatments, followed by

Tukey contrasts for *post-hoc* comparisons. Plots of the GLM were checked by eye for model quality. A Quasibinomial GLM was used to compare the virulence of *S. aureus* in the presence and absence of protection by *E. faecalis* populations. A Quasibinomial GLM was used to account for an over-dispersed binomial model. Plots of the GLM were checked by eye for model quality. Tukey contrasts were used for *post-hoc* comparisons. A mixed effects model with biological replicate and experiment as a random factor was used to compare superoxide production between *E. faecalis* populations. Spearman rank correlation was used to correlate the cost of *E. faecalis* populations against their ability to protect the host from *S. aureus* and to produce superoxide.

Data availability:

The data will be deposited in DRYAD repository.

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Author contributions

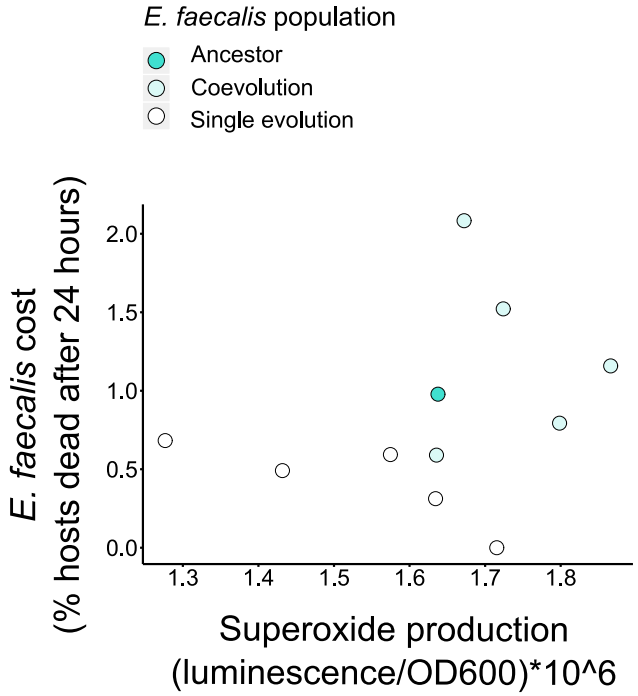
S.A.F. and K.C.K. conceived and designed the project; S.A.F. conducted the experiments and analysed the data; S.A.F. and K.C.K. wrote the paper.

Competing financial interests

The authors declare no competing financial interests.

Supplementary information

Supplementary figure 1



Supplementary figure 1 *E. faecalis* cost and superoxide production

The correlation between the cost of *E. faecalis* populations and their superoxide production.

Supplementary Table 1. Statistical results listed by figure

Figure	Statistical results
3a	**Quasibinomial GLM:** F=106.63 , df=3, P=8.662e-11 Tukey contrasts: Coevolution vs. *S. aureus:* P<0.001 Ancestor vs. *S. aureus:* P<0.001 Single evolution vs. *S. aureus:* P<0.001 Ancestor vs. Coevolution: P=0.9286 Single evolution vs. Coevolution: P=0.0123 Single evolution vs. Ancestor: P<0.001
3b	**Mixed effects model with biological replicate and experiment as random factors:** χ^2=6.45, df=2, P=0.0398 Tukey contrasts: Coevolution vs. Ancestor*:* P=0.363 Single evolution vs. Ancestor*:* P=0.802 Single evolution vs. Coevolution: P=0.0279
4a	**Binomial GLM:** df=2, P= 0.022 Tukey contrasts: Coevolution vs. Ancestor: P=0.71 Single evolution vs. Ancestor: P=0.17 Single evolution vs. Coevolution: P=0.0298
4b	**Pearson's product-moment correlation:** t = 2.7842, df = 9, P = 0.02125 Correlation = 0.68 95 percent confidence interval: 0.1357927 - 0.9091394 R^2= 0.4627
Supp. Fig. 1	**Pearson's product-moment correlation:** t = 0.80655, df = 9, p-value = 0.4407 Correlation = 0.26 95 percent confidence interval: -0.4030130 - 0.7436798

Thesis discussion

Microbes…they giveth and they taketh away.

Many microorganisms are responsible for devastating infectious disease; however we are beginning to discover the diversity of 'defensive' microorganisms that can cure and even prevent these same diseases [1]. The more we look, the more we find [2]. As a result, such defensive microbes are clearly a valuable weapon for their hosts. Research focussing on defensive microbes has grown dramatically over the last decade [2-6], but there is still a lot we do not know about how they interact with pathogens over evolutionary time [1]. This has important implications for how we understand the evolution of pathogens and mutualists in nature and could determine how we approach disease control. In this thesis, I aimed to answer three primary questions surrounding the evolutionary implications of defensive microbes:

Chapter one: *Can defensive microbes drive the evolution of pathogen virulence?*

Pathogens have been shown to adapt rapidly to barriers against infection, and their evolution is often accompanied by changes in pathogen virulence [7-9]. In this chapter, I tested whether defensive microbes can shape the evolution of disease severity. I found that co-passaging a defensive microbe (*E. faecalis*) and a pathogen (*S. aureus*) within *C. elegans* host populations drove the evolution of reduced pathogen virulence as a by-product of resisting the defensive microbe. Genomic and phenotypic analyses showed that the production of fewer iron-scavenging siderophores by the pathogen reduced the fitness of the defensive microbe and underpinned the decline in pathogen virulence. These findings firstly illustrate the ability of defensive microbes to affect the course of evolution taken by the pathogen and impact disease severity. Secondly, I show that the mechanism of pathogen resistance can determine the direction of virulence evolution.

Chapter two: *Do defensive microbes and pathogens have the potential to undergo coevolution?*

Theory posits that defensive microbes and pathogens have the potential to coevolve via fluctuating selection dynamics [5]. Consistent with this prediction, many defensive microbes exhibit genotype-specific interactions with pathogens that explain the variation in host susceptibility to infection [10-15]. These observations indicate a dynamic coevolutionary association between pathogens and defensive microbes, however direct evidence of such coevolution is lacking. In this chapter, I tracked the evolutionary dynamics of co-passaged pathogens and defensive microbes and found evidence of reciprocal adaptation via fluctuating selection. Moreover, I revealed that coevolution with defensive microbes drove patterns of pathogen local adaptation and more rapid and divergent pathogen evolution compared to those passaged through unprotected hosts. Taken together, these results indicate the potential for defensive microbes and pathogens to coevolve, driving interaction specificity and pathogen evolutionary divergence in the absence of host evolution. As such, defensive microbes can be an evolutionarily dynamic arm of the host's response to pathogens in nature.

Chapter three: *Can defensive microbe-pathogen coevolution maintain selection for microbe-mediated defence?*

It has recently been hypothesised that defensive microbes can originate as a by-product of competition with pathogens [3]. Here, defensive microbes arise when the protective benefit outweighs any cost they themselves impose on the host. In this chapter, I investigate what conditions may favour such evolution and how stable the defence may be over time. Firstly, I show that coevolution with a pathogen resulted in the maintenance of the defensive microbe. Secondly, I show that coevolution between a defensive microbe and a pathogen revealed a positive correlation between the cost and the benefit of harbouring the defensive microbe in this system and illustrate that the shape of this correlation likely determined the maintenance

of the net benefit of the defensive microbe. Our results demonstrate that within-host microbial interactions can favour defensive microbes in the absence of direct selection for host protection; however it is likely that the relationship between the cost and the benefit of a defensive microbe can determine when this will be the case.

Thesis conclusions

Defensive microbes shape pathogen evolution

Until recently [1, 5, 6], the role of defensive microbes in shaping the evolution of pathogens has been largely overlooked. As such, it has been an implied assumption in the host-parasite literature that the host is the strongest biotic source of selection acting upon parasites, parasitoids and pathogens. From the findings presented in this thesis we can see that defensive microbes can be a source of strong selection that can significantly affect the evolutionary rate and trajectory of pathogens (chapter one and chapter two). As has been predicted [5], such selective pressure even led to a coevolutionary arms race between the two antagonistic species (chapter two), resulting in interaction dynamics similar to those shown to occur between hosts and their pathogens [16-19]. Our results therefore have interesting implications. For example, where patterns of pathogen adaptation observed in nature may have once been assumed to result from interactions with the host, we now realise that they could indeed be a result from interactions with the defensive microbe. This may include patterns of pathogen local adaptation and interaction specificity [10-15].

Defensive microbes can be an evolutionarily dynamic but stable form of host resistance

This thesis shows that defensive microbes can be an evolutionarily dynamic arm of the host's immune system and so potentially strong candidates for persistent disease control. We have shown that defensive microbes are able to undergo reciprocal adaptation with pathogens within non-evolving hosts (chapter two) and shown that such coevolution did not result in the

transition across the mutualism-parasitism continuum (chapter three). These results have important implications for their use as disease control agents. When faced with the imminent crisis of antibiotic resistance, the pursuit of disease control strategies that can either prevent or circumvent the evolution of resistant pathogen genotypes is of great importance [20, 21]. The ability of defensive microbes to undergo coevolutionary interactions with pathogens suggests the possibility of disease control that can persist in the face of pathogen evolution [22]. That coevolution did not result in the transition of the defensive microbe to become pathogenic itself makes their application in disease control even more attractive.

The importance of mechanism

We have found from the results in this thesis that making predictions on the evolution of both pathogens and defensive microbes may be determined by the mechanisms of their interaction with each other and with the host. Firstly, we showed that the mechanism of pathogen resistance to microbe-mediated defence determines whether defensive microbes could select for pathogens that harm their host more or less (chapter one). Secondly, we demonstrate that if the protective ability of the defensive microbe correlates with the cost they impose on the host then we can predict how selection for increased protection could affect the persistence of the defensive microbe over evolutionary time (chapter three). This predictive value of understanding the mechanism of such microbial interactions is of utmost importance in the optimal design of disease control utilising defensive microbes, e.g. by avoiding the use of defensive microbes that could select for more virulent pathogens or could easily transition to being pathogens themselves.

Future directions

As we start to understand the evolutionary ecology of defensive microbes in nature, we begin to unravel their role in determining patterns of host and pathogen adaptation. A number of questions follow from the conclusions of this thesis.

Firstly: what is the effect of a more complex microbiota on the interactions between defensive microbes and pathogens? This includes assessing whether a more complex environment will inhibit defensive microbe-pathogen coevolution and dilute selection upon pathogen virulence and microbe-mediated defence. Secondly: how does microbe-mediated host protection affect the evolution of the host? In the general introduction of this thesis we state that the defensive microbe could reduce selection pressure on the host to adapt to the pathogen and so result in the evolution of hosts that are dependent on their microbial symbiont [1]. Recent studies have begun to find evidence to support this prediction [23]. Thirdly: is it possible to have tripartite coevolution between hosts, pathogens and defensive microbes? Here, it will be important to determine if one pairwise interaction dominates the network and so negates coevolution between the other partners or if coevolution between all three partners is possible.

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PUBLICATIONS

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Ford SA, Kao D, Williams D, King KC (2016) Microbe-mediated host defence drives the evolution of reduced pathogen virulence. *Nature Communications* 7, 13430. doi:10.1038/ncomms13430

Ford SA, King KC (2016) Harnessing the Power of Defensive Microbes: Evolutionary Implications in Nature and Disease Control. *PLoS Pathogens* 12(4): e1005465. doi:10.1371/journal.ppat.1005465

King KC, Brockhurst MA, Vasieva O, Paterson S, Betts A, **Ford SA**, Frost CL, Horsburgh MJ, Haldenby S & Hurst GD (2016) Rapid evolution of microbe-mediated protection against pathogens in a worm host. *The ISME Journal* 10, 1915–1924. doi: 10.1038/ismej.2015.259

Maiden, Jansen van Rensburg, Bray, Earle, **Ford SA**, Jolley, & McCarthy (2013). MLST revisited: the gene-by-gene approach to bacterial genomics. *Nature Reviews. Microbiology*, 11(10), 728–736. doi: 10.1038/nrmicro3093

COMPETITIVE AWARDS

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(2015) Special grant from St. John's College, Oxford to attend ESEB conference in Switzerland
(2014) **Special grant** from St. John's College, Oxford to attend an evolution workshop in Guarda
(2013) EPSRC RCUK DPhil studentship from Department of Zoology & St. John's College, Oxford
(2012) Special grant from St. John's College, Oxford to work on bacterial genomics
(2011-2013) Casberd Scholarship, University of Oxford, for academic excellence

(2011) Special grant from St. John's College, Oxford to work on bacterial genomics

RESEARCH EXPERIENCE

DPhil with Kayla King & Stuart West, University of Oxford 2013-2016

Carried out a coevolution experiment between *Staphylococcus aureus,* which acts as a pathogen, and *Enterococcus faecalis,* which acts as a defensive microbe, within a non-evolving *Caenorhabditis elegans* host.
Optimised DNA extraction of *S aureus* and *E faecalis* for whole genome sequencing.
Performed a comparative genomics analysis using the Bacterial and Archaeal Genome Analyser (BAGA) on ancestral and evolved populations (clonal and population analysis).

BA Dissertation with Martin Maiden, University of Oxford Summer 2013

Bioinformatics study investigating the distribution of both virulence and antibiotic resistance genes across lineages of *Staphylococcus aureus* (>800 full genome sequences).

Research Project with Martin Maiden, University of Oxford Summer 2011

Bioinformatics study investigating the movement of capsule loci present in *Neisseria meningitidis* across multiple bacterial species.

TEACHING EXPERIENCE

(2016) Training and mentoring new DPhil students in the lab
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Hannah Lepper (2nd year undergraduate dissertation project. Graduated in 2015)
Jack Common (1st year undergraduate project. Will graduate in 2016)
Victoria Pike (2nd year undergraduate dissertation project. Will graduate in 2017)
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(2016) Demonstrating and grading: bioinformatics course for Doctoral Training Centre (DTC) students
(2016) Demonstrating and grading: organismal dissections course for undergraduates, University of Oxford
(2014 & 2016) Demonstrating and grading: experimental biosciences course with *C elegans* and bacteria for Doctoral Training Centre (DTC) students
(2014 & 2016) Demonstrating and grading: evolutionary theories course for Doctoral Training Centre (DTC) students
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(2013-2015) Tutoring on various topics within Evolution to undergraduate Biological Sciences students (2/3 students per tutorial)
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(2014) Demonstrating and grading: cellular and molecular biology techniques course to undergraduates, University of Oxford

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(2015) ESEB, Switzerland – "Experimental coevolution of a parasite and a protective microbe within a host" (oral presentation)
(2014) Department of Zoology, Oxford – "Red Queen Communities: antagonistic coevolution in multi-species communities" (oral presentation)
(2014) BSP, Cambridge – "Red Queen Communities: antagonistic coevolution in multi-species communities" (poster presentation)
(2014) Evolutionary Biology workshop in Guarda, Switzerland - Competitive international summer school ran by Basel University and the ETH Zurich

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Mathematical modelling course. Week-long course ran by University of Stirling (2013).
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PROFESSIONAL CONTRIBUTIONS

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Proceedings B

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SOCIETY MEMBERSHIP

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British Society for Parasitology (BSP) (2014-2015)

Appendix

MOLECULAR ECOLOGY

Molecular Ecology (2016) doi: 10.1111/mec.13906

SPECIAL ISSUE: MICROBIAL LOCAL ADAPTATION

Co-evolutionary dynamics between a defensive microbe and a pathogen driven by fluctuating selection

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Abstract

Microbes that protect their hosts from pathogenic infection are widespread components of the microbiota of both plants and animals. It has been found that interactions between 'defensive' microbes and pathogens can be genotype-specific and even underlie the variation in host resistance to pathogenic infection. These observations suggest a dynamic co-evolutionary association between pathogens and defensive microbes, but direct evidence of co-evolution is lacking. We tested the hypothesis that defensive microbes and pathogens could co-evolve within host populations by co-passaging a microbe with host-defensive properties (*Enterococcus faecalis*) and a pathogen (*Staphylococcus aureus)* within *Caenorhabditis elegans* nematodes. Using both phenotypic and genomic analyses across evolutionary time, we found patterns of pathogen local adaptation and defensive microbe–pathogen co-evolution via fluctuating selection dynamics. Moreover, co-evolution with defensive microbes resulted in more rapid and divergent pathogen evolution compared to pathogens evolved independently in host populations. Taken together, our results indicate the potential for defensive microbes and pathogens to co-evolve, driving interaction specificity and pathogen evolutionary divergence in the absence of host evolution.

Keywords: co-evolution, defensive mutualism, experimental evolution, fluctuating selection dynamics, pathogen

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Introduction

Many microbes can determine host health by defending against pathogenic infection (Ford & King 2016). Such 'defensive' microbes are a valuable addition to the host immune system (Gerardo & Parker 2014) and are found in a diversity of plants and animals, including humans (Ford & King 2016). It is theoretically predicted that defensive microbes and pathogens could undergo co-evolutionary interactions within a host via fluctuating selection dynamics (Kwiatkowski *et al*. 2012). Consistent with this prediction, interactions between defensive microbes and pathogens in nature (including parasites and parasitoids) can show a high degree of specificity and determine the variation in host resistance to pathogenic infection (Oliver *et al*. 2005; Poulsen *et al*. 2010; Koch & Schmid-Hempel 2012; Rouchet & Vorburger 2012; Cariveau *et al*. 2014; Cayetano & Vorburger 2015). These observations suggest the potential for a dynamic co-evolutionary association between defensive microbes and pathogens within host populations. However, direct tests of defensive microbe–pathogen co-evolution are currently lacking (Ford & King 2016).

Co-evolutionary pattern and process can vary across interacting species (Gaba & Ebert 2009; Brockhurst & Koskella 2013), with co-evolution occurring via arms race dynamics or fluctuating selection dynamics. Arms race dynamics can occur when interacting species accumulate generally beneficial traits that can become increasingly exaggerated (Agrawal & Lively 2002). Alternatively, fluctuating selection dynamics occur when traits are advantageous only when rare in the population, resulting in cycles of genotypes through

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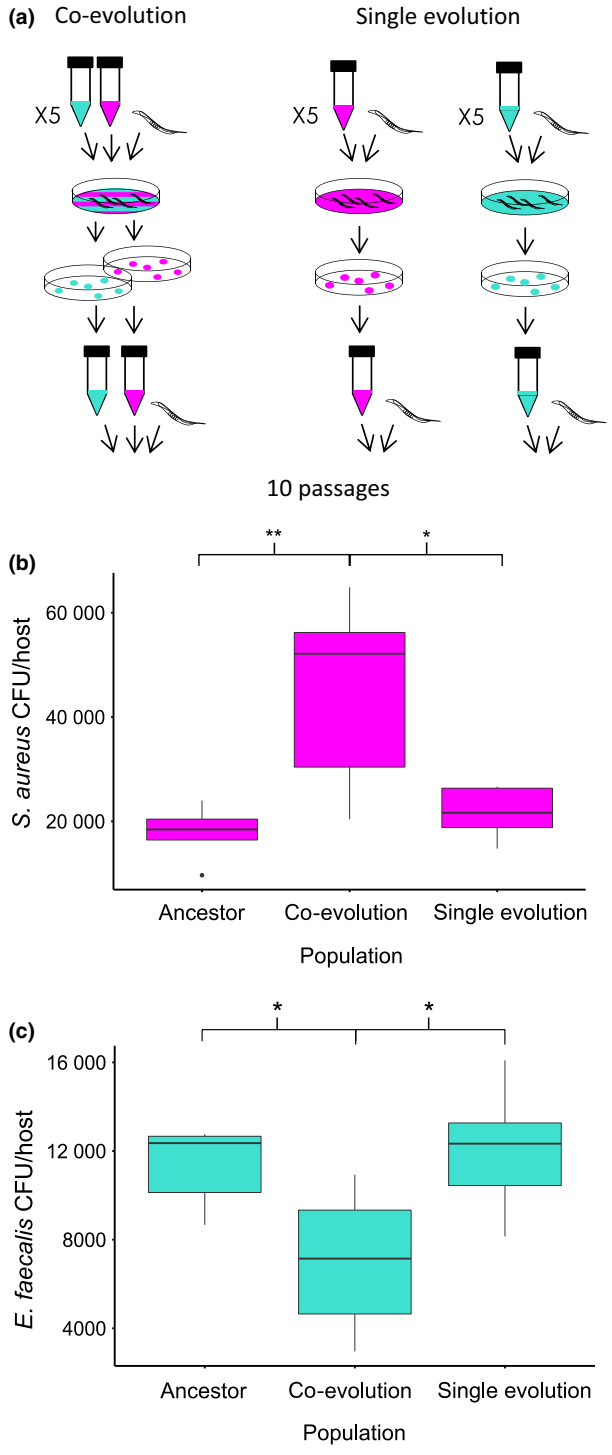


Fig. 1 Experimental co-evolution. (a) Design of evolution experiment: *Staphylococcus aureus (magenta)* and *Enterococcus faecalis* (turquoise) were co-passaged (co-evolution) or passaged independently (single evolution). At each passage, the same genetically homogeneous population of *Caenorhabditis elegans* hosts was exposed to *S. aureus* and/or *E. faecalis*. After 24 h of exposure, 10 colonies of *S. aureus* and/or *E. faecalis* were collected from the pooled guts of 10 hosts using selective media and passaged to the next generation. Each treatment had five replicate populations and was conducted for 10 passages. (b) Fitness of *S. aureus* (CFU/host) under co-colonization with *E. faecalis*. Populations of *E. faecalis* and *S. aureus* were co-colonized from the same replicate populations in each treatment. (c) Fitness of *E. faecalis* (CFU/host) under co-colonization with *S. aureus*. Populations of *E. faecalis* and *S. aureus* were co-colonized from the same replicate populations in each treatment.

time (Agrawal & Lively 2002). Importantly, this rare advantage only results when there is a high degree of specificity in species interactions. As such, the observed specificity in defensive microbe–pathogen interactions in nature (Ford & King 2016) indicates the possibility of co-evolution via fluctuating selection dynamics, consistent with theory (Kwiatkowski *et al.* 2012).

