Title: *E. faecalis* acquires resistance to antimicrobials and insect immunity via common mechanisms

One sentence summary: Host interaction can promote antimicrobial resistance and antimicrobial treatment can promote virulence in *E. faecalis*.

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Abstract:

Enterococcus faecalis is an opportunistic pathogen that infects many animal hosts, including insects and mammals. It is an important human pathogen and is often highly drug resistant. Using experimental evolution, we generated *Drosophila*-adapted *E. faecalis* strains, which exhibited immune resistance, resulting in increased *in vivo* growth and virulence. Resistance was characterised by mutations in bacterial pathways responsive to cell-surface stress. *Drosophila*-adapted strains exhibited changes in sensitivity to relevant antimicrobials, including daptomycin and vancomycin. Evolved daptomycin-resistant strains harboured mutations in the same signalling systems, with some strains showing increased virulence similar to *Drosophila*-adapted strains. Our results show that common mechanisms provide a route to

resistance to both antimicrobials and host immunity in *E. faecalis* and demonstrate that the selection and emergence of antibiotic resistance *in vivo* does not require antibiotic exposure.

Main Text:

Enterococcus faecalis is a normal member of the gut microbiota and an opportunistic pathogen of many animals, including mammals and insects (*1*). This organism is a common cause of nosocomial infections, and is particularly troublesome due to extensive intrinsic and acquired antimicrobial resistance (*2*). It is also a reservoir for antimicrobial resistance genes acquired and deployed by other bacteria (*3*). The biology of *E. faecalis* results from its interaction with multiple host species; this is particularly relevant due to the high level of genetic exchange among isolates from different hosts (*4*).

E. faecalis has been isolated from many insects, including the gut and hemolymph of wild *Drosophila* (*1, 4-6*). Unlike mammals, insect immune responses are often described as being strongly dependent on inducible antimicrobial peptides as immune effectors; *in vitro* selection experiments with other bacteria have borne out the possibility that antimicrobial peptides can select resistance to other antibiotics (*7, 8*). However, intact immune systems—in insects as in other animals—are complex, typically deploying a variety of semiredundant effectors against any given infection, so the actual selective pressure experienced by infecting organisms is not necessarily simple.

We wished to understand the pressures *E. faecalis* faces in an insect host as they will affect its biology in ways relevant to human disease (*4*). To decipher the interaction between *E. faecalis* and insect immunity, we carried out experimental evolution via serial passage of *E. faecalis* Tissier (the type strain) in *Drosophila* (Fig 1A). Evolution was carried out on three occasions, each time with 3-10 parallel replicates. This produced several populations of *Drosophila*-adapted bacteria (Table S1). Single colonies were isolated from these *Drosophila*-adapted populations and used to generate clonal *Drosophila*-adapted strains. These strains exhibited 10-100x increased growth relative to the ancestor over the first eight hours of infection; they also exhibited increased virulence, leading to significantly greater host mortality (Fig

1B, C; Table S1). This increase in virulence was not specific to inbred, laboratory-adapted *Drosophila*: we observed similar virulence in flies from a recently wild-derived outbred population (Fig S1A).

Drosophila humoral immunity against *E. faecalis* is mediated by the Toll signalling pathway (*9, 10*). The final step of this signalling pathway involves nuclear translocation of the transcription factor Dif, where it drives transcriptional changes including induction of genes encoding a variety of antimicrobial effector peptides. The Bomanins are a family of Toll-driven effector peptides essential for the immune response against *E. faecalis* (*11*). To confirm that this mechanism was involved in the selection of our strains, we assayed bacterial fitness in flies lacking *Dif* or most Bomanins. *Dif* and Bomanin mutant hosts exhibited rapid bacterial growth and host death when infected with either ancestral or *Drosophila*-adapted strains (Fig 1D, E; Fig S1B, C). This indicated that Dif-mediated Bomanin induction was the primary selective pressure to which these strains had evolved resistance. To exclude involvement of other antimicrobial peptides in the selection of our strains, we infected flies lacking most other antimicrobial peptides (*12*). We observed that all strains were more virulent in these flies than in wild-type controls, but the difference in virulence between ancestral and *Drosophila*-adapted strains was retained (Fig S1D). Most *Drosophila*-adapted strains exhibited reduced virulence in flies expressing a constitutively active allele of *Toll*, indicating that sufficiently high Toll activation could still protect the host (Fig S1E).

