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 without direct selection for host health, and it was 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 tests and *in vitro* assays. Our results indicate that resident microbes may make the evolutionary transition to mutualism in response to virulent infections, 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 are attacked by the host immune system, but also compete 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; Bourgogne et al., 2008; Martin-Vivaldi et al., 2010; Lawley et al., 2012; Cruz et al., 2013) wherein they can be pathogenic or commensal. Both species 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 to cause host death (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. *E. faecalis* is under selection in this state. Our protocol is in contrast with other studies of coinfecting pathogens that explore changes in virulence throughout the host's lifetime (Peleg et al., 2008; Lopez-Medina et al., 2015).

We tested whether *E. faecalis* resident within *C. elegans* evolved to protect its host 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 in order to enforce within-host competition and 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, may rapidly evolve to defend their hosts in response to more 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 guts have been shown to be co-colonised by multiple pathogens and commensals (Peleg et al., 2008; Portal-Celhay and Blaser, 2011; Montalvo-Katz et al., 2012; 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. Nematode host populations were not evolved throughout the experiment. 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). 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 (LB) broth and kept at 20ºC. The nematodes are able to digest *E. coli* after this bacterium is consumed, but 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 10cm 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* 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. 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 to clean excess bacterial cells off the nematode cuticle. 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 (according to the protocol in Morran et al. (2011)) 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 genetically-fixed *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-independent *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 dead, 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 (Schulenberg 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 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 neighborhoods 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) 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). 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. While these differences in starting conditions changed the absolute values of the results, they did not change the relationships between treatments.

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

245 OD_{600} of 1.00±0.05). A solution of TSB was prepared with 0.25M potassium phosphate buffer
246 containing Superoxide Dismutase E.C. 1.15.1.1 (SOD) from bovine erythrocytes (Sigma-Aldrich) and
247 Catalase E.C. 1.11.1.6 (CAT) from bovine liver (Sigma-Aldrich) each at 0.25mg/ml. An enzyme-free
248 solution of TSB was also prepared as a control, containing the 0.25M potassium phosphate buffer
249 alone. The ancestor and CCE *E. faecalis* (two technical replicates of each replicate population) were
250 mixed in equal ratios with *S. aureus*. From the liquid culture, 6µl was used to inoculate wells in a 96-
251 well plate with 196µl of the TSB solution alongside wells of an *S. aureus* control (*S. aureus* only). The
252 experiment was run in duplicate on the enzyme-free and enzyme-containing media. Cultures were
253 shaken for 24h at 30°C, after which cfu counts were performed.
254
255 **Statistical analyses**
256 All statistical analyses were conducted in SPSS 20.0.
257
258 *Host mortality and bacterial fitness assays*
259 Mortality data met assumptions of normality and equal variances. We performed an ANOVA
260 on untransformed data to test for the difference in nematode mortality caused by *E. faecalis* and *S.*
261 *aureus* independent colonization, as well as their co-colonisation.
262 For the evolution experiment, we examined changes in nematode mortality every G5 in both the
263 SE and CCE selection regimes. We performed a generalized linear model with binomial distribution
264 (and maximum likelihood estimates) on host mortality data in the evolution treatments (with and
265 without the presence of *S. aureus*) over time. Treatment and time (experimental generations) were fixed
266 effects. A separate ANOVA was conducted to test for variation among isolates in within-population
267 protective effects.
268 To examine the spectrum of host protection, we quantified nematode mortality upon co-
269 colonisation by *E. faecalis* and a diverse range of *S. aureus* isolates including both laboratory and

human disease isolates (Fig. 4). Host mortality data met assumptions of normality and equal variances. We tested for whether CCE *E. faecalis* suppressed the virulence of a diversity of invading *S. aureus* genotypes. 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 2-day exposure window of this experiment, with 1% of nematodes dying after 24 hours of colonisation. In contrast, 52% of worms exposed to *S. aureus* died after exposure, indicating that it is highly pathogenic. Colonisation of worms with *E. faecalis* before exposure to *S. aureus* results in intermediate rates of nematode mortality, indicating resident *E. faecalis* has the potential to suppress *S. aureus* virulence (Fig. 2A; ANOVA: $F_{2,15} = 55.571$, $P < 0.001$).

At the end of the evolution experiment, we assessed 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* passaged for five or more host generations (Fig. 2B). While there is some among-population variation in the mortality rates caused by SE *E. faecalis*, none of the replicate populations evolved enhanced protective ability relative to the ancestor (Fig. 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 among isolates (ANOVA: $F_{5,24} = 0.318$, $P = 0.895$; data in Supplementary Table 1).

