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

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Abstract: 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.

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., 2013; McFall-Ngai et al., 2013). As pathogens invade the host, they can 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. *Enterococcus 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 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 two 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 seven 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 Fig. 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. *Enterococcus 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 Fig. 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

Caenorhabditis 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 (University of Minnesota, Minneapolis, MN). 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; Fig. 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 (NGM) plates seeded with 50uL of *Escherichia coli* OP50 in Luria-Bertani broth and kept at 20ºC. 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 28ºC overnight. Lawns of *S. aureus* liquid culture (60µL) were plated onto 9cm petri plates with Tryptone Soy Broth (TSB) agar, and lawns of *E. faecalis* culture (60µL) were also plated on TSB with 100µg/mL rifampicin (in both experimental evolution treatments). This antibiotic is used to select for *E. faecalis* OG1RF from mixed cultures. Bacterial lawns were placed at 28ºC 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 hours, all nematodes were washed off the plate with M9 buffer and centrifuged at 3000 rpm for three minutes. The supernatant was discarded, and then 5mL 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 25ºC. *Enterococcus 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 1.5mL centrifuge tube with 1mL M9 buffer. The tube was centrifuged at 3000 rpm for three minutes, the supernatant was discarded, and 1mL 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 100µg/mL rifampicin to isolate *E. faecalis* – and individual colonies were grown up at 28ºC overnight. Subsequently, 15 colonies of *E. faecalis* were picked from a given replicate population and mixed together in 5mL THB overnight at 28ºC 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 twelve 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 hours 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 hours. 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 hours of exposure at 25ºC, 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 1mL 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 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 (Schulenburg et al., 2001), and importantly the addition of 10 mg/ml 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 200ng of DNA according to the manufactor's protocol and 250bp 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 395Mb and 715Mb 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 SNP 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 SNPs were identified in 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 5µl 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 analysis. The wells were

prepared with 95µl TSB and 100µl 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 hours (for which the kit was optimized by Sigma-Aldrich) and measured every 2.5 minutes 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 (i.e. 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 (OD600) 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). SOD converts superoxide into hydrogen peroxide (H_2O_2), and CAT converts H_2O_2 into water and oxygen. Alone, SOD would remove superoxides by simply replacing it with harmful H_2O_2. Likewise, CAT on its own would only remove the problems caused by H_2O_2 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 0.25M 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 0.25mg/ml. An enzyme-free solution of TSB was also prepared as a control, containing the 0.25M 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, 6µl was used to inoculate wells in a 96-well plate with 196µl 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 24h 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 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 generalized 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 (Fig. 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 generalized 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 colony-forming units (cfu) 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 hours) 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 generalized 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 exposure window of this experiment, with <1% of nematodes dying after colonisation. In contrast, 52% of worms exposed to *S. aureus* died after exposure, indicating that it is highly virulent. Colonisation of worms with *E. faecalis* before exposure to *S. aureus* results in intermediate rates of nematode mortality, suggesting that resident *E. faecalis* has the potential to suppress *S. aureus* virulence (Fig. 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 (Fig. 1). All of the six replicate CCE *E. faecalis* populations evolved to further suppress its virulence. Whilst 18% of worms died within 24 hours 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 (Fig. 2B). While 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 (Fig. 2B; Generalised Linear Model, Treatment: Wald $\chi^2 = 280.783$, $P < 0.001$; 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 (Fig. 2C; Generalized 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 (Fig. 3). In single infections, these *S. aureus* isolates exhibited variation in their virulence towards *C. elegans* (Fig. 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 (Fig. 4). Compared to ancestral *E. faecalis*, CCE *E. faecalis* (assayed at G15) suppressed *S. aureus* viable bacterial counts more (Fig. 4; d vs f) and accumulated marginally more within nematodes (Fig 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* (Fig. 4; a vs b). These SE populations were also associated with higher within-host growth of *S. aureus* compared to CCE *E. faecalis* (Fig 4; Analysis for *S. aureus* cfu: ANOVA across the three treatments: $F_{2,18} = 4.072$, $P = 0.039$; Least Square difference d > f, $P = 0.038$; LSD e > f, $P = 0.019$; Analysis for *E. faecalis* cfu: ANOVA across all three treatments: $F_{2,18} = 3.603$, $P = 0.053$; LSD 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* (Fig. 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 – one 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. *Enterococcus 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 Fig. 1).