E. faecalis could take two routes to escape killing by Toll-induced Bomanins: they could evade detection by the Toll signalling pathway, or they could resist Bomanin-mediated killing. If *Drosophila*-adapted strains evaded immune detection, we would expect a decrease in Toll-induced gene expression compared to the ancestral strain. We assayed antimicrobial target gene expression three and six hours after infection to determine which of these routes our bacteria had taken. We did not observe significant differences in induction of *BomS2* or several antimicrobial peptides (Fig 1F and Fig S2). This indicates that our strains have adapted to resist killing by Bomanin peptides during systemic infection.

Drosophila-adapted strains exhibited a clear growth advantage in fly hemolymph, but it was not obvious what effect this would have on their ability to grow in other environments. We assayed their growth in the *Drosophila* gut, both in the absence of, and in competition with, the ancestral strain (Fig 1G; Fig S3). Bomanins are not expected to play any direct role in gut defence. Consistent with this, no specific trends were observed in this environment.

To identify the genetic changes enabling adaptation to *Drosophila* immunity, we performed whole genome sequencing of our ancestral and *Drosophila*-adapted strains. Variants identified in the *Drosophila*-adapted strains are shown in Table 1. Most *Drosophila*-adapted strains differed from the ancestral strain by only a single mutation. Many implicated genes regulate bacterial surface characteristics (*13-15*), some are known virulence factors in *Enterococcus* or other bacteria (*15-17*), and some are important for antimicrobial resistance (AMR) (*15, 18-20*). Strain A had acquired mutations in *yxdM*, *dcuR*, and *epaQ*; of these three mutations, those in *yxdM* and *epaQ* contributed to the observed increase in virulence (Fig S4A). One mutant allele, $liaF^{S217I}$, was present in seven strains. This was the only mutation identified in these strains, which were genetically and phenotypically indistinguishable; they are collectively referred to here as strain C. Strain E had acquired two mutations, in *croS* and *bgsA*; by screening bacteria collected from an earlier cycle of selection in the strain E population, we were able to isolate a strain carrying only the mutation in *croS* (strain G). Analysis of this strain revealed that both genes contributed to strain E's increased *in vivo* growth and virulence (Fig S4B, C).

These mutations in virulence and AMR-related factors suggested that *Drosophila*-adapted strains might exhibit altered sensitivity to stresses that would be encountered in mammalian hosts. We assayed killing of these bacteria by whole human blood *ex vivo* (Fig 2A) (*21*). Neutrophils dominate the killing of bacteria in these conditions (*22*); however, we observed that all tested *Drosophila*-adapted strains grew more during six hours in human blood than the ancestral strain. We also assayed the ability of *Drosophila*-adapted strains to tolerate bile, a stress encountered in the mammalian gut (Fig S5). We

observed no differences in bile tolerance, except for increased tolerance in strain C. This was not unexpected, as mutations in *liaF* can increase bile tolerance in *E. faecium* (*23*).

Several *Drosophila*-adapted strains had mutations in genes involved in antimicrobial resistance or tolerance, including *mprF2*, *liaF*, *croS*, and *yxdM* (*19, 20, 24, 25*). We tested the minimal inhibitory concentration (MIC) of a panel of antibiotics in our strains (Fig 2B, Table S2). We observed increases and decreases in MIC, with no two *Drosophila*-adapted strains showing the same susceptibility profile. Most changes were modest; however, confirming previous observations, mutations in *yxdM* resulted in increased susceptibility of strain A to bacitracin (*25*), while the *liaF* mutation in strain C conferred increased resistance to daptomycin (*19*). Finally, strain E showed a 4-fold increase in MIC of both vancomycin and teicoplanin, attributable to its mutation in *croS* (Table S2) (*20*). All *Drosophila*-adapted strains showed decreased susceptibility to vancomycin, and half showed decreased susceptibility to cationic antimicrobial peptides and daptomycin.