The lower mortality on *S. aureus* exposure was not associated with a reduction in *E. faecalis* virulence, on average. 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: SE *E. faecalis* vs. CCE *E. faecalis*; 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

318 when tested in the presence of *S. aureus*, when tested alone CCE *E. faecalis* remained mildly
319 pathogenic and therefore costly for the nematode host to possess, clearly demonstrating the context
320 dependence of their fitness effects upon hosts (Chamberlain et al., 2014).
321
322 ***Generality of host protection***
323 All CCE *E. faecalis* populations at G15 were effective at protecting nematode hosts against a
324 range of *S. aureus* isolates (Fig. 3). In single infections, these *S. aureus* isolates exhibited variation in
325 their pathogenicity towards *C. elegans* (Fig. 3; 26-65% mean nematode mortality; ANOVA $F_{6,42}$ =
326 10.505, $P < 0.001$). In co-colonised hosts, all *S. aureus* isolates with ancestral *E. faecalis* produced
327 intermediate rates of host mortality, whereas with all replicate populations of CCE *E. faecalis*,
328 nematode mortality was dramatically reduced to 0-1%. Both treatment and isolate affected the
329 virulence of pathogens on hosts (Generalised Linear Model, Treatment: Wald $\chi^2 = 370.961$, $P < 0.001$;
330 Isolate: Wald $\chi^2 = 303.650$, $P < 0.001$).
331
332 ***Host protection and microbial growth within hosts***
333 We examined whether the evolved *E. faecalis* suppression of *S. aureus* virulence was associated
334 with increased *E. faecalis* proliferation and reduced *S. aureus* growth (Fig. 4). CCE *E. faecalis* (at G15)
335 suppressed *S. aureus* viable bacterial counts compared to its ancestor (Fig. 4; d vs f), and it persisted
336 and grew marginally more within co-colonised nematodes compared to the ancestor (Fig 4; a vs c). By
337 contrast, SE *E. faecalis* populations did not grow to higher titre on average than the ancestor when
338 interacting with *S. aureus* (Fig. 4; a vs b). These populations were also associated with higher within-
339 host growth of *S. aureus* compared to CCE *E. faecalis* (Fig 4; e vs f; Analysis: for *E. faecalis* cfu:
340 ANOVA across the three treatments: $F_{2,18} = 4.072$, $P = 0.039$; Least Square difference d > f, $P = 0.038$;
341 LSD e > f, $P = 0.019$; for *S. aureus* cfu. ANOVA across all three treatments $F_{2,18} = 5.402$, $P = 0.053$;
342 LSD a < c, $P = 0.057$; b < c, $P = 0.023$ a = b, $P = 0.649$).

343 Suppression of *S. aureus* may occur either directly from the presence of *E. faecalis*, be mediated
344 by *E. faecalis*-induced alterations to host biology, or be a product of both direct and host-mediated
345 effects. We assessed the importance of direct suppression using *in vitro* experiments. *In vitro*
346 experiments recapitulated *in vivo* assays showing that CCE *E. faecalis* populations were better able to
347 suppress *S. aureus* growth in liquid culture than ancestral *E. faecalis* (Fig. 5a; Generalised Linear
348 Model, Treatment: Wald $\chi^2 = 3.18 \text{ X } 10^{11}$, $P < 0.001$).
349
350 ***Genomic and biochemical analysis of the mechanism underpinning protection***
351 To investigate the genetic basis of *E. faecalis*-mediated protection, we whole-genome
352 resequenced a randomly picked clone of evolved *E. faecalis* from each of the 12 replicate populations
353 at G15, as well as the ancestor. Each evolved *E. faecalis* clone exhibited a unique set of between one
354 and three mutations compared to the ancestor (Supplementary Table 2). Consistent with the distinct
355 phenotypes that evolved under the two contrasting treatments, the SE and CCE regimes selected for
356 substitutions in different, functionally distinct gene sets. Six of 12 mutations in the CCE *E. faecalis*
357 clones – one per clone per replicated population – were putatively associated with superoxide
358 production, but no mutations associated with this pathway were observed in clones from the SE
359 treatment. *E. faecalis* is known to produce extracellular superoxide (Huycke et al., 2008), mediated by
360 dehydrogenase and fumarate reductase. Mutations were found in two NADH dehydrogenases and four
361 genes associated with the respiratory complex function or purine biosynthesis. Purine biosynthesis
362 represents the major pathway for fumarate production which, if blocked, leads to superoxide
363 production (Supplementary Table 2; Supplementary Fig. 1).
364 We therefore hypothesised that enhanced production of antimicrobial reactive oxygen species
365 (ROS) was the mechanism behind *E. faecalis*-mediated defence. In accordance with this hypothesis, all
366 CCE *E. faecalis* populations produced more superoxide per bacterial cell than the ancestor, in both the
367 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 in the presence of *S. aureus.* 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 $\chi^2 = 8.49 \text{ X } 10^{10}$, $P < 0.001$), 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 $\chi^2 = 7.24 \text{ X } 10^{10}$, $P < 0.001$). 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 defence against virulent 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. Under CCE, a pathogenic bacterium, *E. faecalis*, evolved across the parasite-mutualist continuum to become a host protective mutualist. *Enterococcus faecalis* has been observed in natural microbiomes to possess protective traits (Martin-Vivaldi et al., 2010). Notably, these host-protective effects evolved without any direct selection against host mortality. Instead, a beneficial relationship between the host and a mildly pathogenic, resident bacterium emerged out of interactions with a more 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