In this study, we experimentally co-evolved a microbe with host-protective properties (*Enterococcus faecalis*) and a pathogen (*Staphylococcus aureus*) within nonevolving *Caenorhabditis elegans* host populations (co-evolution treatment, Fig. 1a). We also passaged the defensive microbe and pathogen independently within *C. elegans* populations (single evolution treatment, Fig. 1a). Each treatment consisted of five replicate populations originating from the same clone of each bacterial species such that any evolutionary change was de novo. We conducted the evolution experiment for 10 passages, by which time considerable evolutionary change is known to occur in this system (King *et al.* 2016). *Caenorhabditis elegans* is used as a model for investigating microbial pathogenesis and host–microbiota interactions (Gravato-Nobre & Hodgkin 2005; Cabreiro & Gems 2013; Hodgkin *et al.* 2013; Clark & Hodgkin 2014; Gray & Cutter 2014). Both *E. faecalis* and *S. aureus* are found in the animal microbiota and can colonize the gut of *C. elegans* (Garsin *et al.* 2001). In this experiment, we used bacteria isolated from humans. As a result, their interaction within a nematode host is novel such that we can explore the evolutionary dynamics of defensive symbioses early in their formation. We have previously shown that within-nematode interactions between *E. faecalis* and *S. aureus* mimic those of a defensive microbe and pathogen, respectively (King *et al.* 2016). *Enterococcus faecalis* can suppress *S. aureus* fitness and virulence by producing antimicrobial superoxides (King *et al.* 2016). At the same time, *E. faecalis* also exploits *S. aureus* by thieving the iron-scavenging siderophores it produces (Ford *et al.* 2016). Importantly, both superoxide production by *E. faecalis* and siderophore production by *S. aureus* have been shown to evolve under co-colonization, indicating the possibility of co-evolutionary interactions (King *et al.* 2016; Ford *et al.* 2016).

By experimentally co-passaging *E. faecalis* and *S. aureus* within *C. elegans* hosts, we found direct evidence of

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defensive microbe–pathogen co-evolution. We revealed that the defensive microbe and the pathogen underwent reciprocal adaptation via fluctuating selection dynamics and produced patterns of pathogen local adaptation and genetic diversification. Our results support theoretical predictions and illustrate the potential for defensive microbes to drive patterns of pathogen adaptation in the absence of host evolution.

Materials and methods

Nematode host and bacteria

Caenorhabditis elegans nematodes constantly interact with microbes in their natural habitats (Félix & Braendle 2010), wherein they can act as predators or hosts for numerous microbial species (Clark & Hodgkin 2014; Petersen *et al.* 2015). These animals are thus an established model for microbial colonization and pathogenesis (Gravato-Nobre & Hodgkin 2005; Cabreiro & Gems 2013), and their gut can be co-colonized by multiple pathogens and commensals (Berg *et al.* 2016; Dirksen *et al.* 2016; Samuel *et al.* 2016).

We used the simultaneous hermaphroditic N2 wild-type *C. elegans* strain from the *Caenorhabditis* Genetics Centre (University of Minnesota, Minneapolis, MN). A genetically homogeneous line was generated by selfing a single hermaphrodite for five generations. Populations of these nematodes were frozen in 50% M9 solution and 50% liquid-freezing solution in cryotubes at −80 °C (Hope 1999). Populations were regularly resurrected to prevent the accumulation of de novo mutations in hosts. Nematodes were maintained at 20 °C on nematode growth medium (NGM) plates seeded with *Escherichia coli* OP50. Seeded NGM was made by growing *E. coli* OP50 at 30 °C shaking at 200 rpm overnight in Luria-Bertani (LB) broth and then incubating 100 μl of this culture on NGM plates at 30 °C overnight (Hope 1999). To ensure clean stocks for experimentation and to synchronize the age of individuals in the population, nematodes were regularly treated with a bleach (NaClO) and sodium hydroxide (NaOH) solution that kills both microbes and worms but not worm eggs (Hope 1999).

We used *Staphylococcus aureus* strain MSSA 476 (GenBank: BX571857.1), an invasive community-acquired methicillin-susceptible isolate, and *Enterococcus faecalis* strain OG1RF (GenBank: CP002621.1), an isolate from a human digestive tract. A single ancestral population of each species was grown from a single colony overnight in Todd-Hewitt Broth (THB) shaking at 200 rpm at 30 °C. Bacteria were frozen in a 1:1 ratio of sample to 50% glycerol solution in cryotubes at −80 °C.

Experimental co-evolution

The experiment consisted of two treatments: (i) *S. aureus* and *E. faecalis* were co-passaged under co-colonization within host populations (co-evolution) and (ii) *S. aureus* and *E. faecalis* were passaged separately in host populations (single evolution) (Fig. 1a). Each treatment consisted of five replicate populations and 10 passages. To make exposure lawns, bacteria were cultured overnight in THB shaking at 200 rpm at 30 °C. After standardizing the cultures to an OD600 reading of 1.00, the bacteria were spread onto Tryptic Soy Broth (TSB) plates. We spread a volume of 120 μl of either *S. aureus* or *E. faecalis* for the single evolution treatment, and we spread a mixture of 120 μl of *S. aureus* and 120 μl of *E. faecalis* on the same exposure plate for the co-evolution treatment. Exposure plates were kept overnight at 30 °C.

Approximately 1000 young adult nematodes from the stock *C. elegans* population were placed onto each exposure lawn and incubated at 25 °C. After 24 h of incubation, bacteria from 10 dead nematodes from each lawn were picked to ensure that we took hosts successfully colonized with bacteria. Under co-colonization, the proportion of pathogen collected from the infection did not differ significantly between dead and live worms (Ford *et al.* 2016). Nematodes were considered dead when they did not respond to touch with a platinum wire. Nematodes were washed to remove external bacteria by transferring them between five 5 μl drops of M9 buffer on fresh TSB plates using a platinum wire. Nematodes were then crushed in 20 μl of M9 in a 1 ml Eppendorf tube with a pestle to release internal bacteria. An inoculation loop was used to streak each sample onto selective media (TSB plates with 100 μg/ml rifampicin were used to isolate *E. faecalis*, and Mannitol Salt Agar (MSA) plates were used to isolate *S. aureus*), and the plates were incubated overnight at 30 °C. Ten colonies of *S. aureus* and/or *E. faecalis* were subsequently picked from each replicate and grown in THB overnight, shaking at 200 rpm at 30 °C. These cultures were used to make the exposure plates for the next passage.

Co-colonization dynamics

To determine whether co-passage affected the fitness of the defensive microbe and pathogen under co-colonization, we compared the fitness of the defensive microbe and pathogen from the Co-evolution treatment under co-colonization with the ancestral and single evolution treatment populations under co-colonization. We performed this experiment using populations from passage 10 of the evolution experiment. Exposure plates were made as above. Approximately 1000 young adult

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nematodes from the stock *C. elegans* population were placed onto exposure plates and incubated for 24 h at 25 °C. After 24 h of exposure, three to five dead nematodes were picked from each plate. Nematodes were washed as in the evolution experiment and then crushed in 20 μl of M9 with a pestle to release internal bacteria. After crushing, serial dilutions were plated onto selective media and grown overnight at 30 °C. *E. faecalis* and *S. aureus* colony-forming units (CFUs) per host were counted.

Local adaptation in time

To test whether the defensive microbe or pathogen became locally adapted in time as a result of co-passage, we measured the number of CFUs per nematode of each bacterial species under sympatric-in-time and two allopatric-in-time co-colonization conditions (see Fig. 2a). Exposure plates were made as above. The sympatric condition paired defensive microbe and pathogen populations from the same replicates at passage 10. The two allopatric conditions paired defensive microbe and pathogen ancestors with passage 10 populations. Our assays of local adaptation in time are conducted in a similar fashion to the 'local vs. foreign' and 'home vs. away' comparisons commonly used to measure local adaptation in space (Blanquart *et al.* 2013). Using two allopatric conditions in a single comparison is a conservative method of measuring local adaptation because it allows us to tease apart the evolutionary effects of both interacting species. We also performed the same comparisons using the single evolution treatment populations. Given that both bacterial species evolved independently in the single evolution treatment, the sympatric pairings were 'pseudosympatric', and we did not expect to find a signal of local adaptation.

Time shifts

We looked for evidence of reciprocal adaptation in both pathogen and defensive microbe populations over time by performing time-shift assays (Gaba & Ebert 2009). Here, *S. aureus* populations from passage 7 were allowed to co-colonize the host with *E. faecalis* from past, present and future replicate populations (passages 4–10, see Fig. 3a), and their respective CFUs/host were counted. The reciprocal was carried out for *E. faecalis* (see Fig. 3b). Exposure plates were made, and CFU per host for each species was counted as above.

Genome extraction and analysis

Forty clones were randomly selected from each *S. aureus* and *E. faecalis* replicate population from both selection treatments at passages 5 and 10. Each clone was grown independently overnight in 200 μl THB in a 96-well plate shaking at 175 rpm at

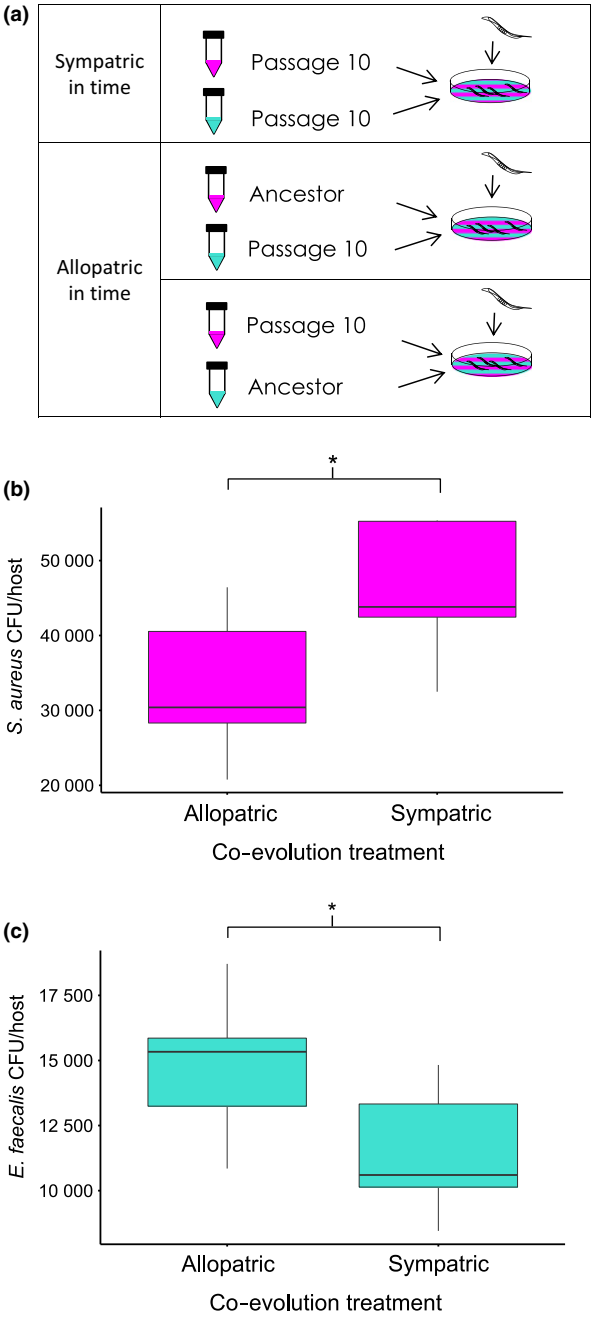


Fig. 2 Local adaptation in time. (a) Sympatric- and allopatric-in-time conditions. Sympatric conditions involve co-colonization of contemporary *Staphylococcus aureus* (magenta) and *Enterococcus faecalis* (turquoise) from the same replicate population at passage 10. Allopatric crosses involve co-colonization of ancestral and passage 10 populations. (b) Fitness of *S. aureus* (CFU/host) under allopatric and sympatric conditions (co-evolution treatment). (c) Fitness of *E. faecalis* (CFU/host) under allopatric and sympatric conditions (co-evolution treatment).

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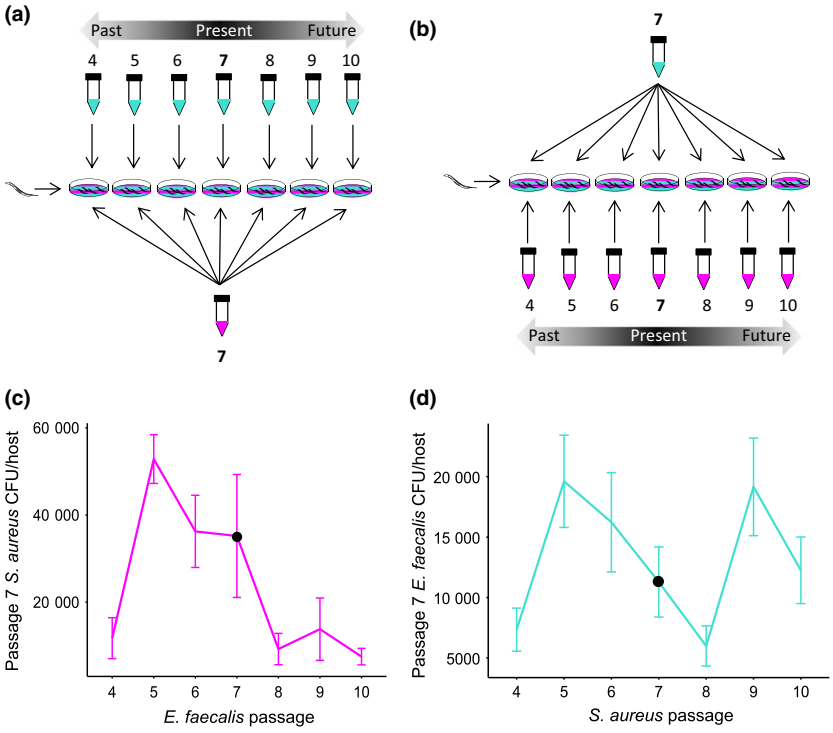


Fig. 3 Time shifts. Design of time-shift assays whereby (a) *Staphylococcus aureus* pathogen (magenta) and (b) *Enterococcus faecalis* defensive microbe (turquoise) replicate populations were paired with the other from the past, present and future time points. (c) Fitness of *S. aureus* (CFU/host) from passage 7 against *E. faecalis* from passages 4–10 (co-evolution treatment). (d) Fitness of *E. faecalis* (CFU/host) from passage 7 against *S. aureus* from passages 4–10 (co-evolution treatment).

30 °C. Subsequently, each set of 40 clones was checked for equal OD600 and pooled in equal volumes, resulting in one sample per 40 clones. A single clone of ancestral *S. aureus* and *E. faecalis* was also grown.

Staphylococcus aureus DNA was extracted by centrifuging 1 ml of culture at 6000 ***g***, removing the supernatant and resuspending the pellet in 160 µl enzymatic lysis buffer (Qiagen), 40 µl lysostaphin (200 µg/ml, Sigma-Aldrich), 40 µl lysozyme (100 mg/ml, Sigma-Aldrich) and 8 µl RNase A (10 mg/ml, Sigma-Aldrich). This mixture was incubated for 1 h at 37 °C. A volume of 25 µl proteinase K (Qiagen) was then added, and 200 µl Buffer AL (without ethanol) was added and vortexed. This mixture was then incubated at 56 °C for 1 h. A volume of 200 µl of ethanol was added and vortexed and DNA purification followed DNeasy Blood and Tissue Spin-Column Protocol (Qiagen). From *E. faecalis*, DNA was extracted by centrifuging 2 ml of culture at 7500 rpm, removing the supernatant and resuspending the pellet in 160 µl buffer B1 (Qiagen), 6 µl of 25KU/ml mutanolysin (Sigma-Aldrich), 40 µl of 200 µg/ml lysostaphin (Sigma-Aldrich) and 40 µln 100 mg/ml lysozyme (Sigma-Aldrich). This mixture was incubated for 1 h at 37 °C. A volume of 25 µl proteinase K (Qiagen) was then added, and 200 µl Buffer AL (without ethanol) was added and vortexed. This mixture was then incubated at 56 °C for an hour. Ethanol (200 µl) was added and DNA purification followed the DNeasy Blood and Tissue Spin-Column Protocol (Qiagen).

DNA was sequenced using the HiSeq4000 platform with 100-bp paired end at the Wellcome Trust Centre for Human Genetics. The project accession no. at the European Nucleotide Archive for the raw read data is PRJEB13385. Sequenced read data in fastq files were trimmed for the presence of Illumina adapter sequences using Cutadapt version v1.9.1 (revision e960cc1 from github.com/marcelm/cutadapt) (Martin 2011). The reads were further trimmed using Sickle version 1.33 (revision f3d6ae3 from github.com/najoshi/sickle) (Joshi & Fass 2011) with a minimum window quality score of 25 and retaining only reads longer than 50 bp after trimming. Single reads remaining from pairs were retained.

Reference genomes for short-read mapping were obtained from the NCBI Assembly database (www.ncbi.nlm.nih.gov/assembly). Short reads of the DNA extractions from the *Staphylococcus aureus* strain MSSA476 isolates were mapped to assembly GCF_000011525.1 using BWA MEM v0.7.12 (revision cc9eef2 from github.com/lh3/bwa) (Li & Durbin 2009) using option -M to flag shorter split hits as secondary ensuring the single best alignment was used. Evolved populations of *E. faecalis* OG1RF were mapped to assembly GCF_000172575.2 using the same aligner and options. Alignments were manipulated using SAMTools v1.2 (revision ac5b8e7 from github.com/samtools/samtools) (Li *et al.* 2009).

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The Genome Analysis Toolkit v3.4 (GATK) (McKenna *et al.* 2010; DePristo *et al.* 2011; Van der Auwera *et al.* 2013) IndelRealigner module was used to realign reads around putative insertions and deletions after which duplicate reads were identified and removed with Picard v1.135 (Carey 2015). Single-nucleotide polymorphism, insertion and deletion discovery was performed with GATK's Haplotype caller module with sample ploidy $n = 40$ as described in Williams *et al.* (2015). Default parameter values were used, however, given diversity was expected to be relatively low due to the experimental duration the priors '–heterozygosity 0.0001 –indel_heterozygosity 0.00001' were used. One replicate population of *E. faecalis* from the co-evolution treatment at passage 10 (C3.10) had high read coverage depth over one region that caused excessive memory usage. This coverage difference was remedied by setting '–downsample_to_fraction 0.8' to decrease the reads used and '–minPruning 2' to simplify the de Bruijn k-mer graph used in variant calling. To distinguish low frequency mutations from sites prone to sequencing error, the ancestral clone was sequenced and variants called with ploidy of 40, as above, and any sites exhibiting apparent polymorphism were filtered out of further analyses. Any sites appearing as polymorphic in more than one population were checked by manual inspection in IGV2.

Genetic distance

A phylogenetic tree was constructed using Euclidean genetic distances (the square root of pairwise differences), which is suggested as an appropriate metric for molecular variation data (Excoffier *et al.* 1992; Paterson *et al.* 2010). It is important to note that a few observed mutations are likely to have arisen in the overnight culture of the ancestral populations used to initiate all populations of all treatments, resulting in some shared mutations from the start of the evolution experiment. Pairwise genetic distances were taken from the distance matrix for statistical analysis. We compared the genetic distances of replicate populations to the ancestor to assess differences in the rate of evolutionary change between treatments. We compared the genetic distances between replicate populations of the same treatments to contrast the diversity in evolutionary trajectories. Finally, to assess differences in rates of genetic turnover of each treatment, we compared the genetic distances of replicate populations between passages 5 and 10.

Statistical analysis

Data analysis was carried out in R v 3.2.0 (http://www.r-project.org/). Parametric tests were used for all data, which met the required assumptions. These assumptions were checked using the Shapiro test to detect whether data were normally distributed and *F*-tests to compare the variances of two samples from normal populations. Nonparametric equivalents were used if the data did not meet those assumptions. Outlying data points were detected and removed using the Dixon test. ANOVAs were used to compare bacterial fitness across treatments under co-colonization, and Tukey contrasts were used for post hoc comparisons. Plots of each ANOVA were checked by eye for model quality. A mixed-effect model was used to measure local adaptation, including replicate population as a random effect and the sympatric/allopatric treatment as a fixed effect. Another mixed-effect model was used to measure reciprocal adaptation across time shifts, including replicate population as a random effect and time shift (passage) as a fixed effect. Tukey contrasts were used for post hoc comparisons. Two-sample *t*-tests were used to compare pairwise genetic distances of bacterial populations. The Welch two-sample *t*-test was used when the assumption of equal variance was broken. The Wilcoxon rank-sum test was used when the assumption of normality was broken. *P*-values generated by comparing pairwise genetic distances were corrected for multiple comparisons for each bacterial species using the FDR method.