Bacterial surfaces are targeted by many host immune effectors and antimicrobials. *E. faecalis* responds to cell-surface stress via several well-characterized signaling pathways. *Drosophila*-adapted strains had mutations in three of these cell-surface stress response systems: *yxdJKLM*, *liaFSR* and *croRS* (*26*). We assayed activity of these systems by measuring expression of well-characterised target genes (*yxdJ*, *liaF*, *croS*, and *dltB*) in *Drosophila*-adapted strains during exponential and stationary phase (Fig 2C; Fig S6A, B) (*19, 20, 24, 25*). Almost all *Drosophila*-adapted strains showed increased stationary phase *croS* and/or *liaF* expression. We observed no general trend in *yxdJ* expression. These cell surface stress signaling pathways influence bacterial surface characteristics such as membrane fluidity and surface charge. Alteration of membrane fluidity is a known mechanism of daptomycin resistance (*27*). Similarly, bacteria increase their surface charge to reduce electrostatic interaction with cationic antimicrobial peptides (*28*). Almost all *Drosophila*-adapted strains had greater membrane fluidity, indicated by decreased generalized polarization when bound to laurdan (Fig 2D). There was also a trend to reduced FITC-poly-L-lysine binding, suggesting increased surface charge, particularly during exponential growth (Fig 2E).

These results suggested fundamental mechanistic similarities in resistance to cell-surface-targeting antimicrobials and insect immune responses. To test this, we performed experimental evolution of *E. faecalis* challenged with increasing doses of daptomycin (Fig 3A). Daptomycin is a lipopeptide antibiotic used to treat vancomycin resistant *Enterococcus* infections (*27, 29*). This evolution experiment produced strains with 16-fold increases in daptomycin MIC (Fig 3B). We then tested the virulence of these daptomycin-adapted strains in *Drosophila* and found that two of five daptomycin-adapted strains exhibited increased virulence (Fig 3C, D). Whole genome sequencing revealed that all daptomycin-adapted strains had mutations in the same regulatory systems, and in some cases the same genes, as *Drosophila*-adapted strains (Table 1) (*20, 25, 30*). The two daptomycin-adapted strains that most strongly increased virulence in the fly both had mutations in *yxdJ*, a sensor kinase that regulates *yxdM* expression (*25*).

Taken together, our results indicate that the mechanisms by which *Enterococcus faecalis* resists insect and human immune responses and cell-surface targeting antibiotics exhibit significant overlap. Immune pressure can promote antibiotic resistance-relevant changes in the bacterium, while daptomycin treatment can select for increased virulence. These observations may have significant implications regarding the interaction of immune function with antimicrobial treatment and the evolutionary origins of intrinsic antimicrobial resistance. They also imply linkage between resistance to antimicrobials and host immune responses, suggesting that the emergence of *Enterococcus faecalis* as a nosocomial pathogen may be driven in part by antimicrobial-selected increases in virulence.

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visualization, writing—review & editing. CDN, formal analysis, investigation, visualization, writing—review & editing. AME, conceptualization, writing—review & editing. MSD, conceptualization, data curation, funding acquisition, project administration, supervision, visualization, writing—original draft, writing—review & editing. Competing interests: none. Data and materials availability: Genome sequence data has been deposited at GenBank and is accessible via BioProject PRJNA863576. Any data not represented in the manuscript are freely available.

List of Supplementary materials:

Materials and methods

Fig S1-S6

Table S1-S25

Figures:

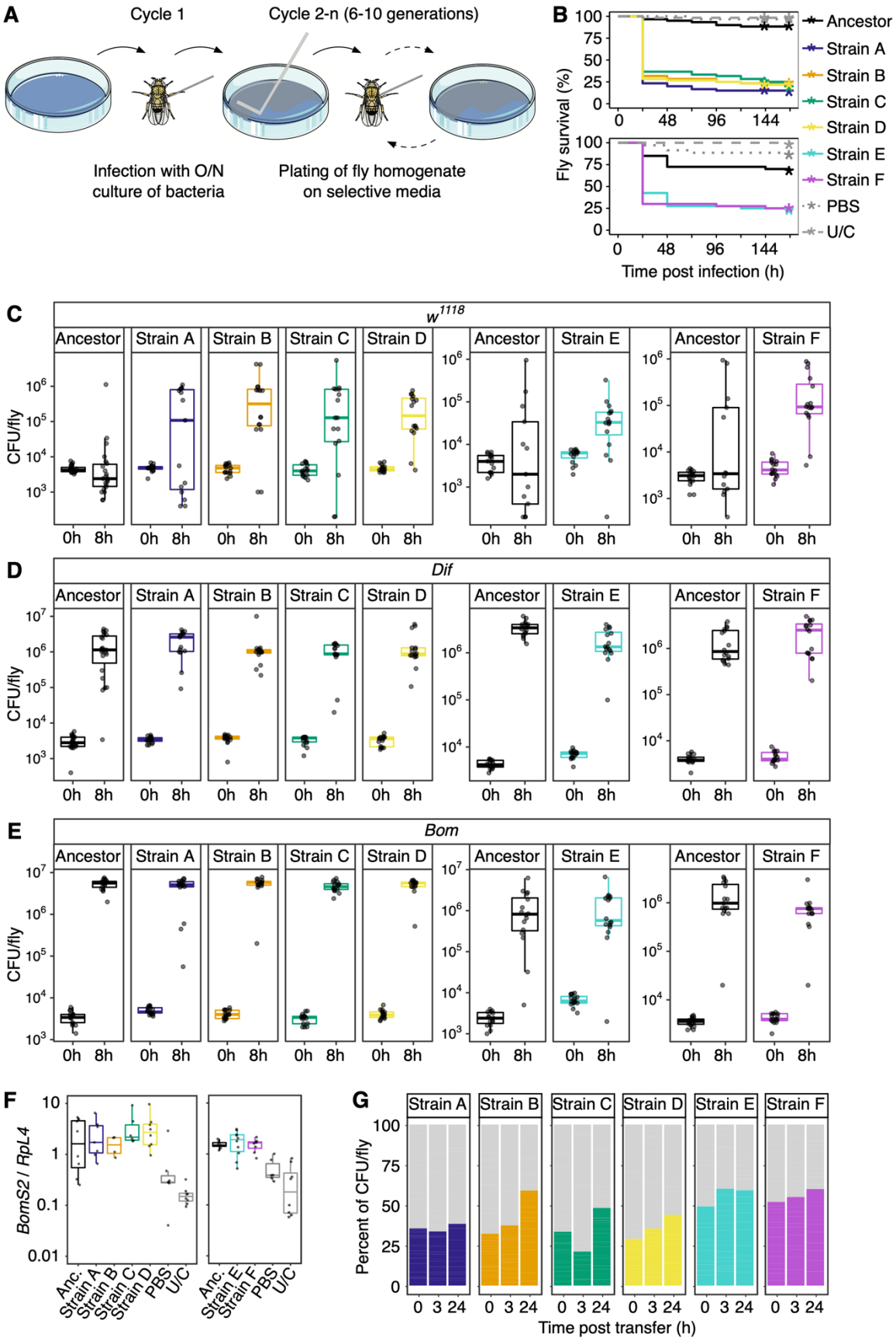
A
Cycle 1
Cycle 2-n (6-10 generations)
Infection with O/N culture of bacteria
Plating of fly homogenate on selective media
B
Fly survival (%)
Time post infection (h)
Ancestor
Strain A
Strain B
Strain C
Strain D
Strain E
Strain F
PBS
U/C
C
w1118
CFU/fly
0h
8h
D
Dif
E
Bom
F
BomS2 / RpL4
Anc.
G
Percent of CFU/fly
Time post transfer (h)

Fig. 1. A. Experimental evolution schematic.

B. Survival of wild-type flies infected with ancestor and *Drosophila*-adapted strains. U/C = uninjected control. P values for survival differences vs ancestor: Strains A-D, P<0.001; PBS and U/C, P=1; Strain E and F, P<0.001; PBS and U/C, P>0.05.

C. *E faecalis* numbers at 0h (input inoculum) and 8h after infection of wild-type (w^{1118}) flies with ancestor and *Drosophila*-adapted strains. CFU = colony forming units. P values for bacterial number differences vs ancestor at 8h: Strain A, P>0.05; Strain B, P<0.001; Strain C, P<0.05; Strain D, P<0.01; Strain E, P<0.05; Strain F, P<0.01**.**

D. *E faecalis* numbers at 0h (input inoculum) and 8h after infection of *Dif*-mutant flies with ancestor and *Drosophila*-adapted strains. P values for bacterial number differences vs ancestor at 8h: Strains A-D, P=1; Strain E, P<0.001; Strain F, P>0.05.

E. *E faecalis* numbers at 0h (input inoculum) and 8h after infection of Bomanin-deficient ($Bom^{\Delta 55C}$) flies with ancestor and *Drosophila*-adapted strains. P values for bacterial number differences vs ancestor at 8h: Strains A-F, P>0.05.