393 retained mild pathology against *C. elegans* when colonising alone, demonstrating the context
394 dependence of their fitness effects on the host (Chamberlain et al., 2014). In an environment where
395 such virulent infection is common, *E. faecalis* would therefore now represent a net mutualist with
396 respect to its impact on host fitness. It thus reflects other protective microbes found naturally, which
397 defend their host whilst retaining pathology (Martinez et al., 2014; Polin et al., 2014).
398 The mechanisms of microbe-mediated protection observed in nature are remarkably diverse.
399 Pathogens may be suppressed by competition for a limiting resource, through direct interference, or
400 through third party 'apparent competition', such as modulation of the host immune system (Gerardo
401 and Parker, 2014). While niche occupation (Dillon et al., 2005), competition for limiting resources and
402 immune system mediation (Abt and Artis, 2013; Hooper et al., 2013; McFall-Ngai et al., 2013) may
403 still play a role in our system, the genomic evidence indicates selection acted predominantly through
404 direct interactions between *E. faecalis* and *S. aureus* during colonisation. We observed parallel
405 evolution of the superoxide-production pathway in CCE *E. faecalis* across all replicate populations.
406 Moreover, we were able to ablate the evolved suppression through enzymatic treatment to remove
407 superoxide radicals. Taken together, these data strongly suggest that antimicrobial superoxide was a
408 key mechanism in the evolved protective phenotype. Superoxide may act to directly suppress *S. aureus*
409 or act indirectly via oxidation of the *S. aureus* auto-inducing pheromone (Rothfork et al., 2004). The
410 lack of genotype specificity we observed is also consistent with a superoxide-mediated suppression
411 system, which represents a generalised form of microbial suppression. While *C. elegans* itself produces
412 ROS in response to pathogen infection (Chavez et al., 2007), ROS produced by resident bacteria may
413 also be a common means of protection against infection, and one that is thus likely to be evolutionary
414 labile in its activity. For instance, lactic acid bacteria in the guts of honeybees are able to suppress a
415 range of pathogens, including *S. aureus* and *Pseudomonas aeruginosa* via ROS production (Olofsson et
416 al., 2014). Our observation is consistent with wildlife and human disease studies showing that
417 defensive microbes can protect against a diversity of pathogen isolates (Koch and Schmid-Hempel,

2011) and species (Martinez et al., 2014), which are distinct from more specific suppression systems (such as bacteriocin secretion).

The extent of the protective phenotype that evolved here, and the rate at which it evolved, were striking: under CCE if nematode hosts were colonised by evolving *E. faecalis*, they were almost universally protected against a pathogen that would otherwise quickly kill the majority of the population. Moreover, whilst 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 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 mammals or trees, 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, mimicking a spillover zoonotic infection whereby the pathogen normally circulates in a different host species. An example is *Salmonella*, whereby some forms commonly reside within the microbiomes of livestock animals, but 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, which may obviate any disease clearance that occurred via evolution of the resident microbial species, 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 multiple resident species on pathogen invasion (Mueller and Sachs, 2015), and on species interactions within the resident microbial community also warrant consideration. Notwithstanding this, the potential for evolution of interactions between resident microbes (even mildly-pathogenic ones) and pathogenic microbes is clear, and future research on microbiome-pathogen interactions should go beyond ecological responses to examine evolved ones.

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The authors declare no competing interests

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549 protease genes and the quorum-sensing locus fsr in *Caenorhabditis elegans* and mice. Infect Immun 70: 5647-5650.

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561 **Figure Legends**

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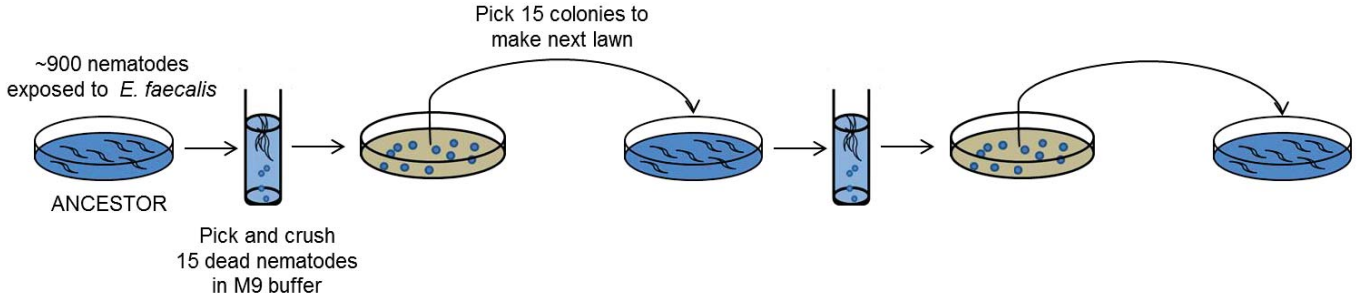
563 Figure 1: Experimental procedure for experimental evolution of *E. faecalis* within *C. elegans*
564 populations. Treatments are shown for a single replicate population. Six populations of *E. faecalis* were
565 independently passaged from a single clone ancestor for 15 experimental host generations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