We therefore hypothesised that enhanced production of antimicrobial reactive oxygen species (ROS) was the mechanism behind *E. faecalis*-mediated defence. In accordance with this hypothesis, all CCE *E. faecalis* populations produced more superoxide per bacterial cell than the ancestor, in both the G5 and G15 generations (Fig. 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 catalase and superoxide dismutase enzymes to growth media ablated the suppression of *S. aureus* growth by *E. faecalis* during *in vitro* interactions (Fig. 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 to the ancestor (Generalised Linear Model, Treatment X 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*.

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 (e.g., in insects, Gerardo and Parker, 2014). We hypothesized that due 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 play 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. While 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). While niche occupation (Dillon et al., 2005), resource competition and

394 immune system mediation (Abt and Artis, 2013; Hooper et al., 2012; McFall-Ngai et al., 2013) may
395 still play a role in our system, the genomic evidence indicates selection acted predominantly through
396 direct *E. faecalis-S. aureus* interactions during host colonisation. Further experiments, however, are
397 required to determine if the microbial interactions observed to evolve here are adaptive to the host
398 environment or if similar evolutionary outcomes would arise *in vitro*. Regardless, we observed parallel
399 evolution of the superoxide-production pathway in CCE *E. faecalis* across all replicate populations, and
400 we were able to ablate the evolved suppression through enzymatic treatment to remove superoxide
401 radicals. These data strongly suggest that antimicrobial superoxides, which may act to directly suppress
402 *S. aureus* or act indirectly via oxidation of the *S. aureus* auto-inducing pheromone (Rothfork et al.,
403 2004), are a key mechanism in the evolved protective phenotype. The lack of genotype specificity we
404 observed is also consistent with a superoxide-mediated suppression system, which represents a broad-
405 spectrum form of microbial suppression. While *C. elegans* itself produces ROS in response to pathogen
406 infection (Chavez et al., 2007), those produced by resident bacteria may also be a common means of
407 broad-spectrum protection against infection, and one that is thus likely to be evolutionary labile in its
408 activity. For instance, lactic acid bacteria in the guts of honeybees are able to suppress a range of
409 pathogens, including *S. aureus* and *Pseudomonas aeruginosa* via ROS production (Olofsson et al.,
410 2014). That our experimental treatment drove the evolution of a broad-spectrum defence mechanism,
411 as opposed to more specific mechanisms of suppression (e.g., bacteriocin secretion), is also consistent
412 with observations from natural disease systems showing that microbes can protect against a diversity of
413 pathogen isolates (Koch and Schmid-Hempel, 2011) and species (Martinez et al., 2014).
414 The extent of the protective phenotype that evolved here, and the rate at which it evolved, were
415 striking. Despite being regularly attacked by pathogens, if nematode hosts were colonised by evolving
416 *E. faecalis*, they were almost universally protected against pathogens that would otherwise quickly kill
417 most of the population. Moreover, whilst the evolution in our experiment occurred during passage
418 through a number of individual worms, the time frame for the evolution of protection by *E. faecalis*

was just five 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 microbial 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.

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The authors declare no competing interests

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Figure Legends

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) Single evolution (SE) repeated passage of *E. faecalis* alone in *C. elegans*, and (ii) Co-colonisation evolution (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 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.

Figure 2: Effects of resident microbes on hosts over evolutionary time. (A) Host mortality with ancestral *Enterococcus faecalis* (blue circle) and *Staphylococcus 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.

Figure 3: Generality of host protection by evolved *E. faecalis* against seven *S. aureus* isolates. Host mortality was evaluated after 24 hours 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. Staphylococcus aureus* is co-colonising with ancestral, SE, or CCE *E. faecalis* populations. Error bars, 1 s.e.

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 catalase (CAT) and superoxide dismutase (SOD), enzymes that remove the presence of reactive oxygen species. (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). Error bars, 1 s.e.

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