F. RNA expression of *BomS2* 3h after infection with ancestor and *Drosophila*-adapted strains. P values for differences in *BomS2* induction vs ancestor: Strains A-PBS, P>0.05; U/C, P<0.001; Strains E and F, P>0.05; PBS and U/C, P<0.001.

G. Relative abundance of *Drosophila*-adapted strains after oral inoculation in competition with ancestor; "Transfer" refers to relative abundances in initial inoculum, and 3h and 24h timepoints are defined relative to time of inoculation. Abundance of ancestor is indicated by grey.

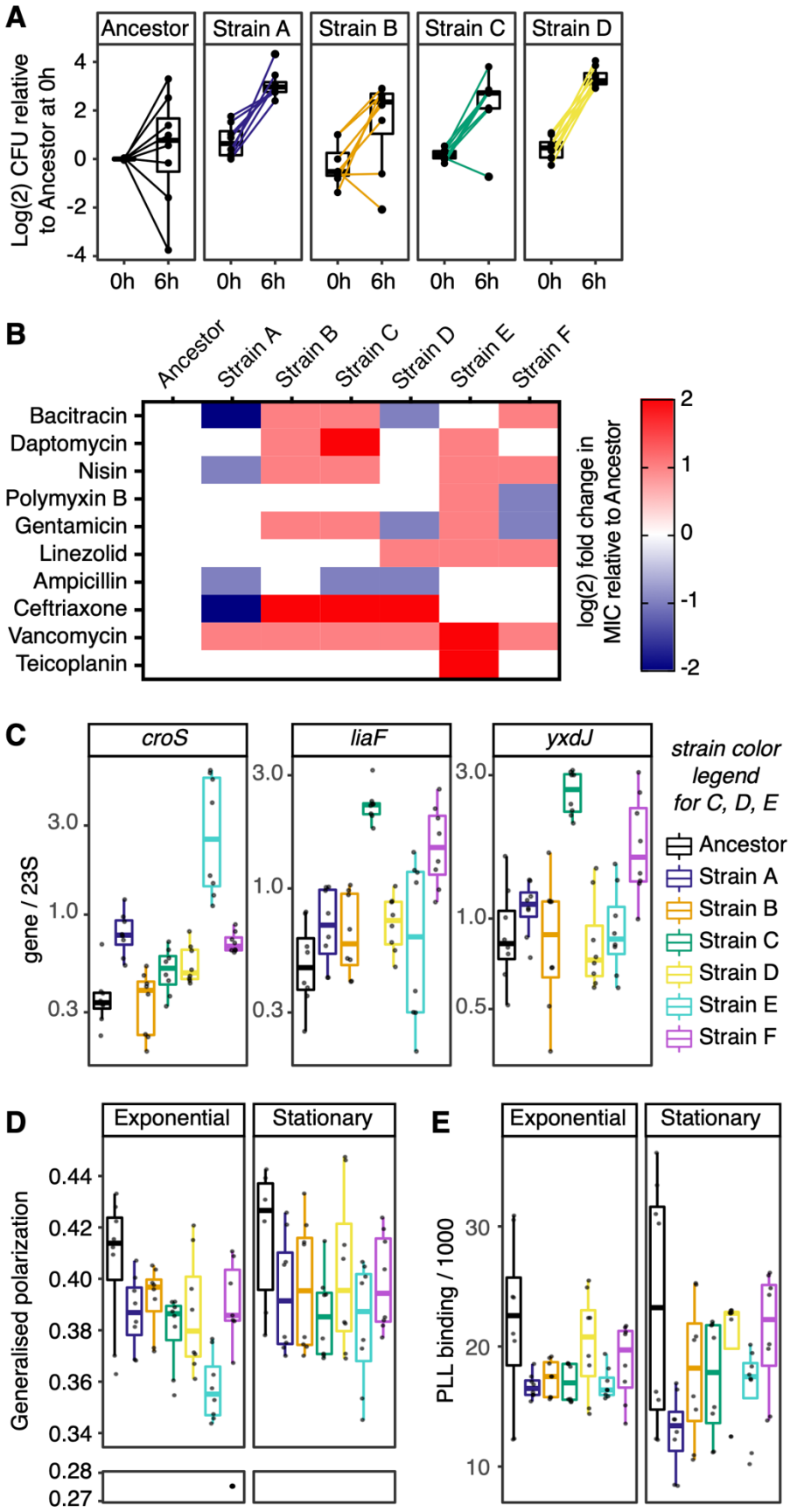
A
Ancestor
Strain A
Strain B
Strain C
Strain D
Log(2) CFU relative to Ancestor at 0h
4
2
0
-2
-4
0h 6h
B
Ancestor
Strain A
Strain B
Strain C
Strain D
Strain E
Strain F
Bacitracin
Daptomycin
Nisin
Polymyxin B
Gentamicin
Linezolid
Ampicillin
Ceftriaxone
Vancomycin
Teicoplanin
log(2) fold change in MIC relative to Ancestor
2
1
0
-1
-2
C
croS
liaF
yxdJ
gene / 23S
3.0
1.0
0.3
0.5
strain color legend for C, D, E
Ancestor
Strain A
Strain B
Strain C
Strain D
Strain E
Strain F
D
Exponential
Stationary
Generalised polarization
0.44
0.42
0.40
0.38
0.36
0.34
0.28
0.27
E
Exponential
Stationary
PLL binding / 1000
30
20
10