574

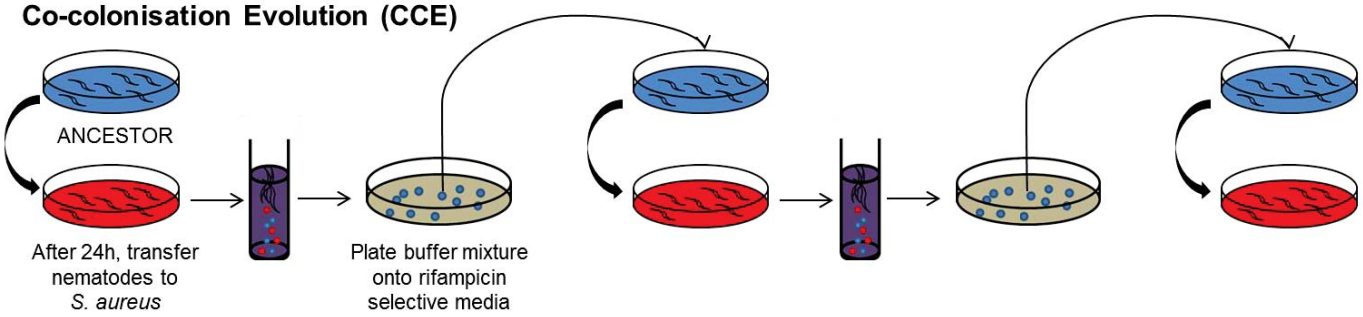
575 Figure 2: Evolutionary dynamics of protective resident microbes. (A) Host mortality with ancestral
576 *Enterococcus faecalis* (blue circle) and *Staphylococcus aureus* (red circle) separate and co-colonising
577 (purple circle) in the nematode. The intermediate level of virulence from co-colonising bacteria species
578 suggested the potential for *E. faecalis* to suppress pathogenic *S. aureus*. Error bars, 1 s.e. (B)-(C)

579 Populations of *E. faecalis* were evolved under two different selection regimes: SE and CCE for 15
580 experimental host generations. To assess the ability of *E. faecalis* to protect hosts from *S. aureus*, host
581 mortality in the (B) presence and (C) absence of *S. aureus* was quantified every G5 for SE (blue
582 cirlces) and CCE (purple circles) *E. faecalis*. Lines connect each of the six replicate populations per
583 treatment across time.
584
585 Figure 3: Generality of host protection by evolved *E. faecalis* against seven *S. aureus* isolates. Host
586 mortality was evaluated after 24 hours of exposure to *S. aureus*. Nematodes were exposed to *S. aureus*
587 alone (red circles), or were previously colonised by ancestral *E. faecalis* (black circles) or CCE *E.*
588 *faecalis* at G15 (purple circles). MSSA476 was used in the evolution experiment. Error bars, 1 s.e.
589
590 Figure 4: Fitness (cfus/nematode) of resident *E. faecalis* bacterial populations and co-colonising *S.*
591 *aureus. Staphylococcus aureus* is co-colonising with ancestral, SE, or CCE *E. faecalis* populations.
592 Error bars, 1 s.e.
593
594 Figure 5: Mechanism of suppression of *S. aureus* (MSSA476) by *E. faecalis*. (A) Mean superoxide
595 production (measure of luminescence controlling for OD_{600}) across exponential growth phase of
596 ancestral, SE, and CCE *E. faecalis* (the latter at G5 and G15). (B) Suppression and enzyme-mediated
597 lifting of suppression of *S. aureus* outside the host. *S. aureus* cfus were counted when the pathogen was
598 grown alone or co-cultured with ancestral *E. faecalis* or CCE *E. faecalis*. Counts were also made upon
599 the addition of catalase (CAT) and superoxide dismutase (SOD), enzymes that remove the presence of
600 reactive oxygen species. Error bars, 1 s.e.

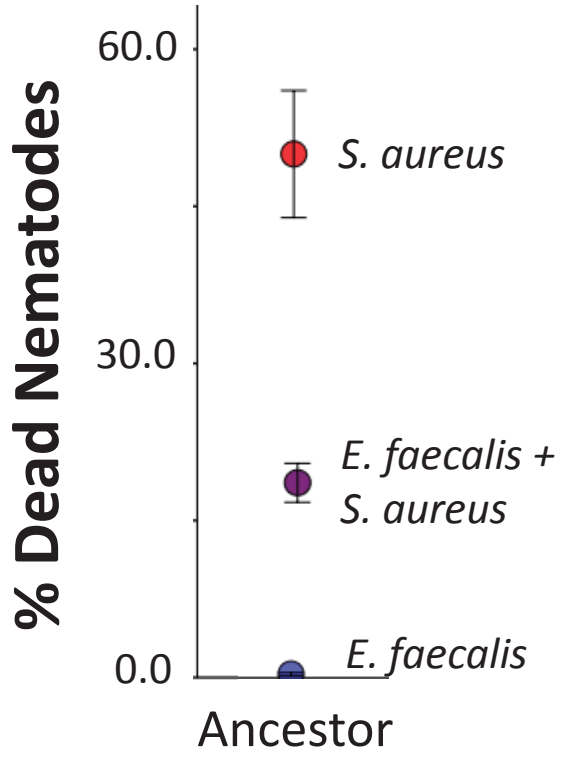
Single Evolution (SE)

