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Supporting Online Material for

Bacteria-Phage Antagonistic Coevolution in Soil

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Supplementary information:

MATERIALS & METHODS

Experimental design. A gentamicin-resistant strain of *P. fluorescens* SBW25 was inoculated into 32 replicate sterile (autoclaved) soil microcosms (10cm x 10cm petri dishes), and, half the microcosms were also inoculated with lytic bacteriophage SBW25ϕ2 (*13*), and half with a soil wash containing the resident microbial community, in a full factorial design. Every 4 days, bacteria and phage were isolated and densities determined by plating onto gentamicin (15 μg/ml) KB agar plates, and soft KB agar containing exponentially-growing ancestral *P. fluorescens*, respectively.

Inoculation and sampling. *P. fluorescens* SBW25 strain with gentamicin resistance (*3*) was grown overnight at 28 ºC in King's media B (KB) in an orbital shaker (180 rpm), and then centrifuged for 10 minutes at 3500 rpm to produce a bacterial pellet, which was resuspendend in M9 buffer to a final concentration of $2x10^8$ colony forming units (cfus)/ml. Thirty two polypropylene trays (10x10cm) with lids containing 100 g of autoclaved compost (John Innes no. 2) soil (soil microcosm) were inoculated with 5 ml of the *P. fluorescens* suspension (10^9 cfu). A natural-soil microbial community ($2.5x10^4$ cfus/g of soil) from a soil-wash (20 g of compost soil/ 100 ml M9 buffer) was inoculated into half of the microcosms; autoclaved wash was added to the others). The virulent bacteriophage SBW25ϕ2 (*13*) ($5x10^6$ plaque forming units (pfus) in 5ml M9) was inoculated into half of the two groups of microcosms, resulting in 4 experimental treatments (natural community and phage present or absent) with 8 replicates. All microcosms were carefully mixed with sterile spatulas, and placed in an environmental chamber at 26 °C and 80 % HR. Every 4 days for 24 days, and 24 days later, soil was mixed with a sterile spatula and samples (2 g) were collected (without replacement with fresh soil) using a sterile spatula and mixed with 10 ml sterile M9 buffer and glass

beads, and vortexed for 1 minute. The resultant soil washes were diluted and plated onto KB agar supplemented with gentamicin (15 μg/ml KB) and incubated for 2 days at 28 ºC to determine cfus per gram of soil. To isolate phages, a sample of each soil wash was vortexed with 10% chloroform and centrifuged at 13000 rpm. The phage supernatant was plated onto exponentially growing ancestral bacteria in 0.6 % KB agar to enumerate pfus (*13*). From each replicate population and time point sampled, six bacterial clones and a phage suspension were stored at -20 ºC in glycerol solution (20 %). Densities of all culturable bacteria were determined from cfus on KB agar plates at every sampling point.

Identification of natural microbial community. To identify the natural culturable bacterial community in the soil-wash, we cultured three samples of the soil-wash in KB medium. After two days at 28 ºC, ten colonies per sample were randomly picked, transferred to new plates, and characterized by analysis of partial 16S rRNA gene using colony PCR [S1] with specific primers; 27f (5´-AGAGTTTGATCMTGGCTCAG-3´) and 1492r (5´-GGTTACCTTGTTACGACTT-3´). PCR amplicons (1465 bp) were separated by electrophoresis, and PCR products were directly sequenced as described previously (*14*). According to analysis of 16S rDNA edited sequences performed with the Ribosomal database project classifier tool (http://rdp.cme.msu.edu/); of 30 isolates, sixteen isolates were assigned to *Bacillus* sp., six isolates to *Stenotrophomonas*, a further five isolates to *Pseudomonas*, another two isolates were assigned to a *Flavobacterium* and one was to *Tetrathiobacter* species. Note that no culturable bacteria were detected that could grow on KB supplemented with gentamicin, nor could they be infected by phage SBW25ϕ2. Moreover, we did not find any phages in the soil wash that were able to infect *P. fluorescens* SBW25.

Resistance assays. Six *P. fluorescens* clones from each population were assayed for resistance by streaking the bacteria against a line of phage on KB agar: growth inhibition indicated sensitivity to the phage (*13, 17, 27*). At each time point, bacterial clones were assayed for resistance against the ancestral phage and their contemporary, co-occurring phage populations. Bacteria from days 24 and 48 were also simultaneously assayed against phages from days 48 and 24 from the same experimental replicate. These additional assays allowed us to determine the resistance of day 24 bacteria to past (ancestral), contemporary and future phage populations, and the infectivity of phages from day 24 to past, contemporary and future bacteria. We chose relatively long time intervals to measure bacteria-phage interactions, because long time intervals increase the chance of distinguishing between different types of coevolutionary dynamic (*6*)

In addition, bacteria from days 24 and 48 were simultaneously assayed against phage from another replicate microcosm from within the same treatment in a paired, reciprocal design (4 independent pairs per experimental treatment). Local adaptation for each pair was calculated as the mean difference between the number of clones susceptible to their local and foreign (from the paired replicate) phages (*14*).

Relative fitness assay. We isolated six bacteria after 48 days evolution in soil from independent replicates for all 4 treatments. We ensured that all bacteria isolated from treatments with phages were resistant to their contemporary phage populations, and that all bacteria isolated from phage-free treatments were susceptible to ancestral phages. The competitive ability of each clone was determined by standard 2 day competition experiments [S2] against a *lacZ*-marked *P. fluorescens* SBW25 both in KB broth and in soil in the absence of phages. Relative fitness (*W*) was calculated from the ratio of the Malthusian parameter (*m*) for each competing type [S2]. Where, $m = \ln(N_f/N_0)$, and N_0 is the initial and N_f the final density of the population at 2 days of competition.