Results

Co-colonization dynamics

We found a significant increase in pathogen fitness (Fig. 1b, ANOVA $F = 7.98$, d.f. $= 2$, $P = 0.0063$, Table S1, Supporting information) and decrease in defensive microbe fitness (Fig. 1c, ANOVA $F = 4.86$, d.f. $= 2$, $P = 0.028$, Table S1, Supporting information) over time in the co-evolution treatment that did not occur in the single evolution treatment. These data suggest that *Staphylococcus aureus* was 'winning' at passage 10 of experimental co-evolution with *Enterococcus faecalis*.

Local adaptation in time

We found that the high pathogen fitness observed at passage 10 in the co-evolution treatment under co-colonization was significantly reduced under allopatric conditions (Fig. 2b mixed-effect model: $\chi^2 = 5.82$, d.f. $= 1$, $P = 0.016$). Conversely, the low defensive microbe fitness recorded at passage 10 in the co-evolution treatment under co-colonization significantly increased under allopatric conditions (Fig. 2c mixed-effect model: $\chi^2 = 5.79$, d.f. $= 1$, $P = 0.016$). These results thus indicate that the co-colonization dynamics observed at passage 10 were a result of interaction

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specificity due to pathogen local adaptation in time and defensive microbe local maladaptation in time.

As expected, there were no significant differences in *E. faecalis* or *S. aureus* fitness between the allopatric and pseudosympatric pairings from the single evolution treatment (Fig. S1a, Supporting information mixed-effect model: $\chi^2 = 1.8$, d.f. = 1, *P*-0.18. Figure S1b, Supporting information mixed-effect model: $\chi^2 = 0.8$, d.f. = 1, *P*-0.36). Thus, the patterns of local adaptation we observe in the co-evolution treatment cannot be reproduced by evolving the bacteria independently within the host.

Time shifts

We discovered strong evidence of reciprocal adaptation between *E. faecalis* and *S. aureus* in replicate populations from the co-evolution treatment. We found that the fitness of *S. aureus* differed significantly when paired with defensive microbe populations from the past, present and future (Fig. 3c mixed-effect model: $\chi^2 = 29.1$, d.f. = 6, $P = 5.819e\text{-}05$, Table S1, Supporting information). Specifically, defensive microbes from the future were significantly better at suppressing pathogens than defensive microbes from the past (8 vs. 5, $P < 0.001$), indicating that the defensive microbe adapted to recently experienced pathogen populations. Critically, as we sampled further back into the past, we found evidence that adaptation was driven by fluctuating selection dynamics. Defensive microbes from the distant past were significantly better at suppressing pathogens than defensive microbes from the recent past (4 vs. 5, $P < 0.001$). From this pattern, we can assume that the defensive microbe genotypes common at passage 8 were also common at passage 4 and were thus fluctuating over time.

From the reciprocal time shift, we found that the within-host fitness of *E. faecalis* differed significantly when paired with pathogen populations from the past, present and future (Fig. 3d mixed-effects model: $\chi^2 = 17.18$, d.f. = 6, $P = 0.0086$, Table S1, Supporting information). Future pathogens were significantly better at suppressing defensive microbes than pathogens from the past (8 vs. 5 $P = 0.011$), suggesting that pathogens were also adapting to recently experienced defensive microbe populations. Consistent with the reciprocal time shift above, we discovered that pathogen adaptation was driven by fluctuating selection dynamics. Here, pathogens sampled further back into the past were significantly better at suppressing defensive microbes than pathogens from the recent past (4 vs. 5 $P = 0.034$). We can assume from this pattern that the pathogen genotypes common at passage 8 were also common in the past at passage 4, consistent with the cycle of defensive microbe genotypes we observed in Fig. 3c. In addition to this apparent cycle, we also discovered that pathogens sampled into the distant future were significantly worse at suppressing defensive microbes than pathogens from the near future (9–8 $P = 0.017$). Interestingly, this fluctuation did not correspond with an equivalent fluctuation in *E. faecalis* genotypes in the reciprocal time shift (Fig. 3c), suggesting that the pathogen was leading a new cycle of adaptation to which the defensive microbes had yet to respond. Together, these phenotypic results provide strong evidence of ongoing, reciprocal adaptation between *S. aureus* and *E. faecalis* by fluctuating selection dynamics.

Co-evolutionary genomics

We plotted the frequency of mutations for each replicate population of the co-evolution treatment over time for both *S. aureus* and *E. faecalis*. Consistent with the results indicating co-evolution via fluctuating selection, we discovered that de novo mutations fluctuated in frequency over time in all *S. aureus* (Fig. 4a) and *E. faecalis* (Fig. 4b) replicate populations from the co-evolution treatment. Most mutations that were common in the populations at passage 5 became rare (or extinct) by passage 10, whilst many rare (or nonexistent) mutations at passage 5 increased in frequency by passage 10. A few mutations were found to increase to near fixation across both time points, indicating either slower fluctuations for these mutations or the existence of simultaneous arms race dynamics.

Several mutations in the co-evolved *E. faecalis* populations were located in genes with putative functions in the production of superoxides (Huycke *et al.* 2001, 2002), a known protection mechanism in this system (King *et al.* 2016) (See Appendix S1). For example, mutations were found in genes involved in purine metabolism and the transport of xanthine, both of which affect the production of superoxides (Huycke *et al.* 2001, 2002). Interestingly, we discovered the same stop mutation in a gene involved in purine metabolism as evolved in a previous experiment whereby *E. faecalis* had evolved increased superoxide production during repeated exposure to a single *S. aureus* genotype (King *et al.* 2016). In addition to this, many of the mutations in the co-evolved *S. aureus* populations were located in genes with putative functions in the siderophore pathway, a pathway important in *S. aureus–E. faecalis* interaction (Ford *et al.* 2016) (see Appendix S2). These included putative siderophore-iron reductases, substrate transporters and proteins involved in glycine metabolism, a key component of ferrichrome (Ford *et al.* 2016).

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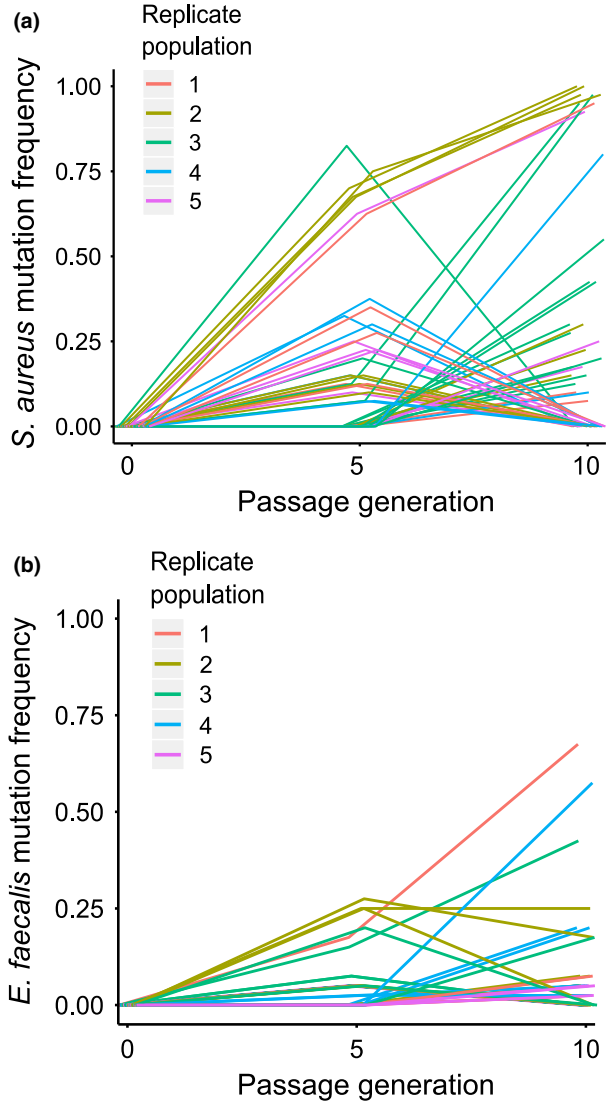


Fig. 4 Dynamics of genomic evolution. The frequency of mutations in 40 sampled clones from (a) *Staphylococcus aureus* and (b) *Enterococcus faecalis* replicate populations at passages 5 and 10 under co-passage (co-evolution Treatment). Each line represents an individual mutation, and colours correspond to replicate populations.

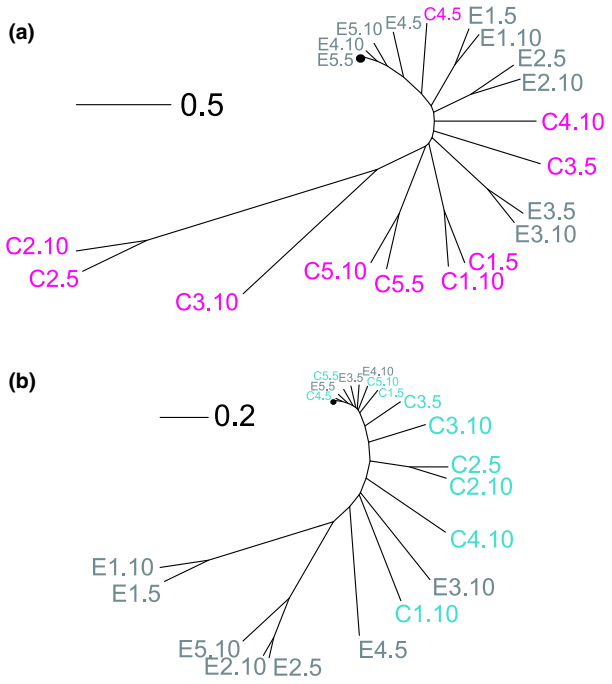


Fig. 5 Genetic distances of pathogen and defensive microbe populations. (a) Phylogenetic tree for *Staphylococcus aureus* populations from the single evolution treatment (E1–5 in grey) and co-evolution treatment (C1-5 in magenta) from passages 5 and 10 along with the ancestor (black circle). (b) Phylogenetic tree for *Enterococcus faecalis* populations from the single evolution treatment (E1–5 in grey) and co-evolution treatment (C1-5 in turquoise) from passages 5 and 10 along with the ancestor (black circle). Genetic distances based on Euclidean distances calculated from the frequency and identity of mutations in each population. Scale bar indicates Euclidean distance.

Genetic distance

Co-passage with the defensive microbe resulted in greater pathogen genetic divergence and turnover than passaging the pathogen through hosts alone. Co-evolved *S. aureus* populations were significantly more genetically distant from the ancestor and thus experienced faster evolutionary change than independently passaged pathogen populations (Fig. 5a two-sample *t*-test, $t = -2.95$, d.f. = 8, fdr-corrected $P = 0.018$). Furthermore, we found significantly greater pairwise genetic distances between each replicate of the co-evolved pathogen populations within the independently passaged pathogen populations (Fig. 5a Welch two-sample *t*-test, $t = -6.97$, d.f. = 12.9, fdr-corrected $P = 3.108\text{e-}05$), suggesting that co-evolving pathogen populations followed more diverse evolutionary trajectories. Co-evolved *S. aureus* populations also exhibited greater genetic distance between passages 5 and 10 than the independently passaged populations (Fig. 5a Wilcoxon rank-sum test W = 25, fdr-corrected $P = 0.012$), indicating greater genetic turnover in the co-evolved pathogens, consistent with fluctuating selection dynamics.

In contrast, we saw no difference in genetic divergence and turnover between *E. faecalis* treatments. Neither the genetic distance from the ancestor nor the pairwise genetic distances among replicate defensive microbe populations differed significantly between the single evolution and co-evolution treatments (Fig. 5b genetic distance from ancestor: two-sample *t*-test, $t = 1.1$, d.f. = 8, fdr-corrected $P = 0.302$; among-replicate genetic distances: two-sample *t*-test, $t = 2.36$, d.f. = 18, fdr-corrected $P = 0.09$). In addition, the co-evolution

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treatment did not differ from the single evolution treatment in genetic distance between passages 5 and 10 (Fig. 5b two-sample *t*-test $t = 1.55$, d.f. = 8, fdr-corrected $P = 0.24$).

Discussion

It is theoretically predicted that defensive microbes and pathogens could undergo co-evolutionary interactions within host populations via fluctuating selection dynamics (Kwiatkowski *et al.* 2012); however, direct tests are currently lacking (Parker *et al.* 2011; Ford & King 2016; Hahn & Dheilly 2016). Here, by experimentally co-passaging defensive microbes and pathogens through host populations, we found evidence of defensive microbe–pathogen co-evolution via fluctuating selection dynamics.

We detected defensive microbe–pathogen interaction specificity as a result of experimental co-passage. Such specificity is a key assumption underlying co-evolution by fluctuating selection dynamics (Agrawal & Lively 2002). At passage 10, all replicate pathogen populations were locally adapted in time to the defensive microbe, whilst the defensive microbe was locally maladapted, resulting in the appearance that the pathogen was 'winning'. This pattern may parallel studies of host–pathogen co-evolution which have found that pathogen local adaptation is more commonly identified than host local adaptation because pathogens have faster evolutionary responses to reciprocal adaptation than hosts (i.e. due to higher rates of pathogen migration and mutation, or shorter generation times) (Gandon *et al.* 2008; Koskella 2014). Although our experimental design did not allow for migration, we observed that all pathogen populations accumulated more mutations than defensive microbe populations, suggesting a faster pathogen evolutionary rate.

By performing time-shift experiments, we found that both pathogens and defensive microbes were constantly and reciprocally adapting to each other throughout the experiment. We show that both species peaked in fitness against antagonist populations recently experienced in evolutionary history, a pattern expected from reciprocal evolutionary change. Here, the fitness of a focal species against their recent enemy is expected to be higher than against their current enemy as the current enemy has begun to respond to the adaptive changes of the focal species (Koskella 2014). Critically, our time-shift data also indicate the dynamics of co-evolution. In line with our finding of interaction specificity, we show that pathogen and defensive microbe adaptation appeared to fluctuate over time, rather than increase directionally, indicating fluctuating selection dynamics (Agrawal & Lively 2002; Gaba & Ebert 2009). For example, the pathogen and defensive microbe genotypes that were common at passage 4 became rare throughout passages 5–7 and then increased in frequency again by passage 8, pointing to a full cycle of genotype fluctuation.

Consistent with our phenotypic evidence of fluctuating selection dynamics, we found direct confirmation of rapid genotype frequency changes across the evolution experiment. We revealed that mutations that had spread rapidly in the population by passage 5 were almost all rare (or extinct) by passage 10, whilst mutations that were rare (or nonexistent) at passage 5 increased in frequency by passage 10. Importantly, many of these mutations were located in genes putatively linked to *Staphylococcus aureus–Enterococcus faecalis* interactions, including siderophore production by *S. aureus* (Ford *et al.* 2016) and superoxide production by *E. faecalis* (King *et al.* 2016). Kwiatkowski *et al.* (2012) show that such oscillations in defensive microbe and pathogen alleles are likely to occur when the host genetic background is evolutionarily stable, as in our experiment. This outcome suggests that defensive microbes could 'take over' from the host and provide rapid co-evolutionary responses to pathogens (Kwiatkowski *et al.* 2012), which could remove or reduce parasite-mediated selective pressure on hosts (Hahn & Dheilly 2016; Martinez *et al.* 2016). We speculate that possessing a defensive microbe might allow hosts with relatively limited evolutionary potential (e.g. due to asexual reproduction, low genetic diversity or long generation times) to maintain resistance to co-evolving pathogens.

An important implication of our findings is that defensive microbes have the potential to be a major source of selection on pathogens, driving fast evolutionary rates and divergent evolutionary trajectories. We observed here that pathogens co-evolving with defensive microbes had higher rates of evolution, greater genetic turnover and more diverse evolutionary trajectories than those evolving independently in host populations. Similar patterns of molecular evolution in pathogens have been found to occur during host–pathogen co-evolution (Paterson *et al.* 2010; Schulte *et al.* 2010). Unlike the pathogen, however, we found no difference in the rate of genomic evolution between the co-passaged and independently passaged defensive microbe populations. It is possible that we saw such asymmetry because *S. aureus* was ahead in the co-evolutionary arms race and, according to our time-shift data, was starting a new cycle to which the defensive microbe was not yet adapted to. It is therefore likely that more evolutionary changes had accumulated in co-evolved *S. aureus* populations relative to independently passaged populations, whilst this was not yet the case for *E. faecalis*. This explanation is consistent with our observations of pathogen local adaptation and

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the higher total number of mutations in the pathogen populations compared to the defensive microbe populations. Given that co-evolution started de novo, we would expect that more evolutionary time, and so more cycles of reciprocal adaptation, would eventually result in greater evolutionary change in both species relative to the independently passaged populations.

Together, our data show the potential for defensive microbes and pathogens to engage in co-evolution driven by fluctuating selection. Our findings suggest that co-evolution may underlie the interaction specificity observed between pathogens and defensive microbes in nature (Oliver *et al.* 2005; Poulsen *et al.* 2010; Koch & Schmid-Hempel 2012; Rouchet & Vorburger 2012; Cariveau *et al.* 2014; Cayetano & Vorburger 2015). Given that studies investigating co-evolution have largely focussed on host–parasite interactions (Agrawal & Lively 2002; Decaestecker *et al.* 2007; Gandon *et al.* 2008; King *et al.* 2009; Morran *et al.* 2011), it is an implied assumption that the host is the strongest source of selection acting upon parasites and pathogens. Although we do not account for additional effects of host immunity on *E. faecalis*–*S. aureus* interactions in this study, it is nevertheless clear that defensive microbes can be as powerful as hosts in imposing strong and divergent selection on pathogens. Identifying that pathogens are able to undergo co-evolution with defensive microbes, instead of the host, has important implications for how we understand the factors shaping patterns of pathogen evolution in nature. Moreover, the ability of defensive microbes to co-evolve with pathogens may have great consequences for their role in disease control. For example, if defensive microbes are able to undergo continual counteradaptation to pathogen resistance, they could be powerful tools to sustainably control infectious diseases of animals and plants (Levin & Bull 2004; Ford & King 2016). In an era of emerging antibiotic resistance, the allure of evolution-proof disease control is at an all-time high (Allen *et al.* 2014; Vale *et al.* 2016).

It is being increasingly revealed that defensive microbes are common within host populations and across numerous host species. As a result, we are only just beginning to realize their immense value to hosts. In this study, we further our understanding of defensive microbes by illustrating their role as an evolutionarily dynamic arm of host immunity and their role in driving pathogen divergence.

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Competing financial interests

The authors declare no competing financial interests.

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Data accessibility

Raw read data for the bacterial genomic sequences were deposited in the European Nucleotide Archive under project accession no. PRJEB13385. The CSV files containing data for each figure are available in the Dryad repository (http://dx.doi.org/10.5061/dryad.0b707) (Ford *et al.* 2016).

S.A.F. and K.C.K. designed the experiments. S.A.F. conducted the experiments and analysed the data. S.A.F., D.W. and S.P. conducted the bioinformatics. S.A.F. and K.C.K. wrote the manuscript.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 (a) Fitness of *Enterococcus faecalis* (CFU/host) and (b) *Staphylococcus aureus* (CFU/host) under sympatric and allopatric-in-time conditions (Single evolution treatment).

Table S1 Statistical results listed by figure.

Appendix S1 Mutations found present in replicate *E. faecalis* genomes across all treatments.

Appendix S2 Mutations found present in replicate *S. aureus* genomes across all treatments.

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Microbe-mediated host defence drives the evolution of reduced pathogen virulence

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Microbes that protect their hosts from pathogens are widespread in nature and are attractive disease control agents. Given that pathogen adaptation to barriers against infection can drive changes in pathogen virulence, 'defensive microbes' may shape disease severity. Here we show that co-evolving a microbe with host-protective properties (*Enterococcus faecalis*) and a pathogen (*Staphylococcus aureus*) within *Caenorhabditis elegans* hosts drives the evolution of reduced pathogen virulence as a by-product of adaptation to the defensive microbe. Using both genomic and phenotypic analyses, we discover that the production of fewer iron-scavenging siderophores by the pathogen reduces the fitness of the defensive microbe and underpins the decline in pathogen virulence. These data show that defensive microbes can shape the evolution of pathogen virulence and that the mechanism of pathogen resistance can determine the direction of virulence evolution.

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Microbes can defend their hosts against pathogenic infection[1]. This phenomenon has been widely observed in nature across plant and animal species, including humans, to provide a strong line of defence beyond the host response[2]. As a result, microbes with protective traits are considered promising disease control agents and alternatives to antibiotics to combat devastating human and crop pathogens[3–6]. Importantly, pathogen adaptation to barriers against infection can drive the evolution of virulence[7], as observed against immune-priming vaccines[8] and competing, co-infecting pathogens[9]. Given that defensive microbes likely impose strong selection on pathogens too[2], they may also play a pivotal role in determining why some infections are more harmful than others. Thus, understanding the effect of defensive microbes on pathogen evolution could ultimately enable us to explain variation in disease severity in nature and to use defensive microbes for optimal disease control.