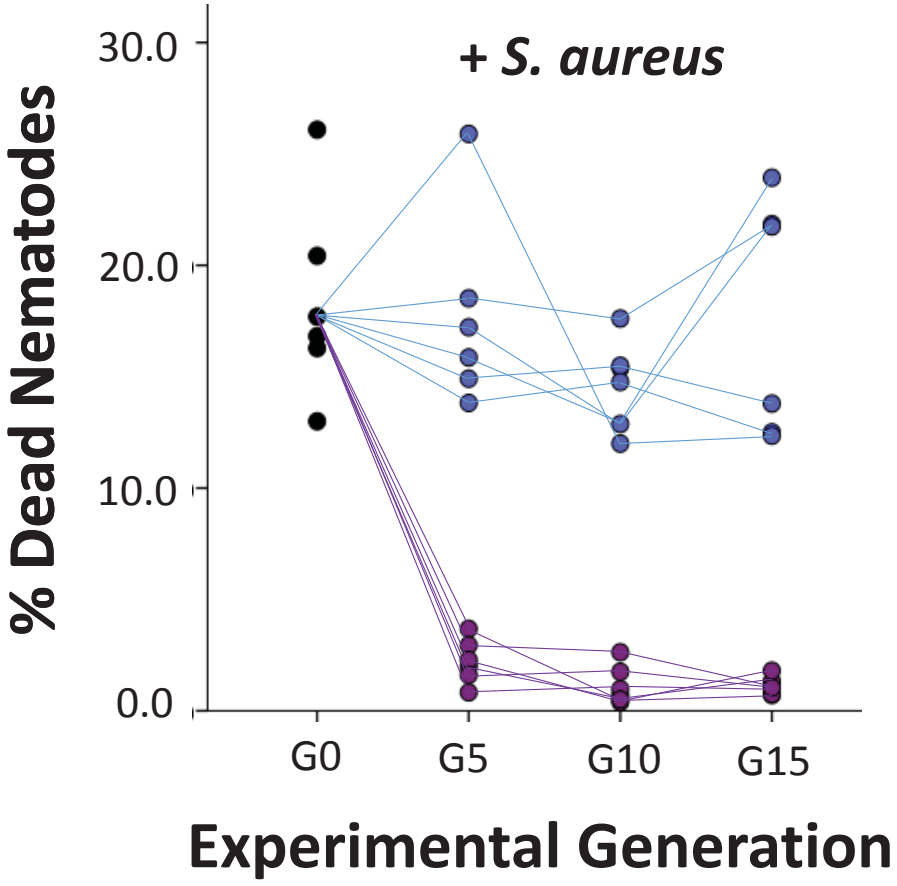


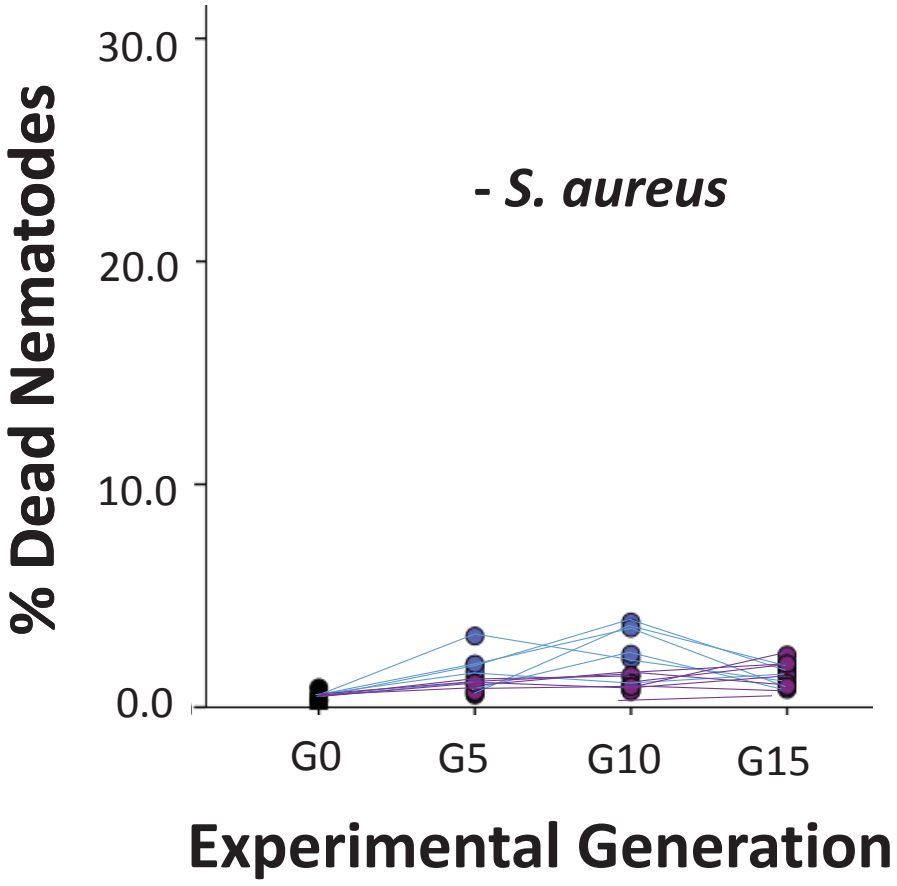
Co-colonisation Evolution (CCE)