Fig. 2. A. Ability of ancestor and *Drosophila*-adapted strains A-D to survive and proliferate over 6h in fresh human blood. P values for differences in bacterial numbers between input and 6h: Ancestor, $P>0.05$; Strain A, $P<0.01$; Strains B and C, $P<0.05$; Strain D, $P<0.01$.

B. Heatmap of changes in MIC of tested antimicrobials on *Drosophila*-adapted strains relative to in-experiment ancestor control.

C. Expression of *croRS*, *liaFRS*, and *yxdJKLM* target genes in ancestor and *Drosophila*-adapted strains at stationary phase. P values for gene expression differences with ancestor: *croS*: Strain A, $P<0.001$; Strains B and C, $P>0.05$; Strain D, $P<0.01$; Strain E, $P<0.001$; Strain F, $P<0.01$. *liaF*: Strain A, $P<0.05$; Strain B, $P>0.05$; Strain C, $P<0.001$; Strain D, $P<0.05$; Strain E, $P>0.05$; Strain F, $P<0.001$. *yxdJ*: Strains A and B, $P>0.05$; Strain C, $P<0.001$; Strains D and E, $P>0.05$; Strain F, $P<0.01$.

D. Membrane fluidity measurements on ancestor and *Drosophila*-adapted strains in exponential and stationary phases. P values for differences with ancestor: Exponential phase: Strains A and B, $P>0.05$; Strain C, $P<0.05$; Strain D, $P>0.05$; Strain E, $P<0.01$; Strain F, $P>0.05$. Stationary phase: Strains A-C, $P<0.05$; Strain D, $P>0.05$; Strains E and F, $P<0.05$.

E. Surface charge measurements on ancestor and *Drosophila*-adapted strains in exponential and stationary phases. P values for differences with ancestor: Exponential and stationary phase: all strains, $P>0.05$.