In this study, we use the term pathogen 'resistance' to describe the pathogen's ability to resist the defensive microbe. We propose that defensive microbes will drive the evolution of pathogen virulence when the mechanism of pathogen resistance to microbe-mediated defence is associated with harm to the host[2,10]. The direction of virulence evolution will depend on the shape of the relationship between resistance and virulence[2,10]. Specifically, we predict that defensive microbes will result in the evolution of increased pathogen virulence if pathogen resistance and virulence are positively associated (Fig. 1a), for example, pathogens could increase in competitive ability against the defensive microbe via a faster growth rate or inducing inflammation, both of which are also detrimental to the host[10–13]. Alternatively, if a trade-off exists between pathogen resistance and virulence traits, for example, resistance is costly and slows pathogen growth rate[14–16], then defensive microbes will drive lower levels of virulence (Fig. 1b). Although the evolution of pathogen resistance to defensive microbes has been theoretically illustrated[10,17] and empirically evidenced[18], studies are few. Our data will thus provide valuable information on the evolution of pathogen resistance in addition to virulence.

Here, we have studied the interactions between *Staphylococcus aureus* and *Enterococcus faecalis* within *Caenorhabditis elegans* hosts which emulate the interactions between a pathogen and a defensive microbe, respectively[19]. Both *E. faecalis* and *S. aureus* co-occur in animal microbiomes and can colonize the gut of *C. elegans*[20]. This nematode species is a model animal system for investigating microbial pathogenesis as well as natural and lab-based host–microbiota associations[21–25]. We used *S. aureus* strain MSSA 476 and *E. faecalis* strain OG1RF, both of which were isolated from humans. Their interaction within a nematode host is therefore novel, allowing us to explore the evolutionary consequences of defensive symbioses early in their formation. We have previously illustrated that *S. aureus* acts as a virulent pathogen within the nematode host[19]. Furthermore, we have shown that although *E. faecalis* imposes a small cost to host longevity while colonizing alone (un-colonized hosts experience no mortality under otherwise the same conditions[19]), this bacterium provides a net benefit to hosts during *S. aureus* infection[19]. By having context-dependent fitness effects on its host, *E. faecalis* reflects symbionts found in nature. In particular, many defensive microbes have been found to impose costs on their hosts in the absence of the enemy[26–31]. Moreover, we know that *E. faecalis* significantly reduces the virulence of *S. aureus* by producing superoxide anions that decrease pathogen fitness[19]. This mechanism of defence is a form of interference competition, whereby defensive microbes produce toxic compounds that may either kill the pathogen or slow its growth rate[2]. Interference competition is currently the most commonly detected mechanism of microbe-mediated defence in nature[2].

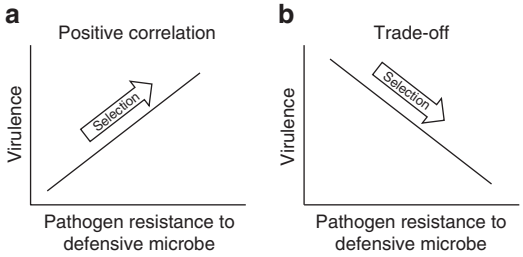


Figure 1 | Proposed associations between mechanisms of pathogen virulence and resistance to microbe-mediated defence. (**a**) Pathogen virulence is hypothesized to increase when it correlates positively with the pathogen's ability to resist microbe-mediated defence. (**b**) Pathogen virulence is hypothesized to decrease when traded off against the pathogen's ability to resist microbe-mediated defence.

To test the hypothesis that defensive microbes can affect pathogen virulence evolution, we co-passaged *S. aureus* and *E. faecalis* (co-evolution treatment) and independently passaged *S. aureus* (single evolution treatment) within non-evolving, genetically homogeneous *C. elegans* populations for 10 passages. Given that defensive microbes and pathogens likely evolve on similar timescales in nature, co-passaging allowed for the realistic possibility of co-evolutionary interactions to shape pathogen evolution. Throughout our experiments, we assay host mortality after 24 h of exposure to ancestral and evolved pathogen populations as a measure of virulence[32]. We use this measure of virulence for several reasons. Host mortality is a widespread measure in theoretical and experimental investigations on the evolution of virulence across a diversity of host–pathogen systems[9,32–37]. Moreover, host mortality is commonly used in *C. elegans*–pathogen experimental evolution studies[38–40] and our previous work shows its relevance in our system[19]. Specifically, we measure host mortality within 24 h to observe adaptation relevant to the exposure window in the evolution experiment.

We find that experimental co-evolution between a defensive microbe and a pathogen within a host population drives the evolution of reduced pathogen virulence as a by-product of adaptation to the defensive microbe. Using both genomic and phenotypic analyses, we discover that pathogen populations evolved to produce fewer iron-scavenging siderophores and that this change lowered the fitness of defensive microbes and underpinned the decline in pathogen virulence. These data show that microbe-mediated defence can shape pathogen virulence evolution and that the mechanism of pathogen resistance can determine the direction of evolution.

Results

Microbe-mediated defence. Before experimental evolution, we show that *E. faecalis* protects the host from virulent infection after 24 h of simultaneous exposure with *S. aureus* (Fig. 2a, Quasibinomial GLM: $F = 41.96$, $df = 2$, $P = 5.193e-09$, Supplementary Table 1) by significantly reducing the infection load (Fig. 2b, Welch two-sample t-test: $t = -4.5$, $df = 16.2$, $P = 0.0003$). We find that the benefit *E. faecalis* provides the nematode host (12% reduction in host mortality after 24 h of exposure to the pathogen, Fig. 2a) is much greater than its cost (0.69% mortality after 24 h of exposure to the defensive microbe, Fig. 2a).

Interestingly, we discover that *E. faecalis* reached significantly higher population sizes in the host when it is infected with *S. aureus* (Fig. 2c, Two sample t-test: $t = -2.8$, $df = 24$, $P = 0.009$) suggesting that, although *E. faecalis* harms *S. aureus*, the pathogen conversely alters the environment in a way that

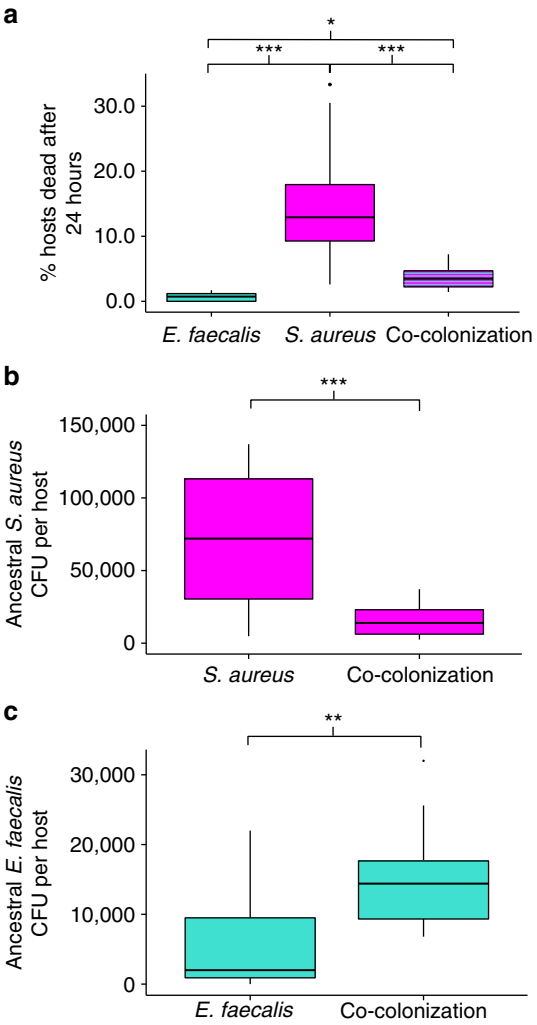


Figure 2 | Ancestral bacterial virulence and within-host bacterial population sizes. (**a**) Virulence (% hosts dead after 24 h exposure) of *E. faecalis*, *S. aureus* and both under co-colonization. Quasibinomial GLM: $F = 41.96$, $df = 2$, $P = 5.193e-09$. Tukey contrasts: *S. aureus* versus *E. faecalis*: $P < 1e-04$; *S. aureus* versus co-colonization: $P < 1e-04$; *E. faecalis* versus co-colonization: $P = 0.025$; Sample size for each treatment: 10 biological replicates. (**b**) Bacterial fitness (colony forming units, CFU per host) of ancestral *S. aureus* under single and co-colonization after 24 h exposure. Welch two sample *t*-test: $t = -4.5$, $df = 16.2$, $P = 0.0003$. Sample size for *S. aureus*: 16 biological replicates. Sample size for co-colonization: 18 biological replicates. (**c**) Bacterial fitness (CFU per host) of ancestral *E. faecalis* under single and co-colonization after 24 h exposure. Two sample *t*-test: $t = -2.8$, $df = 24$, $P = 0.009$. Sample size for *E faecalis*: 11 biological replicates; Sample size for co-colonization: 15 biological replicates. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Colours indicate the bacterial species being measured: magenta, pathogen (*S. aureus*); turquoise, defensive microbe (*E. faecalis*).

facilitates defensive microbe growth. These data therefore reveal a strong antagonistic relationship between the defensive microbe and the pathogen, whereby one species benefits and the other is harmed.

Pathogen virulence. To test whether defensive microbes can affect pathogen virulence evolution, we co-passaged *S. aureus* and *E. faecalis* (co-evolution treatment) and independently passaged *S. aureus* (single evolution treatment) within non-evolving, genetically homogeneous *C. elegans* populations for 10 passages

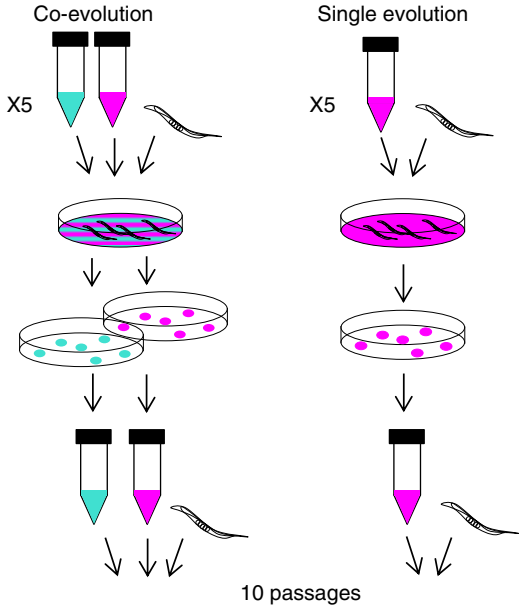


Figure 3 | Experimental co-evolution. Experimental co-evolution: *S. aureus* and *E. faecalis* were co-passaged (co-evolution treatment) and *S. aureus* was passaged alone (Single evolution treatment) within non-evolving, genetically homogeneous *C. elegans* host populations. At each generation, the same *C. elegans* host populations were exposed to mixed bacterial lawns of *S. aureus* and *E. faecalis* or single species lawns of *S. aureus*. After 24 h of exposure, 10 colonies of *S. aureus* and/or *E. faecalis* were collected from the pooled guts of 10 dead hosts using selective media and passaged to the next generation. Each treatment had five replicate populations and was conducted for 10 passages. Colours: magenta, pathogen (*S. aureus*); turquoise, defensive microbe (*E. faecalis*).

(Fig. 3). Both treatments consisted of five replicate populations started from the same clone of each bacterial species such that evolution was *de novo*. Every passage, hosts were exposed to bacterial treatments for 24 h, and 10 colonies of *S. aureus* and *E. faecalis* were collected from the pooled gut contents of 10 dead hosts and used to start the next generation (Fig. 3).

We found that experimental co-evolution with the defensive microbe drove reductions in pathogen virulence across all replicate populations, beyond those seen in the single evolution treatment (Fig. 4a, Quasibinomial GLM, $F = 25.7$, $df = 2$, $P = 4.627e-05$, Supplementary Table 1). This reduction in pathogen virulence correlated with a reduction in *in vitro* pathogen growth rate (Fig. 4b, Pearson's product–moment correlation: $t = 3.38$, $df = 9$, $P = 0.008$, $R^2 = 0.56$), supporting a link between virulence and growth rate, as is commonly assumed[32]. Despite a decrease in growth rate, co-evolved *S. aureus* reached significantly higher infection loads than either the ancestor or single evolution populations in hosts protected by ancestral *E. faecalis* (Fig. 4c, ANOVA, $F = 6.55$, $df = 2$, $P = 0.012$, Supplementary Table 1). This result indicates that the pathogens have adapted to interact with the defensive microbe. Together, these data suggest a trade-off between pathogen virulence and resistance to microbe-mediated defence, supporting our second hypothesis in Fig. 1b.

Siderophore production. We proposed that the mechanism underpinning changes in *S. aureus* virulence and growth rate involved the production of siderophores, compounds produced by microbes to bind and uptake iron[41]. In iron-limited host environments, siderophores can affect pathogen growth rate and so contribute to virulence, as evidenced in *S. aureus* infections[42].

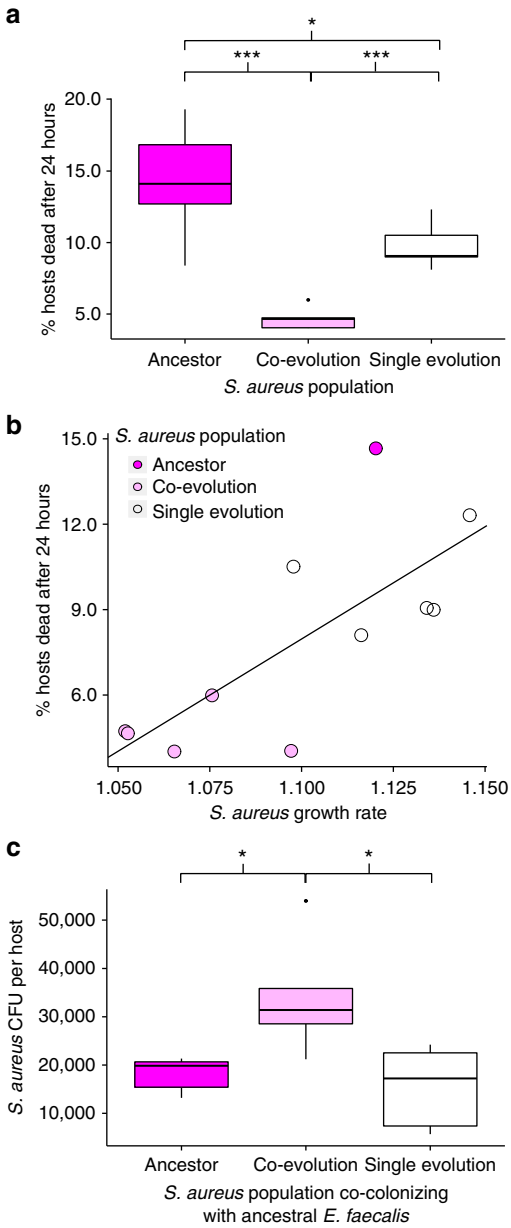


Figure 4 | Pathogen virulence and fitness. (**a**) Virulence of ancestral and evolved *S. aureus* populations in *C. elegans* hosts after 24 h of exposure. Quasibinomial GLM: F = 25.7, df = 2, $P = 4.627e-05$. Tukey contrasts: co-evolution versus Ancestor: $P < 0.001$; co-evolution versus Single evolution: $P < 0.001$; Ancestor versus Single evolution: $P = 0.035$. Sample size for each treatment: five biological replicates (average of two technical replicates). (**b**) Plot of virulence against the *in vitro* growth rate of ancestral and evolved *S. aureus* populations. Pearson's product-moment correlation: $t = 3.38$, df = 9, $P = 0.008$, $R^2 = 0.56$. Sample size for virulence data: as **a** but all ancestral replicates averaged to one biological replicate; Sample size for growth rate data: five biological replicates (average of three technical replicates) for evolved bacteria. One biological replicate for ancestral bacteria (average of eight technical replicates). (**c**) Bacterial fitness (CFU per host) of ancestral and evolved *S. aureus* populations under co-colonization with ancestral *E. faecalis*. ANOVA: F = 6.55, df = 2, $P = 0.012$. Tukey contrasts: Ancestor versus co-evolution: $P = 0.035$; Single evolution versus co-evolution: $P = 0.015$; Single evolution versus Ancestor: $P = 0.883$. Sample size for each treatment: five biological replicates (one technical replicate). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

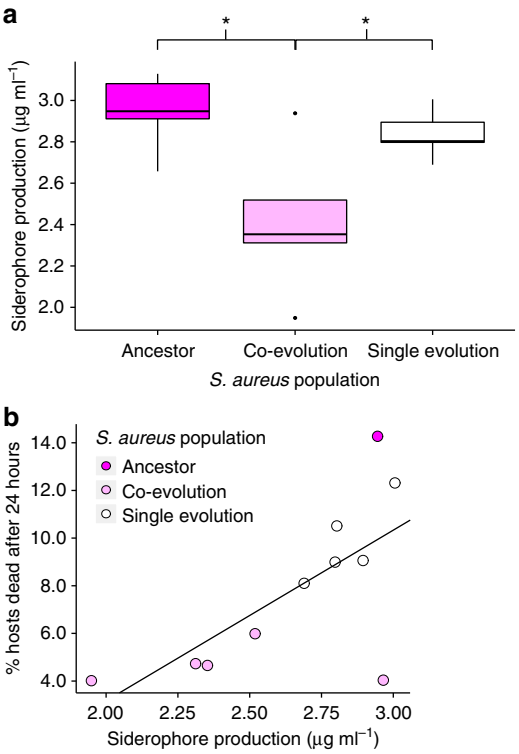


Figure 5 | Siderophore production. (**a**) Concentration (μg ml^{-1}) of siderophores produced by ancestral and evolved *S. aureus* populations *in vitro*. ANOVA: F = 6.681, df = 2, $P = 0.01$. Tukey contrasts: co-evolution versus ancestor: $P = 0.01$; single evolution versus ancestor: $P = 0.8$; single evolution versus co-evolution: $P = 0.04$. Sample size for each treatment: 5 biological replicates (average of 2 technical replicates). (**b**) Plot of virulence against *in vitro* siderophore production (μg ml^{-1}) by ancestral and evolved *S. aureus* populations. Pearson's product-moment correlation: $t = 2.8$, df = 9, $P = 0.02$, $R^2 = 0.5$. Sample size for virulence data: as Fig. 4a but all ancestral replicates averaged to 1 biological replicate. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Crucially, siderophores are also a 'public good' and so can be exploited by neighbouring microbes[41,43]. Theory posits that competition for host resources between unrelated pathogens selects for reduced public good production, and thus reduced virulence[41]. This has been supported by studies of both experimental and natural infections[44,45]. Importantly, when a pathogen is exploited by a defensive microbe, we hypothesize that there would be even stronger selection against public good production, and so virulence, since the exploiting microbe also harms the producer. This extension of current theory would be consistent with our findings that pathogen virulence and growth rate decrease over time in microbe-protected hosts, and would further explain the observed benefit ancestral *S. aureus* confers to *E. faecalis*.

To determine whether the defensive microbe drove a reduction in pathogen virulence as a by-product of selecting for decreased siderophore production, we measured siderophore activity and correlated it with pathogen virulence. By measuring the siderophore concentration produced by *S. aureus* populations *in vitro*, we found that *S. aureus* populations from the co-evolution treatment produced the fewest (Fig. 5a, ANOVA, F = 6.681, df = 2, $P = 0.01$, Supplementary Table 1). We then discovered that siderophore production was positively correlated with pathogen virulence (Fig. 5b, Pearson's product–moment correlation: $t = 2.8$, df = 9, $P = 0.02$, $R^2 = 0.5$). This pattern can be seen both within and between treatments.

Molecular evolution. Given that the production of fewer siderophores by co-evolved *S. aureus* was detected in the absence of *E. faecalis*, we concluded that this change was constitutive. To confirm that it was a result of evolutionary change, we conducted whole-genome resequencing of ancestral and *S. aureus* populations at the end of the evolution experiment. No single mutation was shared between any replicate populations, suggesting the independent evolution of the same phenotype multiple times within each treatment (Supplementary Table 2). Importantly, mutations from both evolution treatments can be linked to siderophore production pathways[46] (the putative functions of each mutation is listed in Supplementary Table 2); however SNPs in the co-evolution treatment were present at much higher frequencies compared with those found in the single evolution treatment, consistent with the distinct phenotypes (Fig. 6). Specifically, at least one non-synonymous mutation in each co-evolved pathogen population that had spread to >80% frequency could be linked to siderophore production (Supplementary Table 2). In contrast, only one mutation in one single evolution replicate population reached 50%. This distribution of mutations suggests that defensive microbes imposed strong selection on *S. aureus* populations which resulted in selective sweeps.