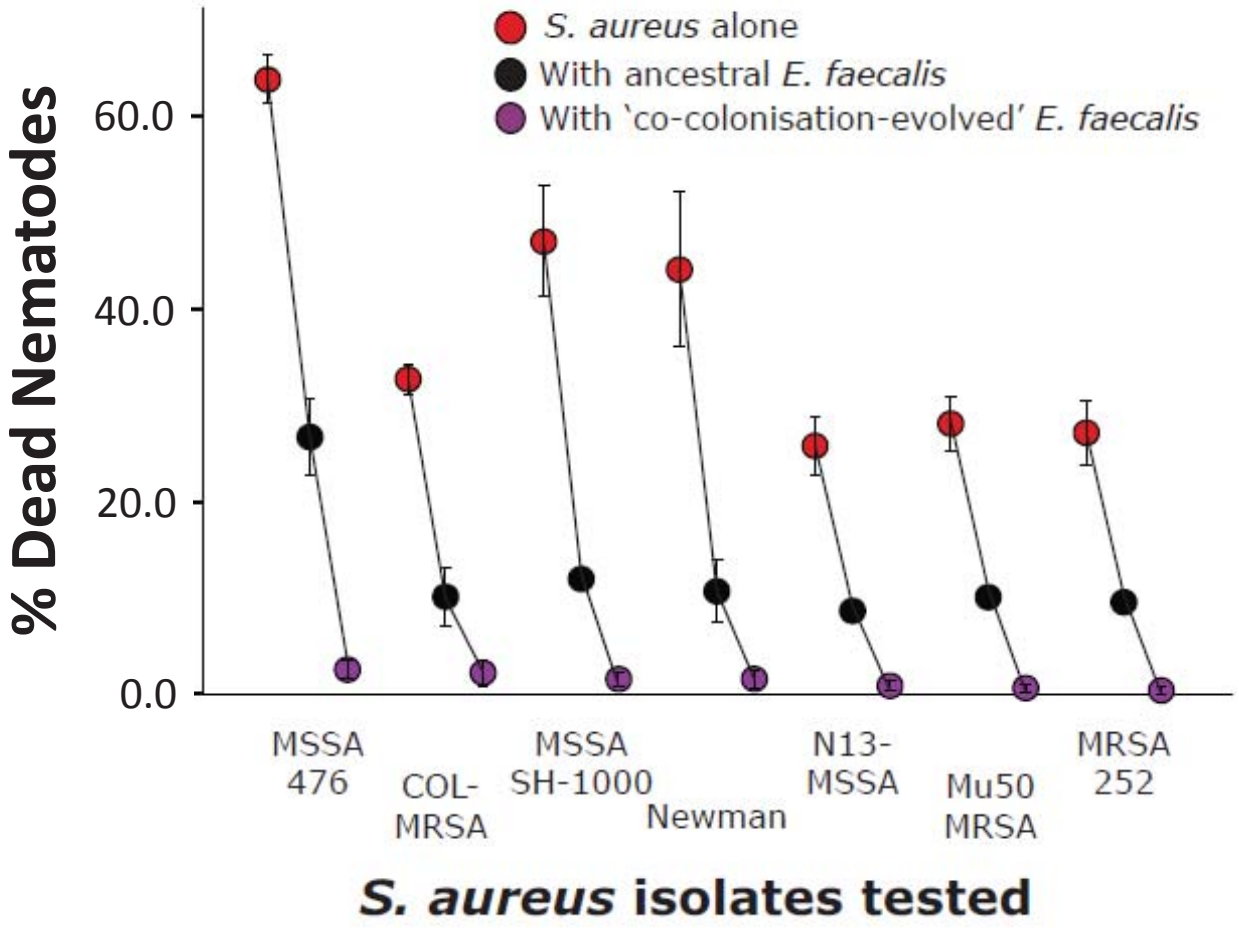


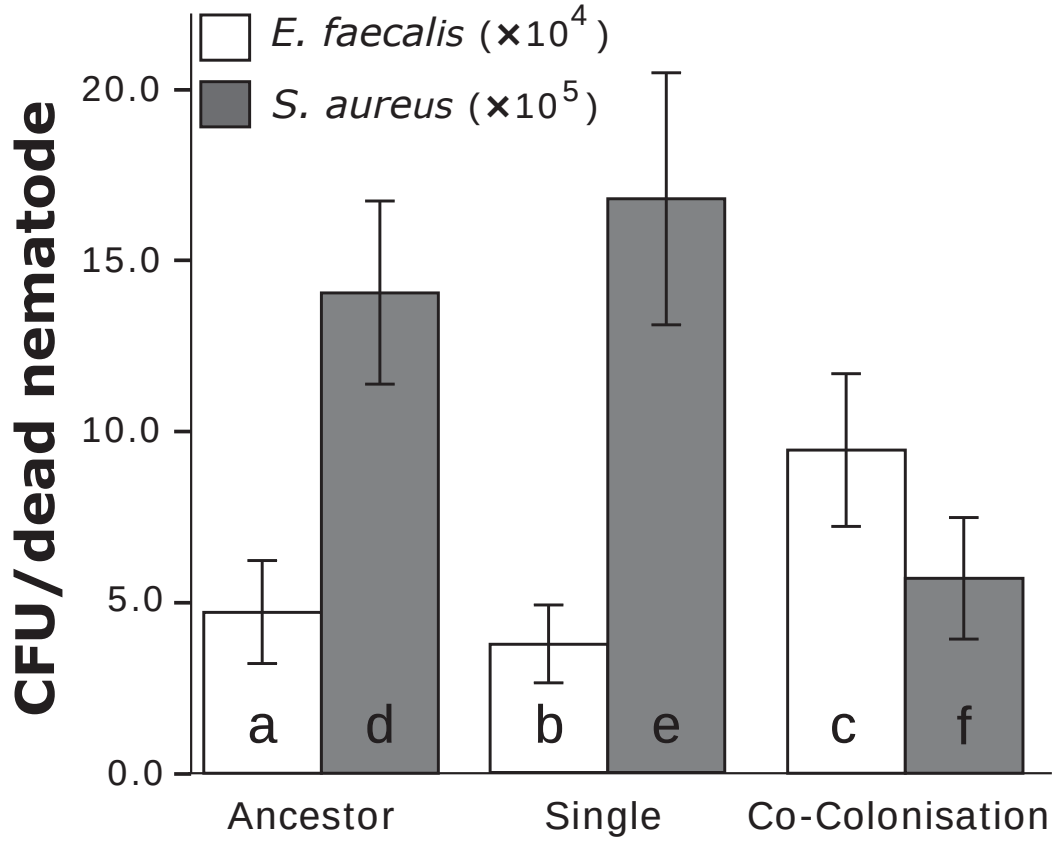
Experimental Host Generation 1 (G1) | G2


% Dead Nematodes
60.0
30.0
0.0
S. aureus
E. faecalis +
S. aureus
E. faecalis
Ancestor