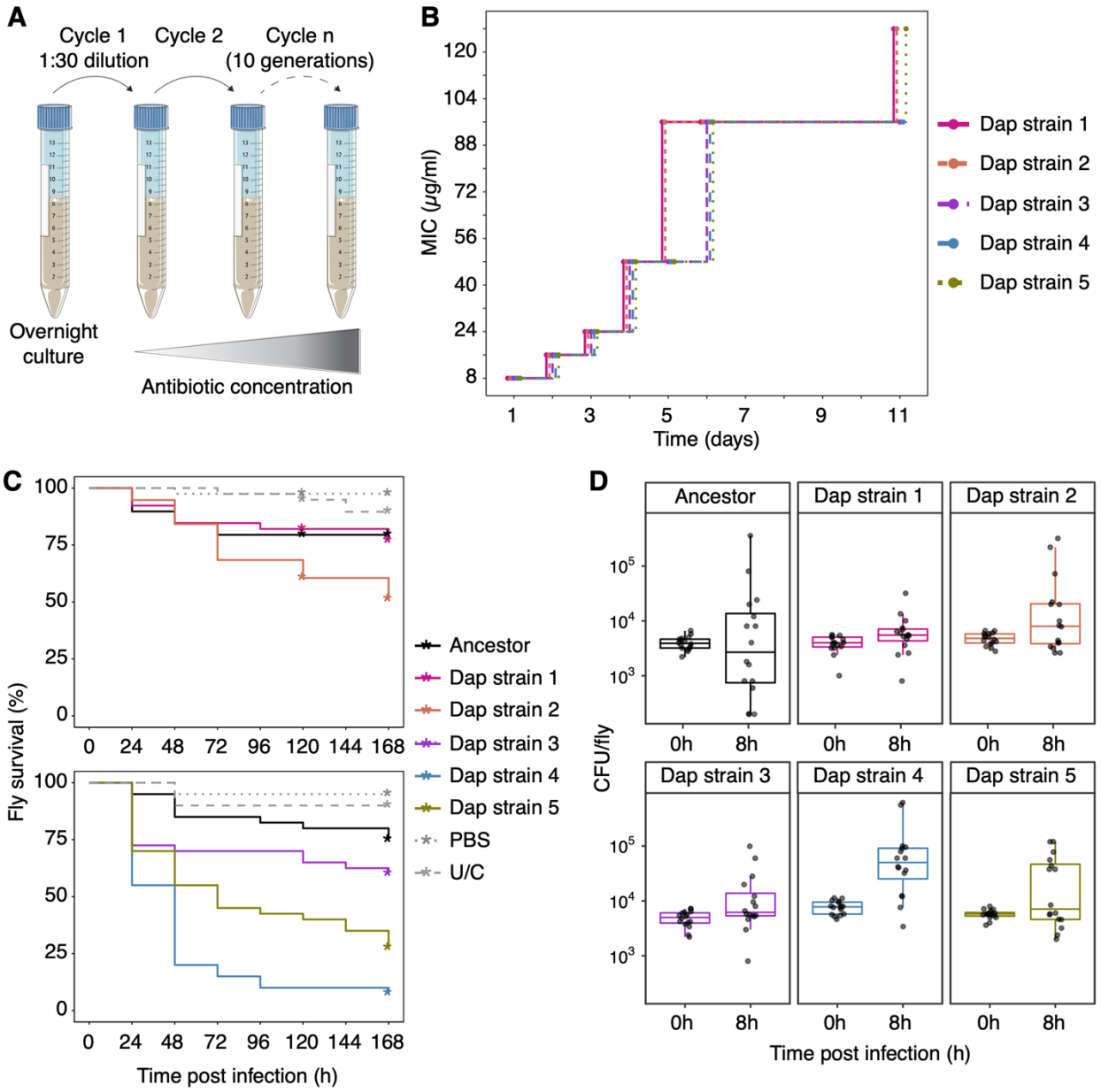

Fig. 3. A. Schematic of selection for daptomycin-resistant strains.

B. Progression of daptomycin MIC's through the course of selection, as assayed by the highest concentration of drug allowing visible growth.

C. Survival of wild-type flies infected with ancestor and daptomycin-adapted strains. P values for survival differences vs ancestor: Dap strains 1 and 2, PBS, and U/C, P>0.05; Dap strain 3, P>0.05; Dap strain 4, P<0.001; Dap strain 5, P<0.01; PBS and U/C, P>0.05.

D. *E faecalis* numbers at 0h (input inoculum) and 8h after infection of wild-type flies with ancestor and

daptomycin-adapted strains. P values for bacterial number differences vs ancestor at 8h: Dap strains 1-3, P=1; Dap strain 4, P<0.001; Dap strain 5, P>0.05.

Table:

Gene	*Drosophila*-adapted strain						Daptomycin-adapted strain					System
	A	B	C	D	E	F	1	2	3	4	5	
yxdM EF2049	Q402*											*yxdJKLM*
yxdJ EF0927										W126C	A136D	
bceB EF2751							M12V	G112D			G112D	
liaF EF2913			S217I									*liaFSR*
liaY EF1752									I99fs			
croS EF3290					K19E				A206S	A206S		*croRS*
dcuR EF1210	N175fs											other
epaQ EF2178	IS6770											
nucF EF1188		A97T										
mprF2 EF1027				IS6770								
bgsA EF2891					G227E							
yihY EF2199						F180L						
EF0797							T4fs					
yqjA EF1910							M267I			E150*		
fmt EF3123								G39V				

Table 1. Mutations acquired during experimental evolution. All identified mutations within transcribed regions are shown. Gene names, where not previously used in *E faecalis*, are derived from homologues in other bacteria. Amino acid locations are relative to the predicted protein in the ancestral strain. "IS6770" refers to mutations caused by new insertions of IS6770 elements in the coding regions of affected genes.