Effect of siderophores on defensive microbe fitness. To determine whether siderophores were public goods exploitable by the defensive microbe, we measured the *in vitro* growth rate of ancestral *E. faecalis* across a range of exogenous siderophore concentrations. We found that siderophore concentration correlated positively with *E. faecalis* growth rate over the natural range of *S. aureus* siderophore production (Fig. 7a, Pearson's product-moment correlation: $t=6.1$, $df=2$, $P=0.026$, $R^2=0.95$). We then found that defensive microbe fitness was directly affected by evolutionary changes in *S. aureus* siderophore production. Here, the fitness benefit *E. faecalis* gained from interacting with the ancestral pathogen (compared with growing independently) disappeared when co-cultured with co-evolved *S. aureus* populations, which produced the fewest siderophores (Fig. 7b, ANOVA, $F=9.2$, $df=3$, $P=0.0009$, Supplementary Table 1).

To test whether changes in siderophore production were sufficient to explain these differences, we measured the effect of surplus iron on *E. faecalis* fitness when co-cultured with ancestral and evolved *S. aureus* populations. Under surplus iron, siderophore production should be downregulated[47]. We found that surplus iron removed the differential effects of *S. aureus* treatment on *E. faecalis* fitness (Fig. 7c, Control ANOVA, $F=8.1$, $df=2$, fdr corrected $P=0.019$; Supplementary Table 1; $+Fe^{3+}$ ANOVA, $F=3.05$, $df=2$, fdr corrected $P=0.092$). This result confirms public good siderophores as the mechanism underlying pathogen adaptation to microbe-mediated defence in this system.

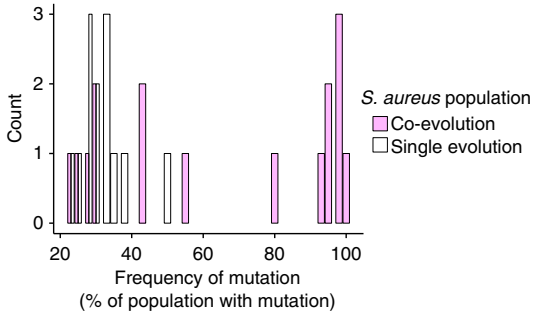


Figure 6 | Molecular evolution. Count of non-synonymous mutations in the co-evolution and single evolution replicate populations at a given frequency in the population (the % of 40 randomly sampled clones).

Discussion

We sought to establish how within-host interactions between defensive microbes and pathogens would affect the evolution of pathogen virulence. We found that the co-evolution between a defensive microbe and a pathogen drove the evolution of reduced pathogen virulence as a trade-off against pathogen resistance to microbe-mediated defence.

We have previously shown that *S. aureus* is a virulent pathogen within *C. elegans* and that *E. faecalis* acts as a defensive microbe by producing superoxide anions that reduce *S. aureus* fitness[19]. In addition to reducing the fitness of the pathogen, we found here that the defensive microbe could exploit the iron-scavenging siderophores produced by *S. aureus,* gaining a fitness benefit under co-colonization. Together, these data reveal a strong antagonistic interaction between the two species (Fig. 8). By experimentally co-evolving *S. aureus* and *E. faecalis* within nematode populations, we discovered that the pathogens from all replicate populations evolved to produce fewer siderophores. These data correspond with the spread of mutations in genes putatively linked with the siderophore pathway. We confirmed that the decrease in siderophore production resulted in the significant reduction of defensive microbe fitness and was associated with a dramatic increase in pathogen infection load within protected hosts. As a by-product of this adaptation, all co-evolved pathogen populations were significantly less virulent than the ancestral and single evolution populations since decreased siderophore production slowed their growth rate in the absence of the defensive microbe. Given that siderophores are widely used by pathogens[44], reduced siderophore production may be a common adaptation to interactions with members of a host's microbiota and may contribute in explaining the presence of siderophore mutants in pathogen populations in nature.

More generally, our results illustrate that just a single component of a host's microbiota could be a major source of selection acting to shape the virulence of pathogens in nature today. As in our experiment, there are many observations in nature of one defensive microbe species protecting against one enemy species[2]. However, defensive microbe–pathogen interactions may often be more complicated[2], for example complex microbiomes are known to protect species as diverse as bumblebees and humans from infection[3,48], and a single defensive microbe species can defend hosts against multiple enemies[49,50]. We have demonstrated the potential for evolutionary interactions between defensive microbes and pathogens and we have highlighted important implications for virulence. Future research will need to establish how these evolutionary interactions can play out in more complex communities.

The trade-off we observe between pathogen virulence and resistance to microbe-mediated defence is an important consideration for disease control. Host-associated microbes that cause pathogens to make this trade-off, either by similar or different mechanisms to that presented here, could be useful tools to tackle infectious disease. This is not only because they protect hosts in the short-term but they would also select for less virulent pathogens in the long-term. For example, lytic phage that act as defensive microbes by killing pathogenic bacteria can select for decreased pathogen virulence when they infect via bacterial receptors necessary for pathogen growth rate or virulence[16]. By altering these receptors, the pathogen trades-off resistance to phage with virulence mechanisms[16]. In this way, microbe-mediated therapy could provide a more powerful strategy to treat disease than drugs designed to block the production of virulence factors[46] by favouring the evolution of pathogens that are less virulent in the first place. Thus, as an alternative to anti-virulence strategies, which can have unforeseen evolutionary implications[51,52], we could design

disease control strategies that aim to direct pathogen evolution in a favourable direction.

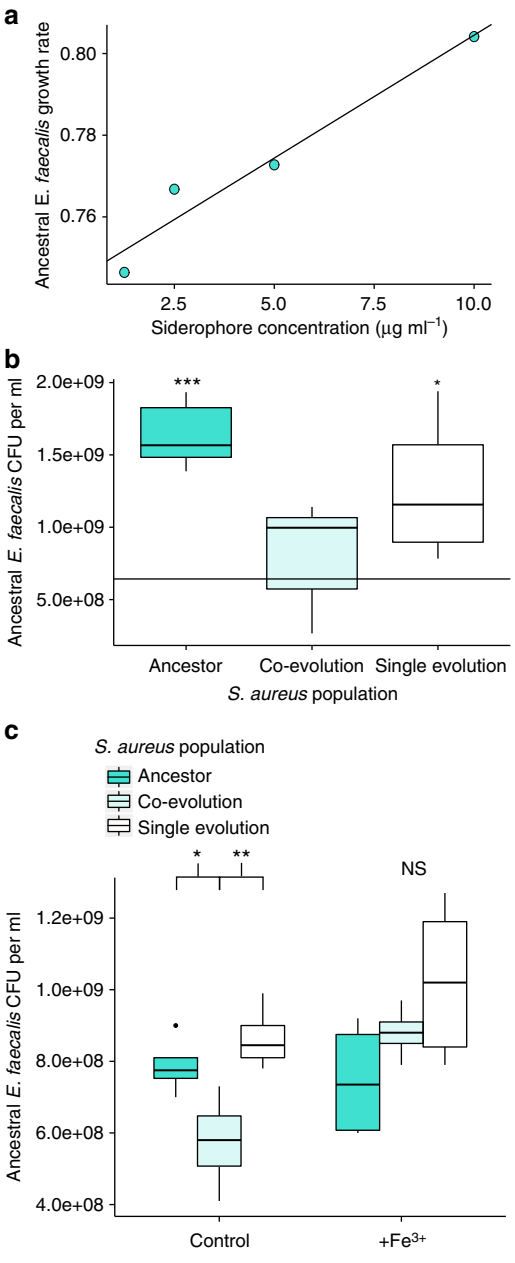
Microbes that protect hosts from infection can shape the evolution of pathogen virulence. Moreover, it is becoming clear that understanding the mechanisms underlying pathogen resistance to microbe-mediated defence may be crucial in explaining the variation in disease severity in nature and in predicting the direction of virulence evolution under applied disease control.

Methods

Nematode host and bacteria. *C. elegans* is a nematode that constantly interacts with microbes in its natural habitat[53], and it can act as a predator or host for numerous microbial species[22,54,55]. These animals are thus an established model for microbial colonization and pathogenesis[21] and their gut can be co-colonized by multiple pathogens and commensals[38,56–58]. We used the simultaneous hermaphroditic N2 wild-type *C. elegans* strain from the *Caenorhabditis* Genetics Centre (University of Minnesota, Minneapolis, MN)[56]. A genetically homogenous line was generated by selfing a single hermaphrodite for five generations. Populations of these nematodes were frozen in 50% M9 solution and 50% liquid freezing solution in cryotubes at −80 °C (ref. 56). Populations were regularly resurrected throughout experimentation to prevent the accumulation of *de novo* mutations in host populations. Worms were maintained at 20 °C on nematode growth medium plates seeded with *E. coli* OP50 (*Caenorhabditis* Genetics Centre, University of Minnesota, Minneapolis, MN). *E. coli* OP50 is grown at 30 °C shaking at 200 r.p.m. overnight in LB and 100 µl of this is spread onto nematode growth medium plates and incubated overnight at 30 °C (ref. 56). To ensure clean stocks and to synchronize the life stages of populations for experimentation, worms were treated with bleach (NaClO) and sodium hydroxide (NaOH) solution which kills everything except unhatched worm eggs[56].

We used *S. aureus* strain MSSA 476 (GenBank: BX571857.1), an invasive community-acquired methicillin-susceptible isolate and *E. faecalis* strain OG1RF (GenBank: CP002621.1), both isolated from humans. Both isolates were sourced from the University of Liverpool. A single ancestral population of each species was grown from a single colony overnight in 6 ml Todd Hewitt Broth (THB) shaking at 200 r.p.m. at 30 °C. Bacteria were frozen in a 1:1 ratio of sample to 50% glycerol solution in cryotubes at −80 °C.

Experimental co-evolution. The co-evolution experiment consisted of two treatments: (i) *S. aureus* and *E. faecalis* were co-passaged under co-colonization (co-evolution treatment), and (ii) *S. aureus* evolution was passaged on its own (single evolution treatment) within *C. elegans* hosts (Fig. 3a). Each treatment had five replicate populations and was conducted for 10 passages. Every passage, bacteria were cultured overnight in 6 ml THB shaking 200 r.p.m. at 30 °C. They were then standardized to an OD600 reading of 1.00, and 120 µl of each species was spread onto Tryptic Soy Broth (TSB) agar plates and kept overnight at 30 °C



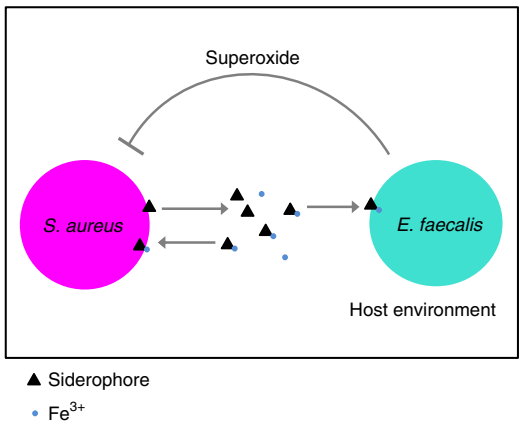


Figure 8 | Model of *S. aureus*(pathogen)—*E. faecalis*(defensive microbe) interactions within the nematode host. *S. aureus* produces iron-scavenging siderophores that *E. faecalis* can exploit. In turn *E. faecalis* produces superoxide anions that suppress pathogen growth[19].

Figure 7 | Effect of siderophores on defensive microbe fitness. (**a**) Exogenous siderophore concentration (µg ml^{-1}) against growth rate of ancestral *E. faecalis in vitro*. Pearson's product-moment correlation: $t = 6.1$, df = 2, $P = 0.026$, $R^2 = 0.95$. Sample size: one biological replicate for each concentration (average of two technical replicates). (**b**) Fitness of ancestral *E. faecalis* (CFU ml^{-1}) after 24 h growth *in vitro* with ancestral or evolved *S. aureus*. The black line indicates the average fitness of *E. faecalis* in the absence of *S. aureus*. Statistical comparisons are made between this black line and each treatment. ANOVA: $F = 9.2$, df = 3, $P = 0.0009$. Dunnett contrasts: co-evolution *S. aureus* and ancestral *E. faecalis* versus ancestral *E. faecalis*: $P = 0.77$; single evolution *S. aureus* and ancestral *E. faecalis* versus ancestral *E. faecalis*: $P = 0.023$; Ancestor *S. aureus* and ancestral *E. faecalis* versus ancestral *E. faecalis*: $P < 0.001$. Sample size for each treatment: five biological replicates (one technical replicate). (**c**) Fitness of ancestral *E. faecalis* (CFU ml^{-1}) after 24 h growth *in vitro* with ancestral or evolved *S. aureus* with ($+Fe^{3+}$) and without (control) surplus iron. Control ANOVA: $F = 8.1$, df = 2, fdr corrected $P = 0.019$. Tukey contrasts: Ancestor versus co-evolution: $P = 0.046$; Single evolution versus co-evolution: $P = 0.009$; single evolution versus ancestor: $P = 0.57$. $+Fe^{3+}$ ANOVA treatment: $F = 3.05$, df = 2, fdr corrected $P = 0.092$. Sample size for each treatment of both ANOVAs: five biological replicates (one technical replicate). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

to make the exposure lawns. Co-culture exposure lawns for the co-evolution treatment were made by mixing 120 µl of each species together such that their population sizes were kept the same across treatments. Approximately 1,000 young adult worms from the genetically homogenous stock were placed onto each exposure lawn and incubated at 25 °C for 24 h.

After 24 h, 10 dead worms were picked from each lawn to ensure we took hosts colonized with bacteria. Under co-colonization dead worms did not differ significantly from live worms in the proportion of pathogen collected from the infection (Supplementary Fig. 1; two sample *t*-test; $t = 1.22$, $df = 10$, $P = 0.25$). Worms were considered dead when they did not respond to touch with a platinum wire[20]. Worms were washed to remove external bacteria by moving them between five 5 µl drops of M9 buffer on fresh TSB plates using a platinum wire, an adapted method[20,38]. Worms were then crushed in 20 µl of M9 in a 1 ml Eppendorf tube with a pestle to release internal bacteria. An inoculation loop was used to streak each sample onto selective media (TSB plates with 100 µg ml^{-1} rifampicin were used to isolate *E. faecalis* from the co-evolution treatment, and Mannitol Salt Agar (MSA) plates isolated *S. aureus* from both treatments) and the plates were grown overnight at 30 °C. Ten colonies of *S. aureus* and *E. faecalis* were picked from each replicate and grown in THB overnight, shaking at 200 r.p.m. at 30 °C, and were used for the next generation of exposures.

Virulence assays. Exposure plates were made in the same way as for the evolution experiment (see above). Approximately 150 young adult worms from the stock *C. elegans* population were placed onto exposure plates and incubated at 25 °C for 24 h. Exposure plates were labelled with a random code so that the treatments were unknown during measurement. The total numbers of worms were counted and the proportion dead calculated as a measure of virulence. Worms were considered dead when they did not respond to touch with a platinum wire[20].

***In vivo* bacterial population sizes.** Exposure plates were made in the same way as for the evolution experiment (see above). Approximately 1,000 young adult worms from the stock *C. elegans* population were placed onto exposure plates and incubated for 24 h at 25 °C. Exposure plates were labelled with a random code so that the treatments were unknown during experimentation. After 24 h of exposure, 3–5 worms were picked from each plate. For Supplementary Fig. 1, live worms were picked for each replicate of the live treatment and dead worms were picked for each replicate of the dead treatment. For all other infection load assays dead worms were picked, reflecting the evolution experiment. Worms were washed to remove external bacteria by moving them between five 5 µl drops of M9 buffer on fresh TSB plates using a platinum wire. Worms were then crushed in 20 µl of M9 in a 1 ml Eppendorf tube with a pestle to release internal bacteria. After crushing, serial dilutions were plated onto selective media and grown overnight at 30 °C. MSA and rifampicin TSB selective media were used to count *S. aureus* and *E. faecalis* colony-forming units (CFU's) per worm host, respectively.

***In vitro* bacterial growth rate.** Bacterial growth rate was measured in 96-well plates. Bacteria were cultured overnight in 6 ml THB shaking 200 r.p.m. at 30 °C. A volume of 2 µl of bacteria was added to 198 µl THB and kept for 10 h in a plate reader set to shake intermittently at 30 °C. OD630 Readings were taken every 1 min and 15 s with Gen5 software and Curve fitter software was used to calculate growth rates of the bacteria in the exponential growth phase.

Siderophore production. Ancestral and evolved *S. aureus* populations were grown in 6 ml THB in falcon tubes with loose cap lids allowing for unlimited oxygen, and so growth, and were shaken at 200 r.p.m. overnight at 30 °C. Overnights were standardized by OD630 and then filtered to remove bacterial cells. One hundred microlitre of each sample was added to 100 µl reaction mixture from the SideroTech (EmergenBio) kit in a 96-well plate and incubated at room temperature for 5 min. OD630 was then measured in a plate reader at room temperature. The concentration of siderophore was estimated from a reference curve of the relationship between OD630 and desferoxamine siderophore (EmergenBio) concentration (Supplementary Fig. 2). This reference curve was made by measuring the OD630 of samples containing desferoxamine siderophore at known concentrations increasing from 0 to 100 µg ml^{-1}. The SideroTech assay works by measuring the changes in OD630 when siderophore removes ferric iron from the reagent complex (composed of a dye, iron and a detergent).

Siderophores and *in vitro E. faecalis* growth rate. The growth rate of ancestral *E. faecalis* was measured in the presence of increasing desferoxamine (EmergenBio) siderophore concentrations in THB. Bacteria were cultured overnight in 6 ml THB shaking 200 r.p.m. at 30 °C. A volume of 2 µl of overnight ancestral *E. faecalis* culture was added to a mixture of 160 µl THB and 40 µl siderophore-diluent solution at final siderophore concentrations of 1.25, 2.5, 5 and 10 µg ml^{-1}. The OD630 was measured every 1 min and 15 s for 10 h in a plate reader set to shake intermittently at 30 °C. OD630 Readings were taken every 1 min and 15 s with Gen5 software and Curve fitter software was used to calculate growth rates of the bacteria in the exponential growth phase.

***In vitro E. faecalis* fitness with and without *S. aureus*.** Bacteria were cultured overnight in 6 ml THB shaking 200 r.p.m. at 30 °C and standardized to an OD630 of 1.00. A volume of 3 µl of ancestral *E. faecalis* and either ancestral, co-evolution or single evolution *S. aureus* populations were added to 194 µl of THB in a 96-well plate. As a control, a volume of 3 µl of ancestral *E. faecalis* was also added to 194 µl of THB without *S. aureus*. The 96-well plate was shaken at 175 r.p.m. for 24 h at 30 °C. Resulting samples were diluted and plated onto rifampicin TSB selective media to count E. *faecalis* CFU per ml as a fitness measure. Plates were labelled with a random code so that the treatments were unknown during quantification.

Surplus iron and *in vitro E. faecalis* fitness with *S. aureus*. THB containing 15 µg ml^{-1} iron (III) sulfate was made by mixing THB and iron (III) sulfate dissolved in H_2O. Control THB was made by mixing the same volume of THB and H_2O without iron. Bacteria were cultured overnight in 6 ml THB shaking 200 r.p.m. at 30 °C and standardized to an OD630 of 1.00. A volume of 3 µl of ancestral *E. faecalis* and either ancestral, co-evolution or single evolution *S. aureus* populations were added to 194 µl of surplus-iron THB or control THB in a 96-well plate. The 96-well plate was shaken at 175 r.p.m. for 24 h at 30 °C. Resulting samples were diluted and plated onto rifampicin TSB selective media to count E. *faecalis* CFU per ml. Plates were labelled with a random code so that the treatments were unknown during quantification.

Genomic extraction. Forty clones were picked from streaked cultures of *S. aureus* replicate populations from both evolved treatments and grown independently overnight in 200 µl THB in a 96-well plate shaking at 175 r.p.m. at 30 °C. Subsequently, each set of 40 clones was checked for equal OD630 and pooled in equal volumes. A single clone of ancestral *S. aureus* was similarly grown.

DNA was extracted from *S. aureus* by taking 1 ml of culture and centrifuging at 7,500 r.p.m., removing the supernatant and re-suspending the pellet in 160 µl enzymatic lysis buffer (Qiagen), 40 µl lysostaphin (200 µg ml^{-1}, Sigma-Aldrich), 40 µl lysozyme (100 mg ml^{-1}, Sigma-Aldrich) and 8 µl RNAse A (10 mg ml^{-1}, Sigma-Aldrich). This mixture was incubated for 1 h at 37 °C. Twenty-five microlitre proteinase K (Qiagen) was then added and 200 µl Buffer AL (without ethanol) was added and vortexed. This was then incubated at 56 °C for 1 h. Two-hundred microlitre of ethanol was added and vortexed and DNA purification followed DNeasy Blood and Tissue Spin-Column Protocol (Qiagen). The extracted DNA was sequenced using the HiSeq4000 platform with 100 bp paired end at the Wellcome Trust Centre for Human Genetics, Oxford.

Genomic analysis. The project accession number at the European Nucleotide Archive for the raw read data is PRJEB13385. Sequenced read data in fastq files were trimmed for the presence of Illumina adapter sequences using Cutadapt version v1.9.1 (revision e960cc1 from github.com/marcelm/cutadapt)[59]. The reads were further trimmed using Sickle version 1.33 (revision f3d6ae3 from github.com/najoshi/sickle)[60] with a minimum window quality score of 25 and retaining only reads longer than 50 bp after trimming. Single reads remaining from pairs were retained.