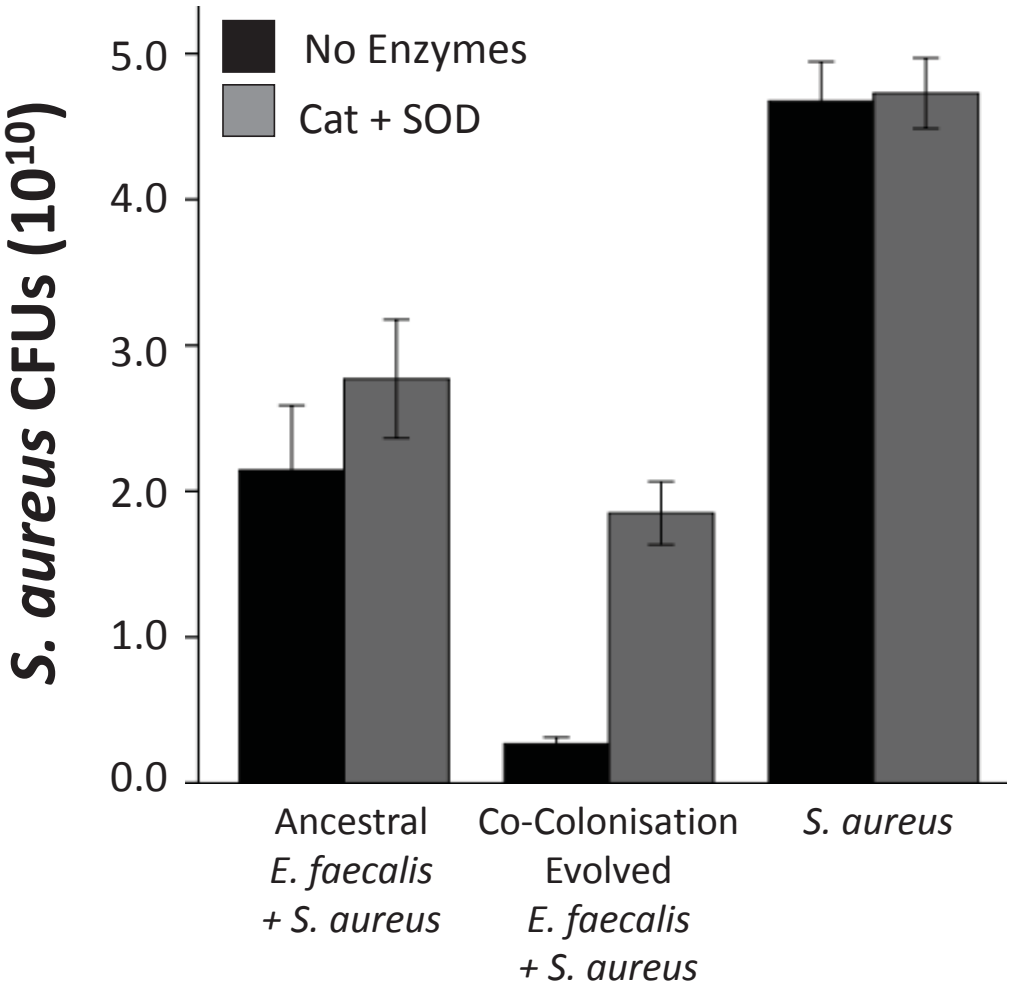

+ S. aureus
% Dead Nematodes
30.0
20.0
10.0
0.0
G0
G5
G10
G15
Experimental Generation

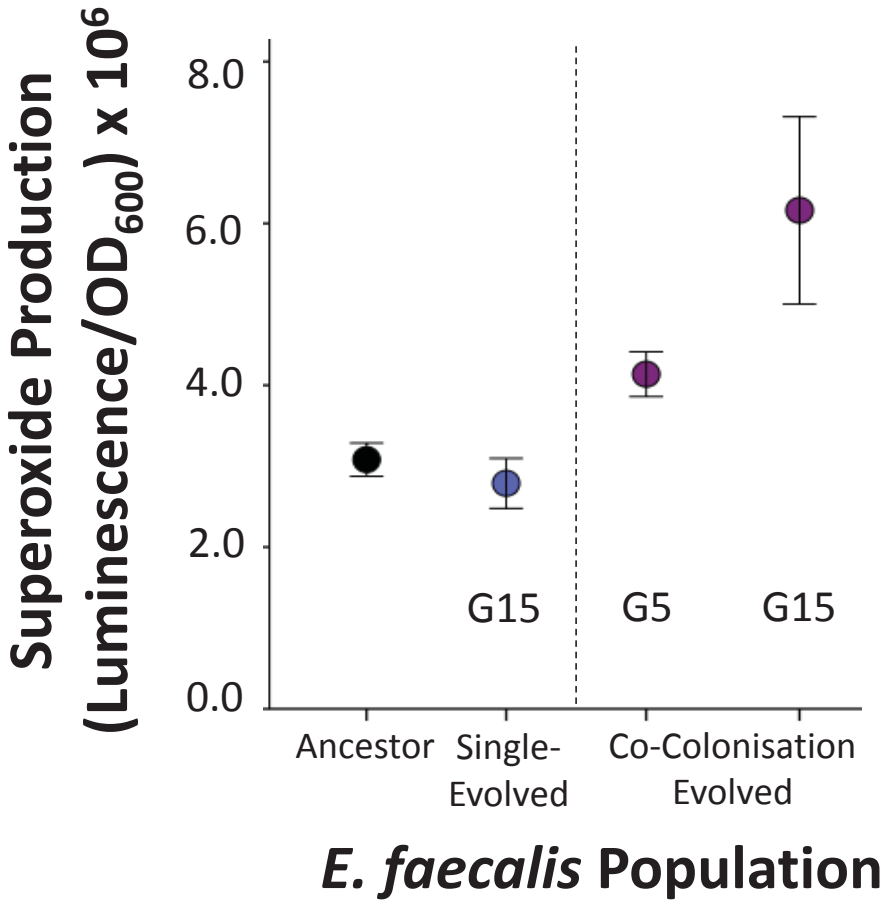

30.0
20.0
10.0
0.0
% Dead Nematodes
- S. aureus
G0
G5
G10
G15
Experimental Generation


S. aureus alone
With ancestral E. faecalis
With 'co-colonisation-evolved' E. faecalis
% Dead Nematodes
60.0
40.0
20.0
0.0
MSSA 476
COL-MRSA
MSSA SH-1000
Newman
N13-MSSA
Mu50 MRSA
MRSA 252
S. aureus isolates tested



***E. faecalis* populations tested with *S. aureus* in nematode hosts**


No Enzymes
Cat + SOD
S. aureus CFUs (10^10)
5.0
4.0
3.0
2.0
1.0
0.0
Ancestral
E. faecalis
+ S. aureus
Co-Colonisation
Evolved
E. faecalis
+ S. aureus
S. aureus


Superoxide Production
(Luminescence/OD600) x 10^6
8.0
6.0
4.0
2.0
0.0
G15
G5
G15
Ancestor
Single-Evolved
Co-Colonisation Evolved
E. faecalis Population