Bacterial population densities were determined plating on Luria–Bertani (LB) agar supplemented with X-gal (40 μg/ ml), in order to score *lacZ*-marked *P. fluorescens* SBW25 strain [S3] and SBW25 populations (*27*). Note that relative fitness of the ancestor SBW25 strain marked for resistance to gentamicin did not significantly differ from SBW25::lacZ ($t = 1.348$, $p = 0.605$).

Statistical analyses. Analysis of the bacteria and phage densities, with General Linear Mixed Models (GLMM) fitted by restricted maximum likelihood method (REML), where the presence or absence of phages and the natural microbial community, and the ancestral, contemporary and future phages were fitted as two or three-level fixed effects, and population fitted as a random effect. Analyses of resistance data were carried out using *t*-tests of angular-transformed data, or non-parametric methods (Wilcoxon, Mann-Whitney, Friedman tests) when parametric assumptions could not be met. Data where averaged through time for these latter analyses to avoid pseudo-replication where appropriate. Relative fitness data was analysed as General Linear Models (GLMs), with resistant/sensitive and the presence/absence of the natural community fitted as fixed factors. All analyses were carried out using JMP® version 8 software.

References and Notes

[S1] D. J. Lane, *In Nucleic acid techniques in bacterial systematics. Ed: Stackebrandt E, Goodfellow M. Chichester, Wiley (UK)*, **115** (1991).

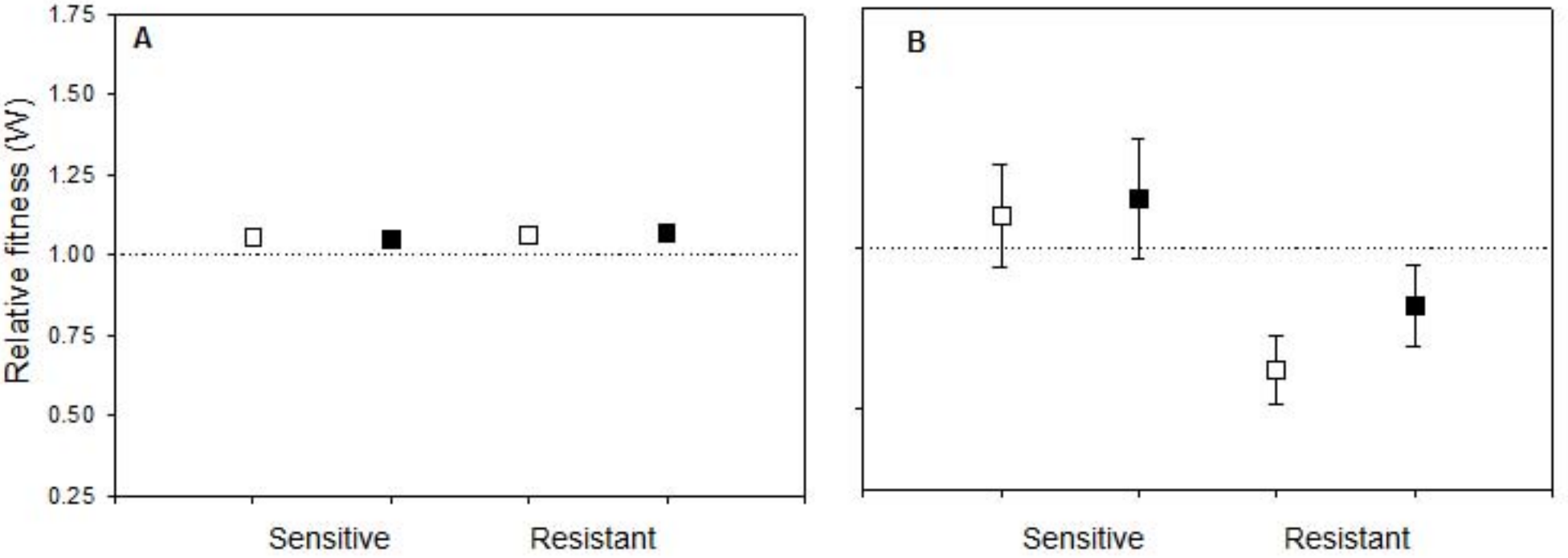
[S2] R. E. Lenski, M. R. Rose, S. C. Simpson, S. C. Tadler, *Am. Nat.* **138**, 1315 (1991).

[S3] M. J. Bailey, A. K. Lilley, I. P. Thompson, P. B. Rainey, R. J. Ellis, *Molecular Ecology* **4**, 755 (1995).

[S4] Partial 16S ribosomal RNA sequences have been assigned by European nucleotide archive under accession numbers FR746065 to FR746094.

Figure legends

Figure S1. Costs of resistance to phages in broth and soil of bacteria. Mean (±SEM) relative fitness after 2 days competition with ancestral SBW25 of resistant and sensitive bacteria clones isolated after 48 days evolving in the absence (□) and presence (■) of the natural microbial community, in nutrient broth (A) and soil (B). Competitions were carried out for 2 days in the absence of the natural microbial community and SBW25ϕ2.


A
B
Relative fitness (W)
1.75
1.50
1.25
1.00
0.75
0.50
0.25
Sensitive
Resistant
Sensitive
Resistant
Bacterial clones