Reference genomes for short read mapping were obtained from the NCBI Assembly database (www.ncbi.nlm.nih.gov/assembly). Short reads of the DNA extractions from the *S. aureus* strain MSSA476 isolates were mapped to assembly GCF_000011525.1 using BWA MEM v0.7.12 (revision cc9eef2 from github.com/lh3/bwa)[61] using option −M to flag shorter split hits as secondary ensuring the single best alignment was used. Alignments were manipulated using SAMTools v1.2 (revision ac5b8e7 from github.com/samtools/samtools)[62].

The Genome Analysis Toolkit v3.4 (GATK)[63,64] Indel Realigner module was used to realign reads around putative insertions and deletions after which duplicate reads were identified and removed with Picard v1.135 (https://github.com/broadinstitute/picard/releases/tag/1.135). Single nucleotide polymorphism, insertion and deletion discovery was performed with GATK's Haplotype caller module with sample ploidy $n = 40$ (ref. 65). Default parameter values were used except the priors '—heterozygosity 0.0001 –indel_heterozygosity 0.00001' were used because diversity was expected to be relatively low given the experiment duration (these values are ten-fold lower than the defaults). Owing to the difficulties in distinguishing called variants at low frequencies from noise caused by sequencing errors, only variants at frequencies greater than 20% were included in this analysis.

Statistical analysis. Data analysis was carried out in R v 3.2.0 (http://www.r-project.org/). Parametric tests were used for all data which met the required assumptions. These assumptions were checked using the Shapiro test to detect whether data was normally distributed and F-tests to compare the variances of two samples from normal populations. Non-parametric equivalents were used if the data did not meet those assumptions. Outlying data points were detected and removed by using the Dixon test. For statistical analyses on passaged bacteria, each replicate population was treated as an independent biological replicate. Thus, there always existed five biological replicates for evolved bacterial population. Quasibinomial GLMs were used for virulence assays to correct for over-dispersion of data, and Tukey contrasts were used for *post hoc* comparisons. *t*-Tests were used for fitness assays of ancestral bacteria within the host. The Welch two sample *t*-test

was used for assays which did not have equal variance and the independent two-sample *t*-test was used when they did have equal variances.

ANOVAs were used for assays comparing ancestral and evolved *S. aureus* treatments measuring within-host bacterial fitness, siderophore production, growth rate and *in vitro* competition of bacteria. Plots of each ANOVA were checked by eye for model quality. For the analysis of ancestral *E. faecalis* in the presence and absence of ancestral and evolved *S. aureus* (Fig. 4b) we performed Dunnett contrasts between treatments with *S. aureus* and the treatment without *S. aureus* as the control. All remaining ANOVAs used Tukey contrasts for *post hoc* comparisons. For the *in vitro* competition assay where a single ANOVA was used for the treatments with surplus iron and a separate ANOVA for the treatments without surplus iron we corrected the treatment *P* values for multiple comparisons using the fdr correction.

Pearson's product–moment correlation was used to test for correlations between *S. aureus* growth rate and siderophore production against virulence, as well as the correlation between siderophore concentration and *E. faecalis* growth rate. The coefficients and R^2 value for the lines of best fit were obtained from a linear regression model. To model the relationship between siderophore concentration and optical density a non-linear curve was fitted by estimating the parameters and standard errors of the parameters as starting estimates. The parameters we estimated included the asymptote, intercept and steepest point in curve, 0.2, −0.53 and 0.039, respectively. All these parameters appeared to be significant in explaining the model and were not extraneous.

Data availability. Raw read data for the bacterial genomic sequences were deposited in the European Nucleotide Archive under project accession number PRJEB13385. CSV files containing data for each figure are available in the Dryad repository (http://dx.doi.org/10.5061/dryad.cb7ns)[66]. The authors declare that all other data supporting the findings of this study are available within the article and its Supplementary Information files, or from the corresponding authors upon request.

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Author contributions

S.A.F. and K.C.K. conceived and designed the project; S.A.F. conducted the experiments and analysed the data; S.A.F., D.K. and D.W. conducted the bioinformatics analysis; S.A.F. and K.C.K. wrote the paper.

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OPINION

Harnessing the Power of Defensive Microbes: Evolutionary Implications in Nature and Disease Control

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Microbes are vital to the functioning of multicellular organisms. This realisation has fuelled great interest in the effects of microbes on the health of plant [1–3] and animal hosts [4–6] and has revealed that microbe-mediated protection against infectious disease is a widespread phenomenon (Table 1) [7–11]. Defensive microbes can protect hosts from infection by parasites (including pathogens and parasitoids) by direct or host-mediated means (Box 1). Such protective traits have made these microbes attractive candidates for disease control. In fact, defensive microbes are already being applied in phage therapy and bacteriotherapy for humans, as well as to control vector-borne and agricultural diseases (Table 2).

Despite the impact defensive microbes can have on host and parasite fitness, our current perspective of host–parasite evolution is largely based upon pairwise species interactions [12]. By combining knowledge of defensive microbe–parasite interactions at the mechanistic level with evolutionary theory, we can predict how defensive microbes might alter the evolution of host and parasite traits, such as resistance and virulence. This will not only shape how we understand patterns of host–parasite coevolution in nature but will inform our decisisons in utilising defensive microbes as disease control agents. We propose three potential evolutionary implications of defensive microbes on host–parasite interactions.

Defensive Microbe–Parasite Coevolution

Microbes can be an evolvable component of host defence (Box 2). Given that they can have short generation times, high mutation rates, and large within-host population sizes, defensive microbes might adapt to parasites much faster than their longer-lived eukaryotic hosts are able [11]. Consequently, coevolution between defensive microbes and parasites could provide "real-time" disease control, whereby evolutionary changes in parasites are met by rapid reciprocal evolution in defensive microbes. For defensive microbes utilising direct mechanisms of interaction with parasites (Box 1), coevolution is likely to depend upon the population size, generation time, and gene flow of the defensive microbe population. For defensive microbes utilising host-mediated mechanisms of interaction with parasites (Box 1), coevolution is more likely to be limited by the rate of host evolution, since those mechanisms employ host-encoded traits. Exploring the potential for defensive microbe–parasite coevolution will be fundamental for predicting the long-term efficacy of microbe-mediated defence in the face of parasite evolution.

There are a variety of methods, traditionally used to study host–parasite interactions, that can be extended to look for evidence of defensive microbe–parasite coevolution. These include: (1) testing for signals of selection at the molecular level [13], (2) identifying coevolutionary

Box 1. Mechanisms of Defensive Microbes

Direct

Hyperparasitism or predation: Microbes can parasitise or predate upon the parasite [39].

Interference competition: Microbes can produce toxic compounds, such as antibiotics or bacteriocins, that may either kill the parasite or reduce its growth rate [40–42].

Resource competition: Microbes can compete with parasites for host resources [10,42], usually via the rate of resource acquisition [40,41].

Host-mediated

Host immune-mediation: Microbes can elicit a host immune response to which the parasite is not resistant [40,42].

Host tolerance-mediation: Microbes can increase the fitness of their host during infection without reducing the fitness of the parasite by enhancing host tolerance (e.g., via tissue damage prevention and/or repair) [43,44].

Box 2. Evolution of Defensive Microbes

Microbe-mediated defence is hypothesised to evolve from two sources of selection [11]. Firstly, microbe-mediated defence can be directly favoured when microbial fitness depends strongly on host fitness [45], such as when microbes are vertically transmitted (e.g., *Wolbachia* in Table 1) or when the host exercises "partner choice" or "host sanctions" and selectively cultures defensive microbes [8, 9, 11]. It is thought that beewolf wasps selectively acquire protective *Streptomyces* bacteria to ensure continued defence [46]. Alternatively, microbe-mediated defence might be selected as a byproduct of intraspecific and interspecific microbial interactions [11,47] (e.g., via competition over resources) or hyperparasitism (e.g., hyperparasitic viruses parasitise fungal pathogens of plants in Table 1). Hosts may also impose partner choice by exploiting microbial competition, termed "competition-based screening" [48].

Ultimately, defensive traits may differ in their efficacy over evolutionary time [11]. It is likely that defensive traits that evolve via partner choice or maternal inheritance are more evolutionarily persistent than those that evolve as a byproduct of microbial interactions. This difference is because host defence is a directly selected trait in microbes in the former but a byproduct of another interaction in the latter. Above all, evolutionary persistence will depend upon the presence of parasites [49,50]. From the defensive microbe's perspective, high parasite prevalence can select for their persistence in the host population [50,51]. However, parasities are not always highly prevalent and can have a natural periodicity (e.g., seasonality) or be spatially variable [15,52]. How this variation alters the intensity of selection for and maintenance of defensive microbes has been explored theoretically [53] and in field patterns [54] but has rarely been tested directly [4].

The host-associated costs of harbouring defensive microbes can affect the potential for such microbes to evolve and, as such, their position on the mutualism–parasitism continuum [55]. In many cases, defensive microbes have been found to be physiologically and ecologically costly to their hosts [4,56–58]. Nevertheless, if the benefit of

protection to the host outweighs any costs, then the evolution of defensive microbes will be favoured [59]. Alternatively, a host–parasite interaction would arise if defensive microbes become more costly than beneficial [59]. For example, a microbe that protects a host as a byproduct of competing for resources with a parasite could itself evolve increased host exploitation, and so virulence, by means of competition. If the cost (virulence) of this microbe now outweighs its benefit (defence), this defensive microbe is now a parasite.

Table 1. Defensive microbes in nature. Defensive microbes are listed with their host(s), the parasite(s) they protect against, their mechanism(s) of defence, their impact on parasite fitness, and their mode of transmission.

Host Group	Host(s)	Defensive microbe (s)	Parasite(s)	Mechanism(s)	Impact on parasite fitness	Transmission mode	Ref(s).
Crustacea	American Lobster	gram-negative bacterium	Fungi	Interference competition	Negative	Unknown	[80]
	Shrimp	*Alteromonas* bacteria	Fungi	Interference competition	Not measured	Unknown	[81]
Insects	Aphids	*Hamiltonella defensa; Regiella*	Parasitoids	Interference competition	Negative	Vertical	[82,83]
	Aphids	*Regiella*; *Rickettsia*; *Rickettsiella*; *Spiroplasma*	Fungi	Unknown	Negative	Vertical	[84–86]
	Beewolf wasp; Subterranean termite	*Streptomyces* bacteria	Fungi	Interference competition	Negative	Vertical and Horizontal	[87,88]
	Bumble bees	*Gilliamella*	Protozoa	Unknown	Negative	Horizontal	[16]
	Dampwood termite	Undefined microbiome	Fungi	Interference competition	Negative	Unknown	[89]
	Fruit flies	*Spiroplasma*	Parasitoids Nematodes	Interference competition	Negative	Vertical	[54,90,91]
	Fruit flies; Mosquitoes	*Wolbachia sp*.	Arboviruses Protozoans Fungi	Resource competition Immune mediation	Negative None	Vertical	[92–95]
	Invasive Whitefly	*Rickettsia*	Fungi	Unknown	Negative	Vertical	[96]
	Leaf cutting ants	*Actinomycete* bacteria	Fungi	Interference competition	Negative	Vertical and Horizontal	[97,98]
	Leaf-rolling Weevil	*Penicillium herquei*	Unknown microbes	Interference competition	Negative	Unknown	[99]
Plants	Cacao Tree	Foliar Endophytic fungi; Arbuscular Mycorrhizal fungi	Fungi Oomycetes	Resource and Interference competition Immune mediation	Negative	Horizontal	[100–103]
	Chestnut trees	Virus	Fungi	Hyperparasitism	Negative	Horizontal	[104]
	Plant species	Mycoparasitic fungi	Fungi	Hyperparasitism	Negative	Horizontal	[3,39]
Vertebrates	Hoopoe birds	*Enterococcus faecalis*	Bacteria	Interference competition	Negative	Unknown	[105,106]
	Humans	Undefined microbiome in breast milk	Bacteria	Unknown	Negative	Unknown	[107]
	Metazoan hosts (incl. humans)	Bacteriophage	Bacteria	Hyperparasitism	Negative	Unknown	[108]
	Metazoan hosts (incl. humans)	*Bdellovibrio bacteriovorus*	Bacteria	Predation	Negative	Horizontal	[109,110]
	North American Bullfrog	Undefined skin microbiome	Fungi	Interference competition	Negative	Unknown	[5]

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dynamics via translocation or time-shift experiments [4,12,14,15], and 3) detecting specificity in defensive microbe–parasite interactions [16–21]. Although tests 2 and 3 cannot be performed in human hosts, it is possible for defensive microbes and parasites of humans to be studied in model organisms, e.g., mice [22] or *Caenorhabditis elegans* [23], in which such studies could be carried out. To date, research has found that defensive microbes often interact specifically with parasites, suggesting defensive microbe–parasite coevolution [16–21]. In the case of aphid protection against parasitoids by *Hamiltonella defensa*, the specificity of microbe-mediated defence [17,19] is associated with a high diversity of toxin genes within the locus responsible for parasitoid resistance [24], indicative of rapid gene turnover due to coevolution [25]. Consistent with these data, mathematical models have revealed conditions under which defensive microbes coevolve with parasites to the point that the host becomes irrelevant [26].

Evolution of Host Dependence on Defensive Microbes

Given that defensive microbes protect hosts from parasite-induced fitness costs, they could reduce selection for costly immune-based or behavioural-based host defence mechanisms [10]. Unless defensive microbes employ host-encoded traits (e.g., host immune or tolerance mediation), selection favouring host defences could wane, making hosts dependent on microbe-mediated protection over evolutionary time. It has been speculated that the loss of immune genes in pea aphids and honeybees that possess defensive microbes are a result of the evolution of host dependence [10,27,28]; however, further research is required to test this hypothesis. Ultimately, a dependence on microbes for defence could increase host susceptibility to infection, making defensive microbe perturbation or loss (e.g., via antibiotics or diet changes) dangerous for the host [29,30].

Phylogenetic comparisons can be used to test the hypothesis that defensive microbes remove selection for host-based defences. Here, the innate resistance of host populations that harbour defensive microbes can be compared with those that do not. We can predict that host populations associating with defensive microbes should have a lower innate resistance to parasites. In support of this prediction, natural *Trachymyrmex* ant populations that harbour protective antibiotic-producing bacteria exhibit significantly reduced cleaning behaviour than populations without the bacteria [31]. The innate resistance of host populations can be assessed by removing the defensive microbe and measuring resistance to parasitic infection and/or the activity level of a host-encoded resistance trait. Given that it is rare to find natural populations that differ only in their association with a defensive microbe, an experimental evolution approach could be used, whereby host populations are coevolved with parasites in the presence or absence of a defensive microbe. The use of lab-tractable host organisms with short generation times and well-studied innate immune systems, such as *C. elegans* [32] or *Drosophila melanogaster* [33], would facilitate such experimentation and allow us to determine whether particular immune system components have been lost or are expressed less over evolutionary time.

Defensive Microbes and Parasite Virulence Evolution

Parasite virulence is defined as the infection-induced decrease in host fitness [34] and is assumed to be an inevitable consequence of host exploitation and so parasite replication [35]. The evolution of virulence can be shaped by interactions between parasites and other microbes within a host; most research to date has focused on the effects of parasite coinfections on virulence evolution (for a full review, see [36]). Critically, as mechanisms of interaction that occur between coinfecting parasites (e.g., resource competition, interference competition, and immune mediation) are similar to those between a defensive microbe and a parasite (Box 1),

Box 3. Case Examples of the Application of Defensive Microbes

Wolbachia and vector-borne parasites

Wolbachia are bacteria that live within the cells of 60%–70% of insect species and can spread vertically via manipulating host reproduction [60,61]. Two *Wolbachia* strains isolated from *Drosophila*, *w*MelPop and *w*Mel, have been found to reduce the infection load of a range of human parasites, including dengue virus, malaria, yellow fever, chikungunya, West Nile virus, and filarial nematodes, when introduced into the novel hosts *Aedes aegypti* and *Anopheles gambiae* [62–68]. Consequently, there is great interest in using *Wolbachia* to halt disease transmission [53,61]. It has been suggested that host immune mediation may be the protective mechanism of *Wolbachia* within mosquitoes [62,69–71]; however, more research is required to assess the role of other mechanisms (Box 1) across different host–*Wolbachia* combinations and over evolutionary time [72]. Trial introductions of *Wolbachia*-infected mosquitoes into natural populations have so far resulted in the successful spread of the *w*Mel strain [73,74].

More information will be critical to predicting the long-term efficacy of *Wolbachia* as a disease control strategy [75]. Specifically, determining whether the intracellular existence and vertical transmission of *Wolbachia* could constrain its evolution will indicate the potential for *Wolbachia*–parasite coevolution and so persistent disease control. Secondly, identifying the mechanism of protection will be vital in predicting whether *Wolbachia* will drive the evolution of mosquito dependence on microbe-mediated defence; if protection is indeed dominated by host immune mediation, *Wolbachia* may not remove the selection pressure for mosquito defences. Finally, characterising the mechanism(s) of *Wolbachia*-mediated defence will be important in predicting whether *Wolbachia* will select for changes in parasite virulence [75].

Human microbiome

Research into the human microbiome has uncovered information regarding microbe-mediated host defence and has highlighted promising routes for disease control (Table 2). Treatments aimed at manipulating the human microbiome commonly rely on the introduction of defensive microbes that suppress parasites by various means (Box 1). Faecal transplantation [6,76] and the introduction of probiotics [77] are two ways to use microbes to restore an unhealthy gut microbiota back to a healthy state. These methods likely rely on microbial competition to eliminate infections. Phage therapy, on the other hand, is a technique that kills targeted bacteria with hyperparasitic lytic phage viruses [78] and is another promising route to manipulate the composition of our microbiota [79]. Importantly, these methods all involve applying defensive microbes that have great evolutionary potential and so could act as persistent disease control agents in the face of parasite evolution.

Despite growing interest in the manipulation of the human microbiome to prevent or cure infectious disease, the evolutionary implications have been largely overlooked. Recent theory has shown that microbiome engineering could lead to the evolution of increased parasite virulence [38]. This prediction is based on the assumption that parasite resistance traits are also virulence traits [38]. This may often be the case, e.g., when the parasite's response to microbe-mediated defence is to elicit host inflammation or to produce a toxin against the defensive microbe that also harms the host. Such mechanisms might be common in the use of faecal transplantation and probiotics, as microbial

competition is likely dominant [38]. In direct contrast to this, studies of hyperparasite–parasite interactions have shown that bacteria become less virulent as they resist phage attack (for full review see [37]), suggesting that phage therapy in the human microbiome may select for decreased, rather than increased, virulence. Here, parasite resistance and virulence traits may instead trade off against each other. Further investigations of the generality of these predictions will be necessary to better understand how different approaches to manipulate the microbiome affect parasite virulence evolution.

Table 2. Applications of defensive microbes in infectious disease control. Defensive microbes are listed with their host(s), the parasite(s) they protect against, their mechanism(s) of defence, their impact on parasite fitness, and their mode of transmission.

Host(s)	Defensive microbe(s)	Parasite(s)	Mechanism(s)	Impact on parasite fitness	Transmission mode	Ref(s).
Fish	Probiotics (oral)	Bacteria	Multiple mechanisms	Negative	Horizontal	[111]
	Faecal transplant undefined microbiome	Bacteria	Multiple mechanisms	Negative	Horizontal	[6,76]
Mammals (inc. humans)	Phage	Bacteria	Hyperparasitism	Negative	Horizontal	[78,79,112]
	Probiotics (oral)	Bacteria Fungi	Multiple mechanisms	Negative	Horizontal	[77,113]
Mosquitoes	*Wolbachia sp*.	Arboviruses Protozoans	Resource competition Immune mediation	Negative	Vertical and Horizontal	[61,74]
Plants	Foliar Endophytic fungi; Arbuscular Mycorrhizal fungi	Fungi Oomycetes	Resource competition Interference competition Immune mediation	Negative	Horizontal	[2,3,103]

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predictions for virulence evolution based on coinfection might be applied to microbe-mediated defence. For example, it is predicted that if a parasite competes over resources under coinfection by evolving increased host exploitation, it will become more virulent [36]. Similarly, this prediction could be made for parasites resisting defensive microbes that function via resource competition. Consistent with this, recent theory on defensive microbes has hypothesised that if parasite resistance to microbe-mediated defence is correlated with parasite virulence, then defensive microbes will alter the evolution of parasite virulence [11,37,38]. It follows that the direction of this correlation will determine the direction of virulence evolution. For example, if parasite resistance measures are also virulent to the host, defensive microbes will select for increased virulence [38]. Alternatively, if parasite resistance and virulence traits trade off against each other, defensive microbes will select for decreased virulence.

The extent to which defensive microbes might affect parasite virulence evolution is of concern in the application of microbe-mediated disease control strategies (Box 3). If microbe-mediated defence via resource competition indeed selects for more virulent parasites, this potential outcome could influence the choice of the defensive microbe being applied (Table 2). This example thus illustrates the significance of linking the mechanism of microbial protection to parasite virulence evolution in the application of defensive microbes.

Conclusions

Defensive microbes are abundant in nature and have important fitness consequences for hosts and their parasites. By considering defensive microbes in the light of evolution, we can identify

traits that enable their coevolution with parasites and reveal their impacts on the evolution of host-based resistance and parasite virulence. Such information will help us to choose defensive microbes for optimal disease control. Ultimately, by combining our understanding of defensive microbes at the mechanistic level with current evolutionary theory, we will be better able to predict the role of defensive microbes in driving host and parasite evolution in nature and in the context of disease control.

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ORIGINAL ARTICLE

Rapid evolution of microbe-mediated protection against pathogens in a worm host

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Microbes can defend their host against virulent infections, but direct evidence for the adaptive origin of microbe-mediated protection is lacking. Using experimental evolution of a novel, tripartite interaction, we demonstrate that mildly pathogenic bacteria (*Enterococcus faecalis*) living in worms (*Caenorhabditis elegans*) rapidly evolved to defend their animal hosts against infection by a more virulent pathogen (*Staphylococcus aureus*), crossing the parasitism–mutualism continuum. Host protection evolved in all six, independently selected populations in response to within-host bacterial interactions and without direct selection for host health. Microbe-mediated protection was also effective against a broad spectrum of pathogenic *S. aureus* isolates. Genomic analysis implied that the mechanistic basis for *E. faecalis*-mediated protection was through increased production of antimicrobial superoxide, which was confirmed by biochemical assays. Our results indicate that microbes living within a host may make the evolutionary transition to mutualism in response to pathogen attack, and that microbiome evolution warrants consideration as a driver of infection outcome.
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Introduction

Microbes can have effects on host biology far beyond their core impacts on digestion (Dillon *et al.*, 2000; Cerf-Bensussan and Gaboriau-Routhiau, 2010; Brucker and Bordenstein, 2013; Lize *et al.*, 2014). Microbes can cause infectious disease, but they can also act to protect hosts from pathogens, a phenomenon observed in a range of animals (Dillon *et al.*, 2005; Dong *et al.*, 2009; Jaenike *et al.*, 2010; Koch and Schmid-Hempel, 2011), including humans (Kamada *et al.*, 2013), and in plants at the root–soil interface (Mendes *et al.*, 2011; May and Nelson, 2014). These protective microbes provide an important complement to the host's defence systems (Abt and Artis, 2013; Hooper *et al.*, 2012; McFall-Ngai *et al.*, 2013). As pathogens invade the host, they can not only be targeted by the host immune system, but also interact with pathogenic and commensal microbial species already present within the host (McFall-Ngai *et al.*, 2013). Resident microbes can therefore provide strong protection against virulent pathogens, and corresponding microbial traits might be evolutionarily advantageous. Evolution of this nature would represent microbes evolving along the parasitism–mutualism continuum (Chamberlain *et al.*, 2014).

The large population sizes and short generation times of microbes also create the potential for the rapid evolution of such defences. Can microbes evolve to protect their host in response to virulent pathogen challenge, and, in doing so make an evolutionary transition to mutualism? It is well established that infecting pathogens can undergo rapid adaptation (Brockhurst and Koskella, 2013) in response to transmission opportunity and mode (Messenger *et al.*, 1999), prior immune exposure (Mackinnon and Read, 2004) and multi-strain coinfection (Garbutt *et al.*, 2011) with host defences known to reciprocally evolve to pathogen adaptation (Schulte *et al.*, 2010; Morran *et al.*, 2011). Despite this, evolutionary responses by resident microbes against pathogen infection have not before been considered.

Here, we use experimental evolution to test whether a mildly pathogenic, resident microbe (*Enterococcus faecalis*) can evolve to defend its host (*Caenorhabditis elegans*) against infection by a more virulent pathogen (*Staphylococcus aureus*), and thus cross the parasitism–mutualism continuum. *E. faecalis* and *S. aureus* frequently occur in animal and human microbiomes (Holden *et al.*, 2004; Martin-Vivaldi *et al.*, 2010; Lawley *et al.*, 2012; Cruz *et al.*, 2013; Kommineni *et al.*, 2015) wherein they can be pathogenic or commensal. Both bacteria can colonise the gut of *C. elegans* (Garsin *et al.*, 2001), a model animal system

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for investigating natural and lab-based host–microbiota associations (Cabreiro and Gems, 2013; Clark and Hodgkin, 2013; Petersen *et al*., 2015) and their evolutionary consequences (reviewed in Gray and Cutter, 2014). Within the lifetime of an individual nematode, both *S. aureus* and *E. faecalis* can be harmful. *S. aureus* is highly virulent, causing 100% host mortality after approximately 2 days of exposure (Sifri *et al*., 2003) by lysing the cells lining the gut wall of nematode hosts (Gravato-Nobre and Hodgkin, 2005). By contrast, *E. faecalis* is lethal to *C. elegans* only over the longer term, requiring more than 7 days of exposure (and no food) to cause total host population reduction (Sifri *et al*., 2002) and is only mildly pathogenic in shorter-term infections. In our experimental set-up, involving 2-day colonisations (described in Figure 1), *S. aureus* is a highly virulent pathogen in single infection, whereas *E. faecalis* is a mildly pathogenic resident of the nematode gut, causing c. $<1\%$ mortality of the host. *E. faecalis* is under selection in this state.

We tested whether *E. faecalis* resident within *C. elegans* evolved to protect against *S. aureus* infection where its host was challenged with the pathogen over 15 experimental host generations. Our experiments examined the following interaction: resident *E. faecalis* was allowed to evolve inside hosts in the presence/absence of a genetically fixed pathogen (supplied from ancestral culture each host generation; experimental procedure in Figure 1), and the properties of *E. faecalis* were compared between the two treatments. Both treatments consisted of six replicate populations started from a single clone of *E. faecalis* that were then independently passaged, and thus any adaptive evolution that occurred was due to *de novo* mutation and selection. We passaged *E. faecalis* from dead hosts to observe evolutionary processes arising from species interactions within hosts, rather than imposing direct selection for host health. We found that host protection against *S. aureus* by resident *E. faecalis* evolved rapidly within nematode hosts in all replicate populations. Genomic and subsequent biochemical analyses pointed to increased production of antimicrobial superoxide as the mechanism. Our results indicate that resident microbes, even mildly pathogenic ones, can rapidly evolve to defend their hosts in response to virulent pathogenic infection.

Materials and methods

Nematode host and bacteria

C. elegans is a nematode that constantly interacts with microbes in its natural habitat (Felix and Braendle, 2010), and it can act as a predator or host for numerous species (Cabreiro and Gems, 2013; Clark and Hodgkin, 2013; Petersen *et al*., 2015). These animals are thus an established model for microbial colonisation and pathogenesis (Gravato-Nobre and Hodgkin, 2005) and their gut can be co-colonised by multiple pathogens and commensals (Peleg *et al*., 2008; Portal-Celhay and Blaser, 2012; Montalvo-Katz *et al*., 2013; Hodgkin *et al*., 2013).

The N2 wild-type nematode strain used herein was obtained from the Caenorhabditis Genetics Center

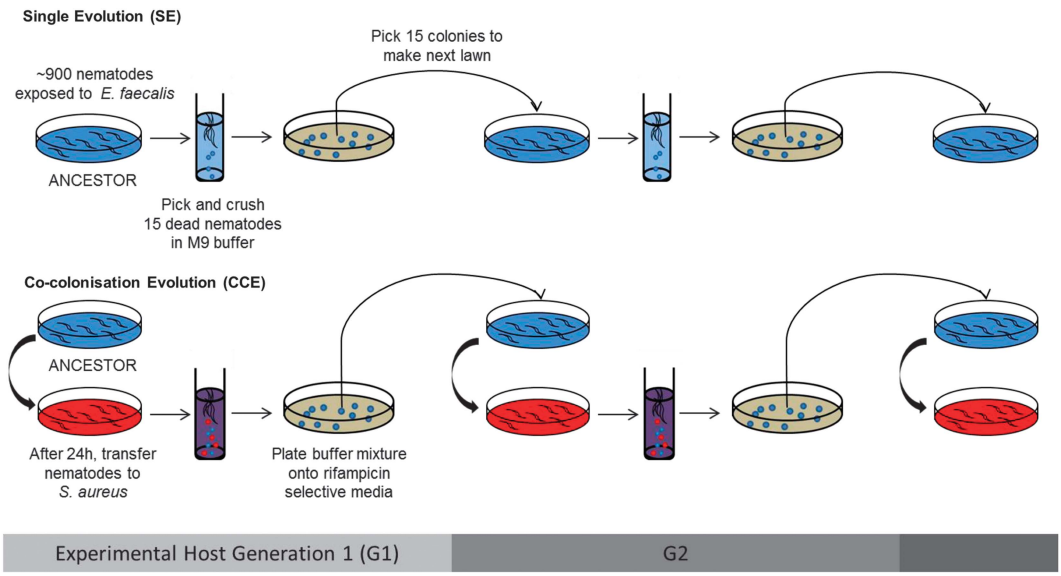


Figure 1 Experimental procedure for experimental evolution of *E. faecalis* within *C. elegans* populations. Treatments are shown for a single replicate population. Six populations of *E. faecalis* were independently passaged from a single clone ancestor for 15 experimental host generations through nematode hosts under one of two different selection regimes: (i) SE repeated passage of *E. faecalis* alone in *C. elegans* and (ii) CCE repeated passage of *E. faecalis* in *C. elegans* with a fixed, non-evolving *S. aureus* isolate. In treatment (i), nematodes were only exposed to *E. faecalis*, while in (ii), nematodes were exposed to *E. faecalis* first, so that the microbe could establish residency, and then to *S. aureus*. We enforced within-host interactions between the bacterial species by propagating *E. faecalis* cells harvested from bacteria-killed nematodes, a method that also avoided direct selection against virulence and for host health. All replicate populations were passaged at the same time during the experiment.

(University of Minnesota, Minneapolis, MN, USA). We used the *E. faecalis* lab strain OG1RF (Garsin *et al*., 2001), an isolate from the human digestive tract, and *S. aureus* strain MSSA476 (Holden *et al*., 2004), a disease-causing pathogen.

Experimental evolution

A single, randomly selected clone of *E. faecalis* was the ancestor for all evolving populations, and stock of a single clone of *S. aureus* was used. Thus, only *E. faecalis* was permitted to evolve in response to species interactions whereby they inhabited the *C. elegans* gut alone (single evolution, SE) or with *S. aureus* (co-colonisation evolution, CCE; Figure 1). Nematodes also remained evolutionarily static throughout the experiment. A stock population of N2 wild-type nematodes was derived by isolating a single hermaphroditic female every generation from the population for five generations to ensure genetic homogeneity. Stock populations of the 'isofemale' line were routinely maintained on nematode growth medium plates seeded with 50 ul of *Escherichia coli* OP50 in Luria-Bertani broth and kept at 20 °C. The nematodes digest *E. coli* after this bacterium is consumed, and it does not accumulate in the gut.

Exposure, transfer and selection

Bacteria were cultured in Todd-Hewitt (TH) broth at 28 °C overnight. Lawns of *S. aureus* liquid culture (60 µl) were plated onto 9 cm petri plates with Tryptone Soy Broth (TSB) agar, and lawns of *E. faecalis* culture (60 µl) were also plated on TSB with 100 µg ml^{-1} rifampicin (in both experimental evolution treatments). This antibiotic is used to select for *E. faecalis* OG1RF from mixed cultures. Bacterial lawns were placed at 28 °C overnight and then cooled at room temperature for several hours prior to use.

For a given replicate, approximately 900 L4 (larval) individuals, previously feeding on *E. coli*, were transferred in M9 buffer to an exposure plate with *E. faecalis*. In the CCE treatment, after 24 h, all nematodes were washed off the plate with M9 buffer and centrifuged at 3000 r.p.m. for 3 min. The supernatant was discarded, and then 5 ml M9 buffer was added to the test tube. This washing procedure was repeated five times to clean excess bacterial cells off the nematode cuticle. Nematodes were in the M9 buffer for <10 min at any given point in time. Nematodes were then transferred to the second exposure plate with *S. aureus* from a frozen culture stock. During exposures, nematodes were placed at 25 °C. *E. faecalis* populations evolved in the absence of *S. aureus* during the SE treatment were simply maintained in *C. elegans* on their plate without transfer during that period.

Twenty-four hours later, 15 bacteria-killed nematode carcasses were picked from a single replicate population and placed in a 1.5 ml centrifuge tube with 1 ml M9 buffer. The tube was centrifuged at 3000 r.p.m. for 3 min, the supernatant was discarded, and 1 ml M9 buffer was added. The wash process was repeated five more times. After the final rinse, the nematode pellet was crushed with a pestle to release the pathogens from inside the carcass. The suspension was streaked onto selective media—TSB agar with 100 µg ml^{-1} rifampicin to isolate *E. faecalis* —and individual colonies were grown up at 28 °C overnight. Subsequently, 15 colonies of *E. faecalis* were picked from a given replicate population and mixed together in 5 ml THB overnight at 28 °C overnight. This liquid culture was then used to make a lawn for the next generation of exposures for that replicate. This procedure was identical for both experimental evolution treatments to control against possible impacts of rifampicin.

The liquid cultures of an ancestral colony (prior to selection) and evolved *E. faecalis* populations were frozen at – 80 °C in 20% glycerol every five generations.

Host mortality and bacterial fitness assays

Host mortality was assayed simultaneously for each population in each treatment at the end of the evolution experiment. We exposed approximately 200 L4 nematodes from the *C. elegans* stock to the ancestral and each of the 12 evolved populations of *E. faecalis* (from the G5, G10 and G15 experimental host generations). If populations were then tested with *S. aureus*, nematodes were washed off the *E. faecalis* exposure plate with M9 buffer into a 15 ml test tube, washed and transferred to the *S. aureus* exposure plate as described above. After 24 h of exposure, we counted the total number of dead nematodes. Nematodes were considered dead if they did not respond to touch with a platinum wire, as is standard in assays of *C. elegans* death. Simultaneously, approximately 200 nematodes were placed on each of six control plates with *E. coli* OP50, but no mortality was observed after 24 h. We also tested for within-population variation in the protective effect exhibited by CCE *E. faecalis*. Four colonies from each of the six replicate populations at G15 were randomly picked, separately grown in THB media and plated. Host mortality in the presence of *S. aureus* was tested as above.

We tested the generality of this protective effect against six pathogenic, genetically divergent *S. aureus* isolates (COL-MRSA, MSSA SH-1000, Newman, N13-MSSA, Mu50 MRSA, MRSA 252), in addition to MSSA476. All isolates were cultured the same way as described above. Similar to the methods above, approximately 50 L4 nematodes from the *C. elegans* stock were exposed to only *S. aureus*, or initially to populations of *E. faecalis* (ancestral or CCE G15) and then to *S. aureus*. After 24 h of exposure at 25 °C, we counted the total number of dead nematodes.

To examine the fitness differences of *E. faecalis* (ancestor vs SE at G15 vs CCE at G15) in co-colonised nematode hosts with *S. aureus*, we measured the number of colony-forming units (cfus) of *E. faecalis*

and *S. aureus*. Five dead nematodes were picked from a plate, placed into 1 ml M9 buffer and washed repeatedly. After the final rinse, the nematode pellet was crushed with a pestle to release the bacterial cells from inside each carcass. The mixture was spread onto selective media to separate *E. faecalis* and *S. aureus* colonies (TSB with 100ug ml^{-1} rifampicin and Mannitol Salt Agar, respectively), and colonies were counted.

Mechanism of pathogen suppression

Genomic analysis. To investigate the genetic basis of host protection conferred by *E. faecalis*, whole-genome resequencing was used for a randomly selected *E. faecalis* clone from each replicate at G15. The phenotype of that clone was confirmed as being consistent with population-level effects on nematodes as assessed above. DNA was isolated using either a DNeasy blood and tissue kit using standard methods for Gram-positive bacteria or using a modified CTAB extraction (von der Schulenburg *et al*., 2001), and importantly, the addition of 10 mg ml^{-1} of Lysozyme (for *E. faecalis*) or Lyostaphin (for *S. aureus*) to the digestion step in both protocols was required. Illumina (San Diego, CA, USA) TruSeq Nano libraries were prepared from 200 ng of DNA according to the manufacturer's protocol and 250 bp paired-end reads generated on an Illumina MiSeq using v2 chemistry. Reads were trimmed for the presence of Illumina adaptor sequences using Cutadapt v1.2.1 and for a minimum quality score of Q20 using Sickle v1.200. The resulting reads (between 395 Mb and 715 Mb per sample) were then mapped to either *E. faecalis* OGIRF (NC_017316) or *S. aureus* MSSA4776 (NC_002953.3 and NC_005951, for main chromosome and plasmid, respectively) using BWA-MEM, duplicate reads were removed using Picard, local realignment and single nucleotide polymorphism calling was performed in GATK and structural variants detected using Breakdancer. Variants found in experimental but not ancestral clones were identified, and SnpEFF was used to predict their functional effects.

The genes with revealed single nucleotide polymorphisms were identified in the SEED database by 'blasting' the corresponding sequences against a collection of *E. faecalis* genomes. The gene annotations were confirmed or suggested by analysis of the sequences and the 20000 bp window neighbourhoods of the corresponding genes. The composition of gene loci of the top 10 homologues in other bacteria was also analysed. STRING database and software was used to reconstruct gene connectivity networks for the detected genes. This application automatically assembles the data on gene positional associations in genomes, genetic, regulatory and physical protein interactions for the input genes that satisfy a set of confidence thresholds.

In vitro biochemical assays. We assessed for a difference in the ability of ancestral and evolved *E. faecalis* to produce superoxides as the mechanism of host protection. Ancestral *E. faecalis,* SE *E. faecalis* (at G15) and CCE *E. faecalis* (at G5 and G15) were grown overnight to stationary phase in TSB. Wells in an opaque, black 96-well plate with a transparent bottom were then inoculated with 5 µl from each overnight culture. Three technical replicates of each replicate population were made. Replicate populations that failed to grow properly in liquid culture were excluded from the analysis. The wells were prepared with 95 µl TSB and 100 µl of a reaction mixture from a superoxide ion assay kit (Sigma Aldrich, St Louis, MO, USA) containing luminol: a reagent that becomes luminescent following oxidation by superoxide, allowing the quantitative and relativistic measure of superoxide production. The inoculated reaction mixtures were monitored over 10 h (for which the kit was optimised by Sigma-Aldrich) and measured every 2.5 min for both OD_{600} and luminescence in a Synergy 2 plate reader (BioTek, Winooski, VT, USA). The actual luminescence produced by a sample is sensitive to starting conditions as it is proportional to bacterial biomass concentration. Bacterial growth is sensitive to several factors (that is, media concentration, population size) and is exponential, translating small differences in growth rate to large differences in luminescence. We thus simultaneously measured bacterial biomass concentration (OD_{600}) and controlled for it in our luminescence data.

To examine the impact of superoxide production by evolved *E. faecalis* on *S. aureus* (and whether this was the source of suppression), we tested the degree to which the evolved enhanced suppression could be removed by the action of catalase (CAT) and superoxide dismutase (SOD). Superoxide dismutase converts superoxide into hydrogen peroxide, and CAT converts hydrogen peroxide into water and oxygen. Alone, SOD would remove superoxides by simply replacing it with harmful hydrogen peroxide. Likewise, CAT on its own would only remove the problems caused by hydrogen peroxide without affecting superoxides. Together, these enzymes create a pathway converting harmful superoxide into harmless products. If exogenous superoxides were responsible for *S. aureus* growth inhibition, we would therefore expect this inhibition to be lifted only when both enzymes are administered. Overnight cultures of all ancestral and CCE populations of *E. faecalis,* as well as *S. aureus*, were grown separately in TSB (standardised to an OD_{600} of 1.00 ± 0.05). A solution of TSB was prepared with 0.25 M potassium phosphate buffer containing Superoxide Dismutase E.C. 1.15.1.1 (SOD) from bovine erythrocytes (Sigma-Aldrich) and Catalase E.C. 1.11.1.6 (CAT) from bovine liver (Sigma-Aldrich) each at 0.25 mg ml^{-1}. An enzyme-free solution of TSB was also prepared as a control, containing the 0.25 M potassium phosphate buffer alone. The ancestor and CCE *E. faecalis* (two technical replicates of each replicate population) were mixed in equal ratios with *S. aureus*. From the liquid culture, 6 µl was

used to inoculate wells in a 96-well plate with 196 µl of the TSB solution alongside wells of an *S. aureus* control (*S. aureus* only). The experiment was run in duplicate on the enzyme-free and enzyme-containing media. Cultures were shaken for 24 h at 30 °C, after which cfu counts were performed.

Statistical analyses
All statistical analyses were conducted in SPSS 20.0.

Host mortality and bacterial fitness assays. Mortality data met assumptions of normality and equal variances. We performed an analysis of variance (ANOVA) on untransformed data to test for the difference in nematode mortality caused by *E. faecalis* and *S. aureus* independent colonisation, as well as their co-colonisation.

For the evolution experiment, we examined changes in nematode mortality every G5 in both the SE and CCE selection regimes. We performed a generalised linear model with binomial distribution (and maximum likelihood estimates) on host mortality data in the evolution treatments (with and without the presence of *S. aureus*) over time. Treatment and time (experimental generations) were fixed effects. A separate ANOVA was conducted to test for variation among isolates in within-population protective effects.

To examine the spectrum of host protection, we quantified nematode mortality upon co-colonisation by *E. faecalis* and a diverse range of *S. aureus* isolates including both laboratory and human disease isolates (Figure 4). An ANOVA was performed on host mortality data collected from single infections of *S. aureus* across the seven isolates to examine the variability in nematode mortality they produced. We then performed a generalised linear model with binomial distribution (and maximum likelihood estimates) on host mortality data with treatment ('alone', 'with ancestral *E. faecalis*' and 'with CCE *faecalis*') and *S. aureus* isolate as fixed effects.

The number of *E. faecalis* and *S. aureus* viable cfus was square-root transformed to meet parametric assumptions. Separate ANOVAs were performed on transformed cfu values for each of *E. faecalis* and *S. aureus* within a dead, co-colonised nematode to test the effects of treatment on bacterial fitness. Least square mean contrasts were performed to test for differences between treatments.

Mechanism of superoxide production and pathogen suppression. Mean superoxide production was compared between ancestral and evolved *E. faecalis* populations from the *in vivo* experiment during the exponential growth phase (6–10 h) of the bacteria using *t*-tests as the data met assumptions of normality. Luminescence measurements were controlled for OD_{600}.

S. aureus growth in liquid culture was compared in the presence and absence of *E. faecalis*. We performed a generalised linear model with Poisson loglinear model (and maximum likelihood estimates) on *S. aureus* cfu values with treatment ('alone', 'with ancestral *E. faecalis*' and 'with CCE *E. faecalis*'), and enzymes (presence and absence) as fixed effects. Their interaction was also evaluated.

Results

Changes in host mortality due to within-host microbial evolution
Ancestral *E. faecalis* is mildly pathogenic within the 2-day exposure window of this experiment, with <1% of nematodes dying after colonisation. In contrast, 52% of worms exposed to *S. aureus* died after exposure. Colonisation of worms with *E. faecalis* before exposure to *S. aureus* results in intermediate rates of nematode mortality, indicating that resident *E. faecalis* has the potential to suppress *S. aureus* virulence (Figure 2a; ANOVA: $F_{2,18} = 51.908$, $P < 0.001$).

At the end of the evolution experiment, we assayed the protective ability of *E. faecalis* evolved in nematodes, either alone or with *S. aureus* co-colonisation (Figure 1). All of the six replicate CCE *E. faecalis* populations evolved to further suppress its virulence. Whereas 18% of worms died within 24 h of *S. aureus* exposure in the presence of the ancestral *E. faecalis* resident, this declined to 1% mortality, on average, in the presence of resident CCE *E. faecalis* from G5 onwards (Figure 2b). Although there is some among-population variation in the mortality rates caused by SE *E. faecalis* upon challenge with *S. aureus*, none of the replicate populations evolved significantly enhanced protective ability relative to the ancestor (Figure 2b; Generalised Linear Model, Treatment: Wald $\chi^2 = 280.723$, $P < 0.001$; Time: Wald $\chi^2 = 97.230$, $P < 0.001$). When four colonies within each replicate population of the CCE treatment were tested at G15, an equivalently enhanced protective effect was observed (ANOVA: $F_{5,24} = 0.318$, $P = 0.895$).

Lower host mortality on *S. aureus* exposure was not associated with a reduction in *E. faecalis* virulence. Rather, whilst mortality remained generally low (<2%) in all replicate populations, *E. faecalis* evolved in both treatments to increase nematode mortality over time when tested alone (Figure 2c; Generalised Linear Model: Treatment: Wald $\chi^2 = 9.126$, $P = 0.003$; Time: Wald $\chi^2 = 22.510$, $P < 0.001$). Thus, although highly beneficial to hosts when tested in the presence of *S. aureus*, on its own, CCE *E. faecalis* remained mildly pathogenic and costly for the nematode host to possess. This result clearly demonstrates the context dependence of the fitness effects of this protective microbe upon hosts (Chamberlain *et al*., 2014).

Spectrum of host protection
All CCE *E. faecalis* populations at G15 were effective at protecting nematode hosts against a broad spectrum of *S. aureus* isolates (Figure 3). In single infections, these *S. aureus* isolates exhibited variation in their

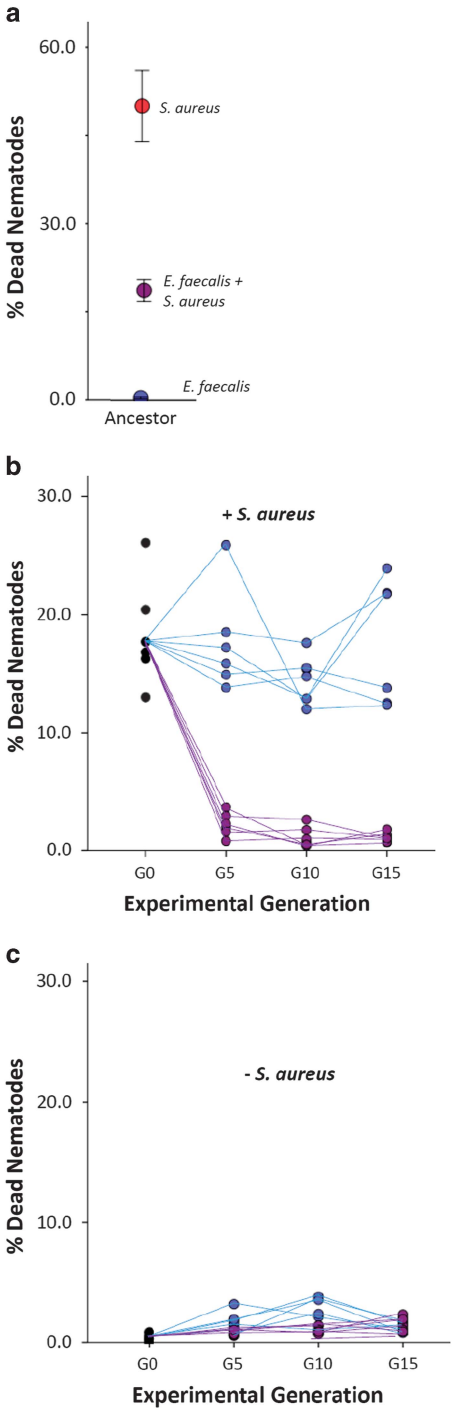


Figure 2 Effects of resident microbes on hosts over evolutionary time. (**a**) Host mortality with ancestral *E. faecalis* (blue circle) and *S. aureus* (red circle) separate and co-colonising (purple circle) in the nematode. The intermediate level of virulence from co-colonising bacteria species suggested the potential for *E. faecalis* to suppress pathogenic *S. aureus*. Error bars, 1 s.e. (**b, c**) Populations of *E. faecalis* were evolved under two different selection regimes: SE and CCE for 15 experimental host generations. To assess the ability of *E. faecalis* to protect hosts from *S. aureus*, host mortality in the (**b**) presence and (**c**) absence of *S. aureus* was quantified every G5 for SE (blue circles) and CCE (purple circles) *E. faecalis*. Lines connect each of the six replicate populations per treatment across time.

virulence towards *C. elegans* (Figure 3; 26–65% mean nematode mortality; ANOVA $F_{6,42} = 10.505$, $P < 0.001$). In co-colonised hosts, all *S. aureus* isolates with ancestral *E. faecalis* produced intermediate rates of host mortality, whereas with all replicate populations of CCE *E. faecalis*, nematode mortality was dramatically reduced to 0–1%. Both treatment and isolate affected the virulence of pathogens on hosts (Generalised Linear Model, Treatment: Wald $\chi^2 = 370.961$, $P < 0.001$; Isolate: Wald $\chi^2 = 303.650$, $P < 0.001$).

Host protection and microbial growth within hosts

We examined whether the evolved *E. faecalis* suppression of *S. aureus* virulence was associated with increased *E. faecalis* proliferation and reduced *S. aureus* growth (Figure 4). Compared with ancestral *E. faecalis,* CCE *E. faecalis* (assayed at G15) suppressed *S. aureus* viable bacterial counts more (Figure 4; d vs f) and accumulated marginally more within nematodes (Figure 4; a vs c). By contrast, SE *E. faecalis* populations did not grow to higher density on average than the ancestor when interacting with *S. aureus* (Figure 4; a vs b). These SE populations were also associated with higher within-host growth of *S. aureus* compared with CCE *E. faecalis* (Figure 4; Analysis for *S. aureus* cfu: ANOVA across the three treatments: $F_{2,18} = 4.072$, $P = 0.039$; Least Square difference d > f, $P = 0.038$; Least Square difference e > f, $P = 0.019$; Analysis for *E. faecalis* cfu: ANOVA across all three treatments: $F_{2,18} = 3.603$, $P = 0.053$; Least Square difference a < c, $P = 0.057$; b < c, $P = 0.023$ a = b, $P = 0.649$).

Suppression of *S. aureus* may occur either directly from the presence of *E. faecalis*, be mediated by *E. faecalis*-induced alterations to host biology or be a product of both direct and host-mediated effects. We assessed the importance of direct suppression using *in vitro* experiments. *In vitro* experiments recapitulated *in vivo* assays showing that CCE *E. faecalis* populations were better able to suppress *S. aureus* growth in liquid culture than ancestral *E. faecalis*

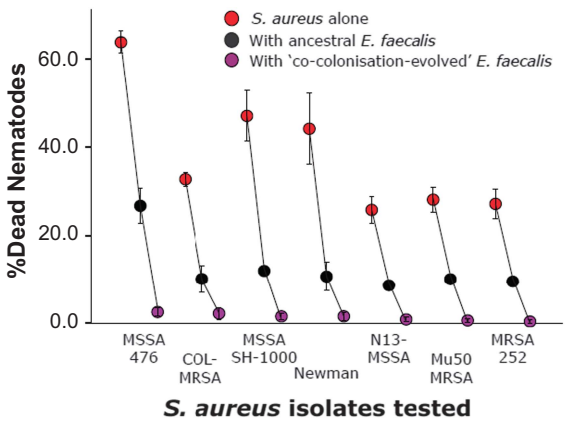


Figure 3 Generality of host protection by evolved *E. faecalis* against seven *S. aureus* isolates. Host mortality was evaluated after 24 h of exposure to *S. aureus*. Nematodes were exposed to *S. aureus* alone (red circles) or were previously colonised by ancestral *E. faecalis* (black circles) or CCE *E. faecalis* at G15 (purple circles). MSSA476 was used in the evolution experiment. Error bars, 1 s.e.

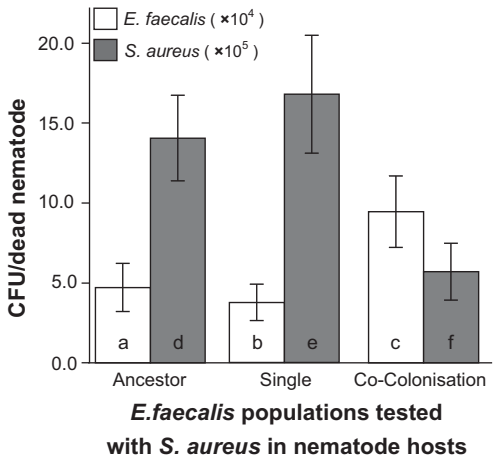


Figure 4 Fitness (cfus/nematode) of resident *E. faecalis* populations and infecting *S. aureus*. *S. aureus* is co-colonising with ancestral, SE, or CCE *E. faecalis* populations. Error bars, 1 s.e.

(Figure 5a; Generalised Linear Model, Treatment: Wald $\chi^2 = 3.18 \times 10^{11}$, $P < 0.001$).

Genomic and biochemical analysis of the mechanism underpinning protection

To investigate the genetic basis of *E. faecalis*-mediated protection, we whole-genome resequenced a randomly picked clone of ancestral *E. faecalis* and evolved *E. faecalis* from each of the 12 replicate populations at G15. Each evolved *E. faecalis* clone exhibited a unique set of between one and three mutations (Supplementary Table 1). Consistent with the distinct phenotypes that evolved under the two contrasting treatments, the SE and CCE regimes selected for substitutions in different, functionally distinct gene sets. Six of 12 mutations in the CCE *E. faecalis* clones—1 per clone per replicated population—were putatively associated with superoxide production, but no mutations associated with this pathway were observed in clones from the SE treatment. *E. faecalis* is known to produce extracellular superoxide (Huycke *et al*., 2011), mediated by dehydrogenase and fumarate reductase. Mutations were found in two NADH dehydrogenases and four genes associated with the respiratory complex function or purine biosynthesis. Purine biosynthesis represents the major pathway for fumarate production which, if blocked, leads to superoxide production (Supplementary Table 1; Supplementary Figure 1).

We therefore hypothesised that enhanced production of antimicrobial reactive oxygen species was the mechanism behind *E. faecalis*-mediated defence. In accordance with this hypothesis, all CCE *E. faecalis* populations produced more superoxide per cell than the ancestor, in both the G5 and G15 generations (Figure 5b; *t*-test: Ancestor vs G5, $t = -3.056$, $df = 31.385$, $P = 0.005$; Ancestor vs G15, $t = -2.619$, $df = 14.888$, $P = 0.019$). Moreover, there was no difference in superoxide production between SE and ancestral *E. faecalis* ($t = 0.788$, $df = 20.329$, $P = 0.440$) suggesting that this trait only evolved during *S. aureus* challenge. The addition of CAT and SOD enzymes to growth media ablated the suppression of *S. aureus* growth by *E. faecalis* during *in vitro* interactions (Figure 5a; Generalised Linear Model, Enzymes: Wald $\chi^2 = 8.49 \times 10^{10}$, $P < 0.001$), and had a greater effect at reducing suppression during interactions with CCE *E. faecalis* compared with the ancestor (Generalised Linear Model, Treatment × Enzymes: Wald $\chi^2 = 7.24 \times 10^{10}$, $P < 0.001$). Together these data strongly point to increased superoxide production by evolved CCE *E. faecalis* as the mechanism of suppression of *S. aureus*.

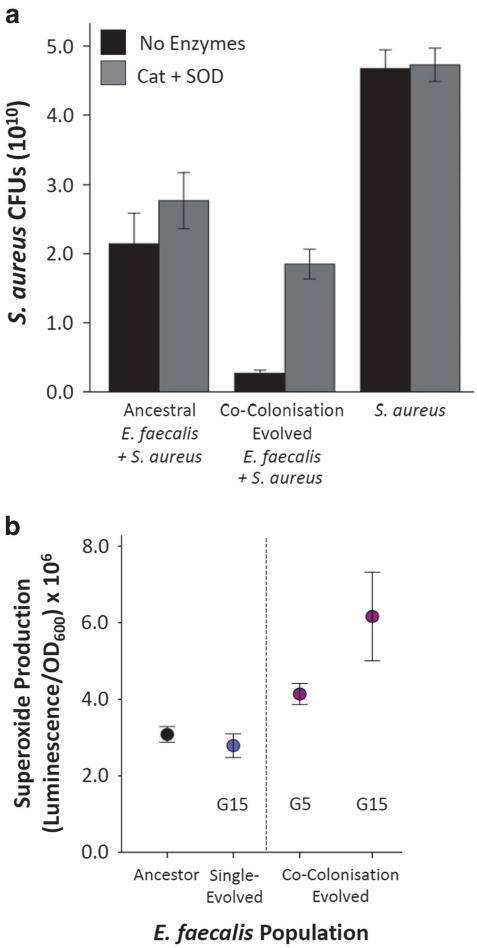


Figure 5 Evolved mechanism of suppression of *S. aureus* by *E. faecalis*. (**a**) Suppression and enzyme-mediated lifting of suppression of *S. aureus* outside the host. *S. aureus* cfus were counted when the pathogen was grown alone and co-cultured with ancestral or CCE *E. faecalis*. Counts were also made upon the addition of CAT and SOD, enzymes that remove the presence of reactive oxygen species. Error bars, 1 s.e. (**b**) Mean superoxide production (measure of luminescence controlling for OD_{600}) across exponential growth phase of ancestral, SE and CCE *E. faecalis* (the latter at G5 and G15).

Discussion

The role of microbes in protecting their host against virulent pathogens has traditionally focused on ecological sources of protection, namely niche occupancy

and competition for resources (for example, in insects, Gerardo and Parker, 2014). We hypothesised that owing to the high evolutionary potential of microbes —associated with their short generation times and large within-host population size—rapid *de novo* microbial evolution could have a role in shaping host resistance against infection. We observed the evolution of host-protective effects during microbial experimental evolution within nematode hosts in all independently passaged CCE populations, thus confirming the potential for this process to occur. Thus, *E. faecalis*, a microbe that has been observed in natural microbiomes to possess protective traits (Martin-Vivaldi *et al.*, 2010; Kommineni *et al.*, 2015), evolved across the parasite-mutualist continuum to become a host-protective mutualist upon pathogen attack. Notably, these host-protective effects evolved without any direct selection against host mortality. Instead, a beneficial relationship between the host and the resident bacterium emerged out of interactions with a virulent pathogen and selective processes acting upon the resident microbial populations. Although CCE *E. faecalis* populations evolved the ability to attenuate the high mortality caused by *S. aureus*, they also retained mild pathology against *C. elegans* when colonising alone, demonstrating the context dependence of their fitness effects on the host (Chamberlain *et al.*, 2014). In an environment where such virulent infection is common, *E. faecalis* would therefore now represent a net mutualist with respect to its impact on host fitness. This result reflects observations of other protective microbes found naturally, which defend their host whilst retaining pathogenicity (Martinez *et al.*, 2014; Polin *et al.*, 2014).

The mechanisms of microbe-mediated protection observed in nature are remarkably diverse (Gerardo and Parker, 2014). Although niche occupation (Dillon *et al.*, 2005), resource competition and immune system mediation (Abt and Artis, 2013; Hooper *et al.*, 2012; McFall-Ngai *et al.*, 2013) may still have a role in our system, the genomic evidence indicates that selection acted predominantly through direct *E. faecalis*–*S. aureus* interactions during host colonisation. Further experiments, however, are required to determine whether the microbial interactions observed to evolve here are adaptive to the host environment or whether similar evolutionary outcomes would arise *in vitro*. Regardless, we observed parallel evolution of the superoxide production pathway in CCE *E. faecalis* across all replicate populations, and we were able to ablate the evolved suppression through enzymatic treatment to remove superoxide radicals. These data strongly suggest that antimicrobial superoxides, which may act to directly suppress *S. aureus* or act indirectly via oxidation of the *S. aureus* auto-inducing pheromone (Rothfork *et al.*, 2004), are a key mechanism in the evolved protective phenotype. The lack of genotype specificity we observed is also consistent with a superoxide-mediated suppression system, which represents a broad-spectrum form of microbial suppression. While *C. elegans* itself produces reactive oxygen species in response to pathogen infection (Chávez *et al.*, 2007), reactive oxygen species produced by resident bacteria may also be a common means of broad-spectrum protection against infection, and one that is thus likely to be evolutionary labile in its activity. For instance, lactic acid bacteria in the guts of honeybees are able to suppress a range of pathogens, including *S. aureus* and *Pseudomonas aeruginosa* via reactive oxygen species production (Olofsson *et al.*, 2014). That our experimental treatment drove the evolution of a broad-spectrum defence mechanism, as opposed to more specific mechanisms of suppression (for example, bacteriocin secretion), is also consistent with observations from natural disease systems showing that microbes can protect against a diversity of pathogen isolates (Koch and Schmid-Hempel, 2011) and species (Martinez *et al.*, 2014).

The extent of the protective phenotype that evolved here, and the rate at which it evolved, were striking. Despite being regularly attacked by pathogens, if nematode hosts were colonised by evolving *E. faecalis*, they were almost universally protected against pathogens that would otherwise quickly kill most of the population. Moreover, although the evolution in our experiment occurred during passage through a number of individual worms, the time frame for the evolution of protection by *E. faecalis* was just 5 days of co-colonisation. This short timescale presents the possibility of the evolution of microbe-mediated protection within the lifetime of a longer-lived host, such as a mammal or tree, in which evolution is likely potentiated by larger population sizes.

Future research will need to establish how within-host evolution of microbial species would alter disease progression. A key simplification in our experiment is that the virulent pathogen is genetically fixed, thus mimicking a spillover zoonotic infection whereby the pathogen normally circulates in a different host species. An example is *Salmonella*. Some isolates of this bacterium commonly reside within the microbiomes of livestock animals, but can cause serious infections if transmitted to humans. Within a host individual, however, it is possible that pathogen evolution would also occur on a similar timescale, obviating any evolved protective abilities in resident microbial species and setting the stage for coevolutionary interactions. Our experiment also considers only a binary microbial interaction, whereas natural microbial communities are often highly diverse. The impacts of the evolution of the microbiome on pathogen attack (Mueller and Sachs, 2015) and on interactions within the microbiome also warrant consideration. Notwithstanding this, the potential for evolution of interactions between resident microbes and pathogens is clear, and future research on microbiome–pathogen relationships should go beyond ecological responses to examine evolved ones.

Conflict of Interest

The authors declare no conflict of interest.

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